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Molecular ultrasound imaging using microbubble contrast agents

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1. ABSTRACT

Molecular imaging is a new field in bioscience which, by virtue of utilizing a contrast agent or reporter, facilitates early detection of the disease processes before phenotypic changes become apparent. Molecular imaging with ultrasound utilizes contrast agents that bear adhesion ligands designed to bind tissue markers specific for a disease process. Such agents can be detected by ultrasound with a great degree of sensitivity, providing both anatomical reference information as well as additional data such as molecular characteristics of the interrogated region. This review summarizes current applications and challenges of molecular ultrasound imaging and offers future technologies which likely follow as an improvement to the existing techniques. Due to the widespread availability of ultrasound and ease of use, molecular ultrasound imaging is likely to emerge as a powerful diagnostic technique.

2. INTRODUCTION TO ULTRASOUND CONTRAST AGENTS

Image contrast achieved during ultrasound imaging is a function of differences in density and compressibility between different tissue types and structures. The larger the difference in density and compressibility of different tissues, the larger the reflected echo which can be detected by the imaging system.

Typically, the scattered signal from blood is about 30-40 dB lower than that from tissue, hence the signal from blood is fairly difficult to detect, particularly in small vessels (1). Thus, without assistance, measurements of blood perfusion with ultrasound are difficult - a challenge which is overcome by the use of ultrasound contrast agents. Ultrasound contrast agents (UCAs) were first conceptualized by Gramaik and Shah in 1968, who observed that bubbles resulting from injection of a dye into the heart were observable on an echocardiogram (2).

2.1. Microbubble contrast agents

Today, FDA approved UCAs are encapsulated microbubbles with mean diameters on the order of several μm. This diameter range permits passage through the pulmonary circulation and results in contrast agents with resonance frequencies overlapping imaging frequencies utilized with clinical imaging systems (3). These microbubbles are filled with air or with a gas with low water solubility than air such as a perfluorocarbon. Typical filling gases include octafluoropropane (C_3F_8), decafluorobutane, (C_4F_{10}), and sulfur hexafluoride (SF_6) (4). The microbubble shell, designed to reduce gas diffusion into the blood, can be made of lipids, denatured albumin, or polymers. Shell thickness can vary from 10 to 200 nm, depending on the material. Polyethylene glycol can be incorporated into the microbubble shell, particularly in the case of lipid-shelled agents, in order to enhance stability and reduce immune system recognition.

Both the shell and the gas core affect the behavior of the microbubble. Microbubble contrast agents are highly echogenic due to the large difference in material properties (density and compressibility) between the gas in the microbubble and the fluid surrounding it, and because they exhibit resonant oscillations at clinically-used ultrasound frequencies. The magnitude of the echogenicity of microbubble agents is such that even a single agent, with a volume on the order of femtoliters (10^{-15} liter), can be detected by a clinical imaging system (5, 6).

2.2. Other types of contrast agents

Microbubble agents are the most common type of UCA in the United States, and currently the only agent type which is FDA approved. However, other types of contrast agents are being tested in the preclinical environment. Two other agents have demonstrated promise for ultrasound molecular imaging: perfluorocarbon nanoparticles (7-11) and echogenic liposomes (12-16). Microbubbles are the most widely used ultrasound contrast agents, and we will focus our discussion on microbubble contrast agents in this review,

3. CHARACTERISTICS OF CONTRAST ENHANCED ULTRASOUND IMAGING

Ultrasound is traditionally thought of in the context of structural imaging. However, the development of UCAs has extended the use of ultrasound into the fields of functional and molecular imaging. Contrast-enhanced ultrasound imaging is performed in real-time using contrast-specific imaging modes. A typical procedure entails administering a small volume (microliters to milliliters) of contrast agents in solution into the vasculature. In humans, a typical dose is on the order of 10^8 - 10^{10} microbubbles, which translates to a total encapsulated gas volume of on the order of microliters. Contrast agent solution is administered into a peripheral vein in humans and large experimental animals, whereas in small animals administration via the jugular vein, tail vein, or retroorbital sinus is used. UCAs are rapidly distributed by blood flow to the targeted imaging site, and contrast enhancement is typically apparent within seconds. Many microbubble contrast agents exhibit flow characteristics similar to erythrocytes *in vivo*, a property which has been exploited as a mechanism for detecting and quantitating vascular perfusion (17-19). The contrast effect can persist for tens of minutes, dependent upon the administered dose and physiochemical properties of the agents. Microbubbles are removed from circulation by several mechanisms, including filtration by the reticuloendothelial system, engulfment by phagocytic cells, and entrapment in the lung and other dense capillary beds (20, 21). Additionally, microbubble UCAs are readily destroyed by ultrasound energy at higher acoustic pressures. Blood flow imaging typically requires a constant venous infusion of contrast, (22, 23) although bolus administration is appropriate for some settings (24, 25). The relatively rapid contrast clearance of microbubble agents can enable repeated administration, adding tremendous convenience in molecular imaging applications.

The use of ultrasound contrast agents for imaging blood flow has been demonstrated in multiple clinical settings, particularly diagnostic cardiology (26-28).

Table 1. Modalities for molecular imaging

Modality	Spatial Resolution	Scan Time	Contrast Sensitivity	Scanner Price[11]	Comment
MRI	25-100 um	minutes-hours	10-1000 mmol	+++	High spatial resolution at the expense of long scan time, high equipment cost, and poor contrast sensitivity
PET[1]	1 mm	minutes	1-10 pmol	++	Limited spatial resolution and access to contrast; excellent contrast sensitivity and ability to image intracellular events
SPECT[1]	0.2 mm	minutes	10-100 pmol	++	
Optical	mm-cm	seconds-minutes	nmol-fmol	+	Excellent contrast sensitivity and low cost, but limited penetration and spatial resolution
Ultrasound	0.05 – 0.5 mm	seconds-minutes	Single particle	+	Real-time imaging at good resolution and high contrast sensitivity; intravascular only

[1]requires ionizing radiation

Additionally, ultrasound contrast agents have demonstrated diagnostic efficacy in various radiological applications; for example, microbubbles have been shown to enhance the detection of blood flow in both abdominal and peripheral vascular structures (29). Evaluation of microvascular blood flow is a clinical goal in many organ systems, such as kidney, pancreas, and liver, especially after subsequent to transplantation. In the setting of cancer, microbubbles have been shown to facilitate the detection of intratumoral blood vessels in liver, kidney, ovary, pancreas, prostate, and breast tumors (30).

3.1. Comparison with other imaging modalities

Ultrasound molecular imaging offers several advantages over competing imaging modalities (Table 1). In both clinical and preclinical setting, low equipment cost and high equipment mobility are key advantages of ultrasound unsurpassed by other modalities. Currently, ultrasound molecular imaging is confined to the preclinical sector, although contrast enhanced ultrasound is proving to be a strong research tool. In a preclinical setting, only optical imaging offers similar equipment cost. Under some conditions, spatial resolution can be comparable to that of MRI or CT, and is significantly greater than that of nuclear imaging methods (PET and SPECT) and optical imaging. Contrast sensitivity is high for microbubble contrast agents, and signal-to-noise ratio is on the same order of that of PET and SPECT. Ultrasound imaging is not inherently tomographic, although three-dimensional reconstruction imaging schemes are available on commercial scanners (31). Ultrasound boasts excellent temporal resolution, and imaging is performed in real-time. Finally, the rapid clearance of many ultrasound contrast agents allows for repeated administration and imaging of multiple molecular targets in the same subject.

4. PHYSICS OF THE CONTRAST EFFECT

An ultrasound image is reconstructed from echoes returned to the imaging transducer as they are reflected from tissue features. The amplitude of the sound reflected from is a function of the acoustic impedance (a function of density and compressibility) of the media. A larger acoustic impedance mismatch will reflect more sound, which correlates with "more sensitivity" of the imaging system to this interface. Typically, the impedance mismatch between different physiologic tissues is on the order of 1%. However, the impedance mismatch between a gas and blood or tissue is on the order of 10,000 fold (32). Hence, sound waves are scattered from microbubble contrast agents substantially more efficiently than from tissue boundaries or blood components.

In addition to this impedance mismatch, the nonlinear behavior of microbubble contrast agents when insonified makes them unique contrast agents. When excited by an acoustic pulse, highly compressible microbubble agents undergo cycles of expansion and compression in response to the acoustic pressure change (Figure 1). As these microbubbles oscillate, they act as active acoustic sources, and can produce harmonics of the imaging frequencies as well as frequencies related to their inherent resonant frequency (33-35). Perhaps even more significantly, the oscillatory response of contrast agent microbubbles is nonlinear in relation to acoustic pressure, frequency, and phase. These unique qualities have allowed the development of imaging strategies which allow the detection of small quantities of contrast agent and discrimination of echoes from contrast from those of tissue.

5. IMAGING STRATEGIES FOR CONTRAST SPECIFIC IMAGING

Microbubble agents can be detected by conventional imaging methods, although detection sensitivity can be greatly increased with contrast-specific imaging schemes. In conventional ultrasound, the imaging system transmits and receives using the same frequency band, commonly referred as fundamental imaging. Fundamental imaging involves signal processing which does not distinguish between the scattered echoes from tissue and contrast agents. Utilizing this method, microbubbles in small vessels such as capillaries are difficult to detect because of overlapping of echoes from microbubbles and tissue in the time and frequency domain, and also due to the limited number of microbubbles available in small vessels.

Therefore, clinical contrast imaging often takes advantage of signal processing to extract the unique signatures of microbubble contrast agents. Contrast-specific imaging methods have been developed to distinguish microbubbles from tissue based on the nonlinear response of microbubble contrast agents.

As microbubble contrast agents are excited by the imaging system, they expand and contract in an oscillatory fashion. Their magnitude, phase, and rate of oscillation are

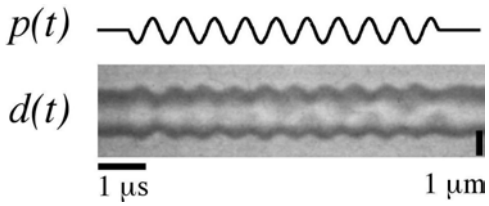

Figure 1. Microbubble Contrast Agent Response to an Ultrasound Pulse. High-speed photography of an oscillating microbubble presented as a diameter vs. time "streak" image *d(t)*. *p(t)* illustrates ultrasound pressure waveform in relation to microbubble oscillation. Dayton *et al.*, unpublished image.

nonlinear with respect to the ultrasound wave pressure and frequency (15, 16, 36-42). This results in the scattering of echoes from microbubble contrast agents which are uniquely different than echoes from tissue, which scatters echoes in a more linear fashion.

Contrast imaging methods can be generally divided into two categories, single pulse or multi-pulse strategies, based on whether a single imaging pulse or multiple pulses are applied. Single pulse contrast imaging strategies include subharmonic imaging, second harmonic imaging, super-harmonic/ultra-harmonic imaging, and multiple pulse strategies include phase inversion imaging and contrast pulse sequence (CPS) imaging.

5.1. Single pulse imaging strategies

5.1.1. Second harmonic imaging

Second harmonic imaging involves signal processing where the received ultrasound signal is filtered to retain only frequencies which are approximately twice the imaging center frequency (43-46). This imaging method relies on detecting harmonics, or higher multiples of the imaging frequency, which are produced by oscillating contrast agents. One advantage to this method is that second-harmonic imaging has improved resolution because of the higher frequency produced by the microbubbles, however, this method is limited for microbubble detection as tissue also produces higher harmonics at moderate pressures, limiting the conrast-to-tissue ratio (CTR).

5.1.2. Subharmonic imaging

Subharmonic imaging involves signal processing where the received ultrasound signal is filtered to retain only frequencies which are approximately half of the transmission center frequency (33, 47-49). When a population of contrast agent microbubbles is insonified, some of the contrast agents will scatter energy at half of the imaging frequency. Two types of subharmonic signal are described in the literature, one where the contrast agents are excited by the imaging frequency at their natural resonant frequency, and a second where the contrast agents are excited by the imaging center frequency at their harmonic (33). Both mechanisms result in microbubbles producing echoes with frequency components at half of the imaging frequency. Insonified tissue does not generate subharmonic echoes, and scatters only frequencies the same or higher than the transmission frequency. The result is that this imaging technique can achieve a better CTR than traditional fundamental imaging. One drawback of subharmonic imaging is that the spatial resolution is reduced since the only the lower frequencies are preferentially retained.

5.1.3. Power Imaging

Power imaging, power Doppler imaging, or energy imaging, as it is sometimes referred to, involves detecting the decorrelation of the ultrasound signal over successive pulses. Although this imaging method was designed to detect blood flow, it is also very effective at detecting moving or breaking contrast agents (50, 51). However, this method is most effective at higher acoustic pressures where the contrast agents are destroyed, so a constant refreshment of microbubbles in the tissue to be imaged is required.

5.1.4. Super-harmonic, ultra-harmonic, and transient imaging

Oscillating contrast agents produce a broadband response which contains energy at integer multiples (3, 4 …) and rational multiples (3/2, 5/2, …) of the fundamental frequencies. Since tissue signal are mainly located in fundamental and second harmonic frequency band, imaging methods which selectively detect energy in higher frequency bands can provide better significantly better CTR compared to fundamental imaging, as well as much better spatial resolution compared to subharmonic imaging (52-54). The major difficulty associated with these methods is the wide bandwidth requirement, which can be over 100% relative bandwidth. A commercial ultrasound system generally has a relative bandwidth of ~70% to 80%, therefore transducer technology will need to be improved before these imaging techniques can be optimized for clinical imaging systems.

5.2. Multiple pulse imaging strategies

5.2.1. Phase inversion imaging

Phase inversion, also called pulse inversion, is a technique in which two transmitted pulses of opposite phase are transmitted one after the other separated by a delay (37, 55, 56). A linear scatterer will reflect the original and inverted pulses similarly, and the two opposite phase pulses will cancel when summed. A non-linear scatterer, such as a contrast agent microbubble, will respond differently to the different phase pulses, and the sum of the two will not be zero. This technique achieves tissue suppression in exchange for a reduction in imaging frame rate.

5.2.2. CPS

Cadence Contrast Pulse Sequencing, as it is referred to by Siemens, is a multiple pulse imaging method first proposed in the US patent by Brock-Fisher (57). This method improves upon phase inversion by modeling tissue echoes as a polynomial to accommodate for nonlinear propagation and utilizing both the phase and amplitude response of contrast agents. This method assumes that echoes from tissue are a function of a deterministic memoryless nonlinear system and echoes from bubbles are

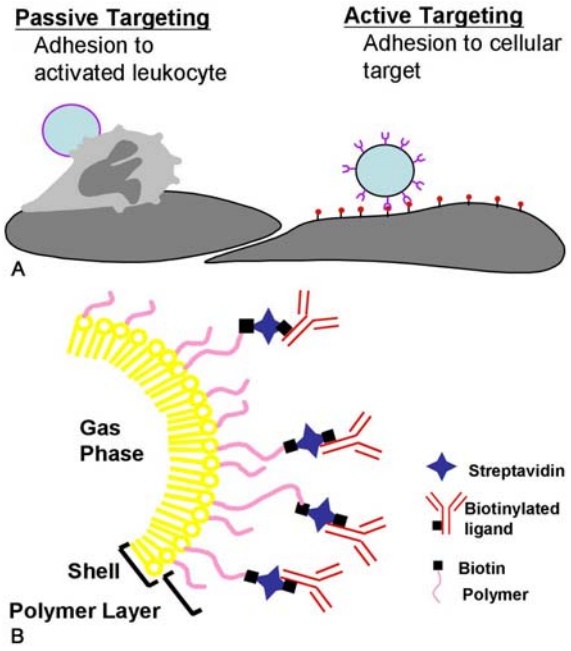

Figure 2. Mechanisms of Microbubble Targeting. A) Two general targeting strategies have been described: passive targeting, in which the physiochemical properties of the microbubble are selected to enable retention to or uptake by cellular infiltrates (such as activated leukocytes), and active targeting, in which a targeting ligand specific for a molecular target is conjugated to the microbubble surface. B). Schematic of a Typical Targeted Ultrasound Contrast Agent. The gas phase is encapsulated by a lipid, polymer, or protein shell, which may be stabilized by a polymer layer. Targeting ligands can be immobilized on the distal surface of the polymers using biotin-streptavidin or other coupling chemistries.

a function of an indeterminate nonlinear system with memory, and additionally, that both processes are separable in the return echoes. As it was originally proposed, this method involves transmission of three pulses which have the same shape but different amplitude and phase. Upon receive, three calibrated gain factors calculated to suppress one or more tissue harmonics (including the fundamental) are applied to the received echoes, and the result is summed. This method can achieve tissue suppression greater than phase-inversion imaging, but also results in reduced frame rate and more susceptibility to tissue motion.

6. ULTRASOUND MOLECULAR IMAGING

Molecular imaging is typically defined as the non-invasive application of an imaging modality to discern changes in physiology on a molecular level. Traditional B-mode ultrasound imaging, which relies on detecting differences in the density and compressibility of tissue, is by definition an anatomical imaging modality. However, the development of molecularly-targeted contrast agents and contrast-specific imaging schemes have expanded the use of ultrasound to molecular imaging applications. As most ultrasound contrast agents are confined by size to the intravascular space, relevant molecular targets for this technique must be expressed on the vascular lumen or on intravascular cells. Thus, ultrasound molecular imaging is able to detect the state of the vasculature, but is generally unable to image intracellular and genetic events. Fortuitously, many pathophysiological conditions induce the expression or up-regulation of cell surface biomarkers which may be used as molecular targets for ultrasound molecular imaging.

6.1. Targeted contrast agents

Ultrasound contrast agents were initially developed for blood pool imaging, and used as tracers to detect blood flow. Blood pool ultrasound contrast agents generally cannot be used for molecular imaging, although several surprising characteristics pertaining to selective retention of some blood pool agents have been reported. The use of contrast enhanced ultrasound for imaging blood flow and perfusion has been reviewed elsewhere in depth (58, 59).

Ultrasound molecular imaging requires the selective retention of the contrast agent to the intended target. Two strategies have been investigated for site-targeted imaging using UCA (Figure 2); active targeting, in which a ligand specific for the molecular target is immobilized to the agent surface, and passive targeting, in which the physiochemical properties of the agent are used to achieve retention at the target site. Passive targeting generally does not exhibit molecular specificity, and may be best classified as functional, rather than molecular, imaging. Both strategies have shown promise in numerous animal models of disease, although ultrasound molecular imaging has yet to be approved for clinical use.

Like most molecular imaging techniques, ultrasound molecular imaging requires a dwell period after administration, typically 4-15 minutes, during which the agents accumulate at the target site and circulating agents are cleared. Contrast agents accumulated at the target site are then imaged, sometimes using a destruction-subtraction algorithm (60) to measure the residual circulating contrast signal. The entire imaging procedure typically lasts no more than 30 minutes. Data analysis is typically performed off-line, and may consist of digital subtraction and frame alignment, region-of-interest quantification, and color map post-processing.

6.2. Passive targeting

Passive targeting has been explored in the context of inflammation, lymph node imaging, and detection of liver lesions. Early observations of albumin-encapsulated microbubbles revealed delayed clearance and retention in dysfunctional vessels (61) and to inflamed endothelium (62). Subsequent research determined that leukocyte and complement mediated adhesion was largely responsible for this behavior (63, 64). The role of microbubble surface charge, shell composition, and surface architecture have each been explored in the context of passive microbubble targeting. Passive targeting of albumin-encapsulated microbubbles can occur via adhesion to beta-2 integrins on activated leukocytes (65) and complement fragments (64).

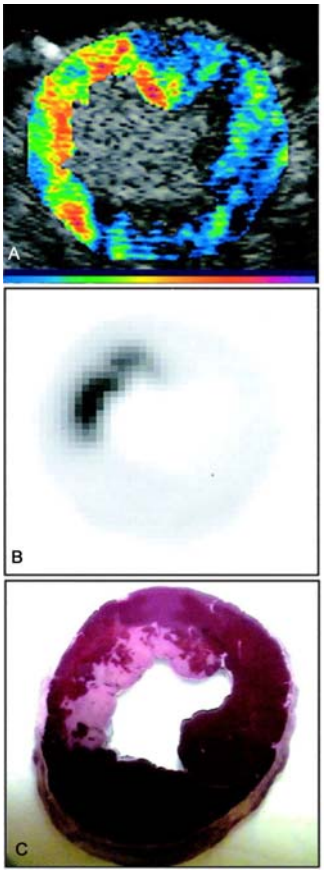

Figure 3. Contrast-enhanced Ultrasound Imaging of Myocardial Ischemia/reperfusion Injury by Passive Targeting of Activated Leukocytes. A contrast-enhanced ultrasound signal is observed in the post-ischemic bed (A), and corresponds to that observed by 99mTC-labeled leukocyte accumulation (B) and TTC staining of infarcted myocardium (C). Reproduced with permission from 70.

For lipid-encapsulated microbubbles, surface charge has been shown to be involved in passive targeting, with anionic microbubbles exhibiting significantly greater adhesion (66). The presence of a layer of stabilizing polymer, such as polyethylene glycol (PEG) on the microbubble surface can inhibit passive adhesion, and microbubbles lacking such as protective layer exhibit adhesion to inflamed endothelium under some conditions (66).

6.2.1. Passive targeting of inflammation

In light of the adhesive mechanisms described above, imaging the inflammatory process is a natural application for passive targeting. This technique does not provide molecular-level information regarding inflammation, but rather enables detection of leukocyte adhesion secondary to endothelial activation. Unlike other imaging methods, the confinement of microbubble contrast agents to the vascular compartment enables detection of only activated, endothelium-bound leukocytes, the presence of which may have particular prognostic significance. Complement-avid phospholipids, such as phosphatidylserine, have been used to construct leukocyte-targeted microbubbles. A decrease in acoustic signal from cell-bound microbubbles has been observed *in vitro* (67), although recent work has suggested that cellular adhesion increases microbubble stability by diminishing gas exchange across the shell (68). Microbubbles retained by activated leucocytes will be phagocytosed, causing a reduction of acoustic signal at low to moderate acoustic powers (69).

Microbubbles composed partially of a phosphatidylserine lipid shell have been used to image leukocyte adhesion in the context of post-ischemic inflammation dog myocardium (Figure 3) (70). Subsequent work using lipid-shell microbubbles enabled detection of leukocyte adhesion in the context of cardiac allograft rejection in rats (71), presumably via-charge mediated retention. Retention of albumin-encapsulated microbubbles has been observed in hypertriglyceridemia and balloon injury models of arterial dysfunction in swine (72). These microbubbles, composed of sonicated dextrose and albumin, were recently used to detect atherosclerotic plaque in a mouse model of atherosclerosis (64). In this study, microbubble retention was also observed in hyperglycemic mice and atherosclerosis-prone rats in the absence of histologically evident plaque and endothelial dysfunction, suggesting that this technique may enable early stage detection of atherosclerosis.

6.2.2. Passive targeting of lymphatics

Lymph node imaging is an emerging application for contrast enhanced ultrasound imaging. One key application for lymphosonography is detection of sentinel lymph nodes, which are the first nodes to receive lymphatic drainage from a neoplasm. Both sub-micron (73) and microbubble (74, 75) contrast agents have been shown to accumulate in the lymphatics, with preferential accumulation at sentinel, but not second-order, lymph nodes. Uptake of microbubbles following subcutaneous (76), sub-mucosal (74), and parenchymal (77) administration has been demonstrated in multiple species . Although the precise mechanism for transport into the lymphatic system has not been fully described, it may be related to the avidity of certain microbubble preparations for macrophages.

6.2.3. Passive targeting of liver

In liver lesions, characteristic contrast enhancement patterns caused by variations in blood flow enable discrimination of lesions from parenchyma. This multiphasic enhancement is thought to be due in part to retention of microbubbles in healthy liver tissue, with recent research implicating Kupfer cell adhesion and phagocytosis (78, 79). Contrast-enhanced ultrasound imaging has shown detection efficacy similar to CT in detection of liver lesions in clinical trials, and Sonazoid, a liver-avid microbubble, was recently approved for this indication in Japan.

6.3. Active targeting of inflammation

Inflammation is a hallmark of many disease processes, and detection of the inflammatory response at a molecular level can potentially improve the differential

diagnosis and guide treatment for many diseases. Inflammation is mediated in part by several receptors found on luminal endothelium (80), and thus accessible to ultrasound contrast agents. Active targeting strategies for inflammation have focused primarily upon endothelial cell adhesion molecules, which are expressed or up-regulated under inflammatory conditions. Ultrasound molecular imaging has been examined in numerous animal models, and inflammation imaging is rapidly emerging as a particular strength of this technique.

6.3.1. Active targeting of post-ischemic injury

Inflammation is a critical component of ischemic injury, and ultrasound molecular imaging shows particular promise in its detection. Post-ischemic injury has been investigated in the mouse kidney using lipid shell microbubbles targeted to P-selectin. (60). Low-frequency harmonic imaging of lipid-shell microbubbles targeted to P-selectin using an anti-P-selectin monoclonal antibody revealed a strong contrast signal, with significantly lower non-specific signal in sham treated kidney or with microbubbles targeted with an isotype control antibody. Intravital microscopy of the cytokine-treated cremaster muscle in mouse revealed that these microbubbles adhere to endothelial P-selectin, as well as to activated leukocytes and platelets.

Post-ischemic injury in myocardium has been investigated in a rat model of ischemic memory using lipid-shell microbubbles targeted to the selectins (81). In this study, the oligosaccharide sialyl Lewis^x (sLe^x), which mediates leukocyte adhesion to P- and E-selectin, was utilized as a targeting ligand. This disease model was intended to simulate acute coronary syndrome without infarction, although some myocardial necrosis was present. The heart was imaged at 30 and 60 minutes after reperfusion, and an approximately 10-fold enhancement in acoustic signal was observed in post-ischemic myocardium relative to unaffected regions of the myocardium at both time points. A strong linear relationship was observed between post-ischemic risk area size and the size of acoustically enhanced myocardium region. The acoustic signal from targeted microbubbles was approximately 3-fold higher post-ischemic myocardium compared to that from microbubbles targeted with a non-specific negative control ligand.

6.3.2. Active targeting of transplant rejection

Intravascular cell adhesion molecule-1 (ICAM-1) is a 90 kDa protein expressed on vascular endothelium, erythrocytes, activated lymphocytes, and macrophages (82). Specific retention of lipid shell microbubbles bearing and anti-ICAM-1 monoclonal antibody was reported on a substrate of coronary arterial endothelial cells (83, 84), and these agents were subsequently used to image acute cardiac allograft transplant rejection in rats (85). Relatively high spatial variability in the contrast signal was observed in rejecting myocardium, which was attributed to inhomogeneous ICAM-1 expression or variable vascular delivery of contrast agents. An approximately 10-fold increase in contrast video intensity was observed in rejecting myocardium relative to control hearts, which was greater than the specificity observed *in vitro* (85). This study compliments work done by Kondo and colleagues (71), which used passive targeting to image leukocyte adhesion in a similar transplant rejection model.

6.3.3. Active targeting of inflammation in the central nervous system

ICAM-1 has also been investigated in the context of imaging inflammation in the central nervous system. The retention of polymer shell microbubbles targeted to ICAM-1 using the same monoclonal antibody as in (85) was examined in a rat model of adoptive transfer autoimmune encephalitis (86). A strong periventricular acoustic signal was observed after administration of ICAM-1 targeted microbubbles, with minimal signal in healthy control animals. Animals treated with methylprednisolone after adoptive transfer exhibited a minimal targeted signal (86, 87).

6.3.4. Active targeting of inflammatory bowel disease

Ultrasound molecular imaging has been proposed as a tool for detecting periodic flare-ups and for staging in chronic inflammatory bowel disease. Microbubbles targeted to the gut-specific marker mucosal addressin cellular adhesion molecule-1 (MAdCAM-1) were investigated by Bachmann and colleagues (90), and shown by intravital microscopy to bind specifically in the Peyer's Patch in a mouse model of Crohn's disease (Figure 4). Transabdominal ultrasound imaging revealed an approximately 5-fold enhancement of MAdCAM-1 targeted microbubbles relative to microbubbles targeted with an isotype control antibody. A strong linear correlation was observed between targeted acoustic signal and histologically-determined disease severity.

6.4. Active targeting of atherosclerosis

Endothelial cell adhesion molecules have also been targeted for imaging chronic inflammatory disorders. Vascular cell adhesion molecule-1 (VCAM-1), like ICAM-1, is a member of the immunoglobulin superfamily and is up-regulated in the setting of chronic inflammation. VCAM-1 has been used as a molecular target for imaging atherosclerotic plaque development in the ApoE$^{-/-}$ mouse model (88, 89). Cho and colleagues (89) have reported specific retention of multi-targeted microbubbles targeted bearing ligands against both P/E-selectin and VCAM-1 in a mouse model of fulminant atherosclerosis.

6.5. Active targeting of thrombosis

Thrombus imaging has received significant attention in the ultrasound field due to its broad etiological significance. Unger and colleagues have explored several lipid shell microbubble preparations useful for imaging thrombosis. These microbubbles bear a covalently bound peptide containing an Arginine-Glycine-Asparate (RGD) sequence (91). The RGD sequence and its analogues are known to broadly bind various cell surface integrins, including GPIIb/IIIa, which is responsible for agonist-induced platelet aggregation. These agents were tested using fundamental imaging in canine models of thrombosis in the femoral artery and left atrial appendage (92, 93), and induced a ~20% increase in videointensity relative to non-

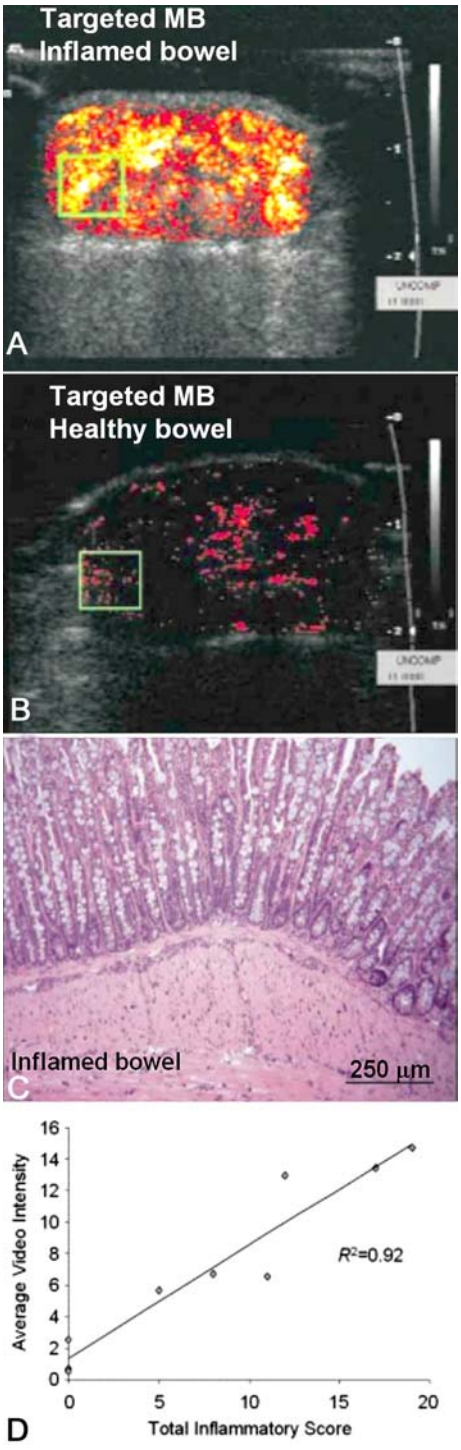

Figure 4. Ultrasound Molecular Imaging of Inflammatory Bowel Disease. Contrast-enhanced ultrasound images using microbubbles targeted to MadCAM-1 in (A) the SAMP/Yit model of spontaneous intestinal inflammation and (B) healthy control mice. Illeal tissue obtained from inflamed bowel showed gross morphological changes (C), and a strong linear correlation was observed between targeted CEU signal videointensity and inflammatory score assessed by histology. Reproduced with permission from 90.

targeted control agents. Schumann and colleagues (94) subsequently used intravital microscopy to show specific retention of GPIIb/IIIa-targeted microbubbles to thrombus in mouse cremaster microcirculation, and that the number of retained microbubbles scaled linearly with clot size.

6.6. Active targeting of angiogenesis

Neovascularization is involved in many physiological processes, and often contributes to disease progression or resolution. Angiogenesis in the setting of solid tumor development is correlated to progression to malignancy, while in chronic ischemic disease it may be therapeutic. Contrast-enhanced ultrasound is particularly relevant for angiogenesis imaging, as both molecular components of the process and, using blood-pool contrast agents (58), blood flow can be measured. Contrast enhanced ultrasound may become an important tool in both research and clinical imaging settings for assessing the response to therapies targeted at vascular remodeling.

Tumor angiogenesis has been explored using microbubbles targeted to several molecular markers (Figure 5). Korpanty and colleagues (95) recently reported imaging tumor progression in an orthotopic model of pancreatic cancer in mice using avidinated dextrose/albumin microbubbles. A monoclonal antibody against the VEGF:VEGF receptor bond complex was used as a ligand in this study. Microbubbles were detected using fundamental B-mode imaging, and the authors were able to track the response of the tumor to cytotoxic or anti-angiogenic therapy. Angiogenesis was imaged in a similar subcutaneous tumor model using microbubbles bearing antibodies against VEGF receptor 2 (VEGFR2) and endoglin.

Microbubbles targeted to the alpha-v integrin family have been used to image angiogenesis in several animal models. The $alpha_v$-$beta_3$ integrin has been implicated in the formation of new blood vessels (96) and is known to bind multiple extracellular matrix components via the RGD peptide motif. In this setting, imaging of $alpha_v$ integrins is unlikely to be confounded by perivascular integrin expression, as microbubbles are purely intravascular tracers. Optical-acoustic observations have confirmed specific retention of microbubbles bearing anti-$alpha_v$-$beta_3$ antibodies or RGD peptides to endothelial cells *in vitro* (67). A 20-50 fold increase in adhesion was observed for targeted relative to control microbubbles in this system.

Microbubbles bearing the disintegrin echistatin, which binds to several integrins, or a monoclonal antibody against the $alpha_v$ integrin subunit, have been used to image angiogenesis in mice implanted with subcutaneous matrigel plugs or fibroblast growth factor-2 (FGF-2) microcapsules (97). Significant microvascular retention of targeted microbubbles was observed by intravital microscopy in FGF-2 treated skeletal muscle, and an approximately 3-fold greater acoustic signal was observed for targeted relative to control microbubbles in matrigel plugs.

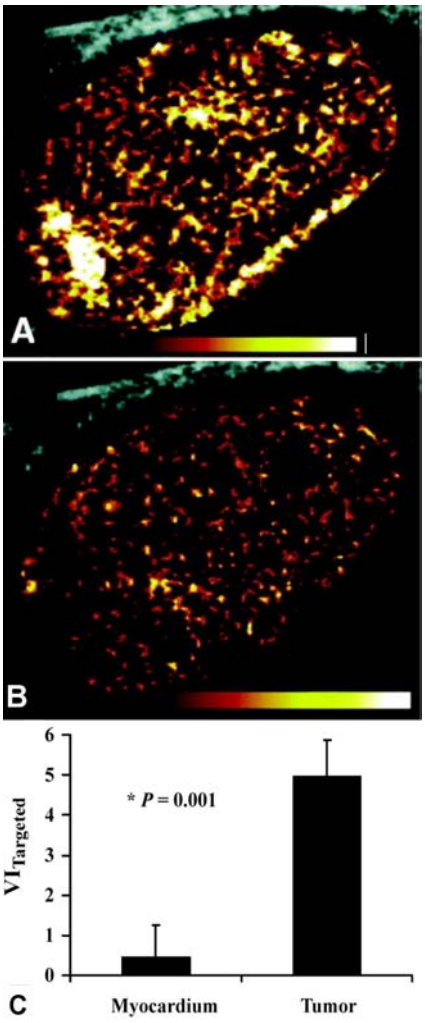

Figure 5. Contrast-enhanced Ultrasound Imaging of Tumor Angiogenesis. Microbubbles bearing a cyclic RRL-containing peptide were used to image angiogenesis in a subcutaneous tumor model in mice. Significant accumulation was observed for microbubbles bearing the RRL peptide (A), with reduced accumulation of microbubbles bearing a negative control peptide (B). Quantification of targeted signal videointensity of RRL-bearing microbubbles within the tumor and (non-angiogenic) myocardium is shown in (C). Reproduced with permission from 99.

Similar microbubbles bearing echistatin were subsequently used to image tumor growth over several weeks in a rat glioma model (98). In this study, the acoustic signal from target-adherent microbubbles was normalized by the ultrasound-derived parametric blood flow (22) to account for the role of blood flow in delivering microbubbles to the targeted vasculature. Targeted ultrasound imaging slightly overestimated tumor size when compared with histological measurement, although ultrasound imaging was able to detect small metastases in several cases. Weller and colleagues (99) used a microbubble bearing a cyclic RRL peptide motif, which has been shown to bind preferentially to tumor endothelium (100), to image tumor angiogenesis in a mouse model. Healthy myocardium was used as a non-angiogenic control, and targeted microbubbles exhibited minimal non-specific adhesion..

Microbubbles bearing echistatin have also been used to image therapeutic arteriogenesis in the setting of peripheral vascular disease (101). In this study, hindlimb ischemia was produced by ligating the iliac artery in rat. Ultrasound molecular imaging was able to detect a biphasic response to the induced ischemia over four weeks, which was accelerated and enhanced by FGF-2 treatment. Comparison to the timecourse of non-capillary microvessel recruitment revealed that ultrasound molecular imaging detected the angiogenic response at the very early stage of ischemia-induced remodeling.

6.7. Active targeting of lymph nodes

Active targeting in the setting of lymphosonography has been examined by Hauff and colleagues (102). MECA-79 antigen, an L-selectin ligand involved in lymphocyte homing and expressed on endothelium within peripheral lymph nodes (103), was selected as a molecular target in this study. Polymer shell microbubbles were coated with the MECA-79 antibody and administered intravenously in mice and dogs. High-MI destructive imaging revealed microbubbles in popliteal lymph nodes and the spleen within 30 minutes. Microbubbles bearing a control ligand showed infrequent adhesion in the lymph nodes, but consistent adhesion in the spleen.

7. TARGETED CONTRAST AGENT LIMITATIONS

7.1. Microbubble adhesion

Although the current generation of targeted microbubbles has shown promise in a wide variety of experimental conditions, critical limitations remain. In some conditions, targeted contrast enhanced ultrasound imaging suffers from a relatively low signal-to-noise ratio. To remedy this, it is desirable to maximize the differential retention of targeted microbubbles to targeted and control tissue (84). This may be achieved by careful selection of appropriate ligands and molecular targets, by modification of the contrast agent structure to reduce non-specific retention, and by enhancing the stability of targeted agents. The second limitation is related to the absolute degree of retention. The accumulation of a sufficient number of agents at the target is required for the production of a robust ultrasound signal, and limited microbubble retention may possibly result in false negative interpretation. As will be discussed below, hemodynamic and anatomical conditions, as well as the target site density, contribute to the efficiency with which a contrast agent is able to achieve targeted adhesion. Potential mechanisms for enhancing absolute microbubble adhesion efficiency will be discussed in section 8.

7.1.1 Microbubble adhesion in blood flow

The intravascular behavior of a microbubble contrast agent can influence its efficacy for targeted

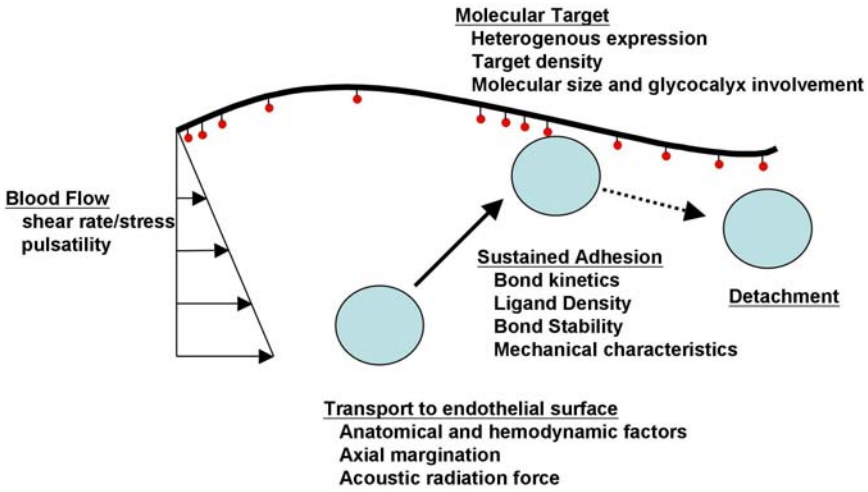

Figure 6. Factors Influencing Targeted Microbubble Adhesion. Multiple factors influence a microbubbles ability to undergo sustained adhesion to an endothelial molecular target, including the inherent physical properties of the microbubble, intrinsic characteristics of the target:ligand bond pair, and the local hydrodymanic conditions.

imaging. Many microbubble formulations have been reported to exhibit rheological behavior similar to erythrocytes *in vivo*, and thus tend to remain close to the axial center of the blood vessel (17, 104, 105). For microbubbles used as blood flow tracers, this is a desirable characteristic. However, targeted imaging requires that the microbubble encounter target receptors on the endothelial surface or associated cells (Figure 6). Microbubbles that preferentially migrate to the axial center of the vessel may thus exhibit infrequent contact with the intended molecular target. This behavior is related to the size, geometry, and compressibility of the particle, as well as the vessel and blood flow characteristics. It should be noted that the axial migration of a targeted microbubble is predicted to occur primarily in large vessels, and not necessarily in the small and branching vessels of the microcirculation (106). In large vessels where these hemodynamic factors are largely absent, microbubble transport may present a significant deficiency and microbubble retention may be reduced. Selective targeting in large arterial vessels has been demonstrated (64, 88, 89), although the role of axial microbubble distribution in targeting efficiency has not been explored in detail.

7.1.2. Microbubble bond formation

Targeted microparticle retention to luminal vasculature requires the rapid formation of adhesive bonds. The ability of a ligand:target pair to form a bond is known to be dependent upon the molecular contact time, the force with which the bond is loaded, and the intrinsic kinetic properties of the bond pair (107). Kinetic properties of bond pairs are commonly expressed in terms of off- and on-rate constants (k_{off} and k_{on}, respectively), the quotient of which is equivalent to the bond dissociation constant (K_D). *In vitro* flow chamber studies have revealed that microbubble adhesion efficiency is strongly dependent upon the fluid flow rate and target site density (84, 108). The selection of targeting ligands possessing kinetic properties (e.g. rapid k_{on} and slow k_{off}) conducive to bond formation under a wide variety of flow and target conditions is desirable in a robust molecular imaging contrast agent.

7.1.3. Microbubble bond force

The ability of an adhesive bond pair to withstand the dislodging force of blood flow is also pertinent to targeted microbubble retention. Once a microbubble achieves contact with the endothelium and is bound to the target, it experiences a dislodging force imparted by the incident blood flow. This force is distributed to the load-bearing molecular bonds and generally serves to reduce bond stability. Bond stability has been observed to increase with applied force in some cases, although this behavior appears to be limited to a select group of adhesive bond pairs (109, 110). The magnitude of the force applied to the adhesive bond is proportional to the fluid shear rate and size of the particle (111, 112), and may result in the rapid dissociation of microbubble:endothelium bonds. *In vitro* measurements have revealed that a significant proportion of microbubble adhesive events may be short lived, with adhesion lasting for less than 10 seconds at even a moderate magnitude of shear flow (135).

7.2. Signal quantification and interpretation

Selective targeting has been demonstrated for various ultrasound contrast agents and in multiple animal models. However, the meaning of the parameter that is being measured by this technique has not been precisely established. In most settings, video enhancement averaged over a two-dimensional region of interest is measured off-line using imaging processing software and presented in units of decibels or mean pixel amplitude. Microbubble contrast has been shown to be linear over the range of several thousand particles per mL (6, 68) *in vitro*, and increasing video intensity is therefore expected to correspond to increasing microbubble concentration within the ultrasound beam. Relating the quantity of adherent microbubbles to disease severity, however, may be confounded by the complexity of the vasculature and target

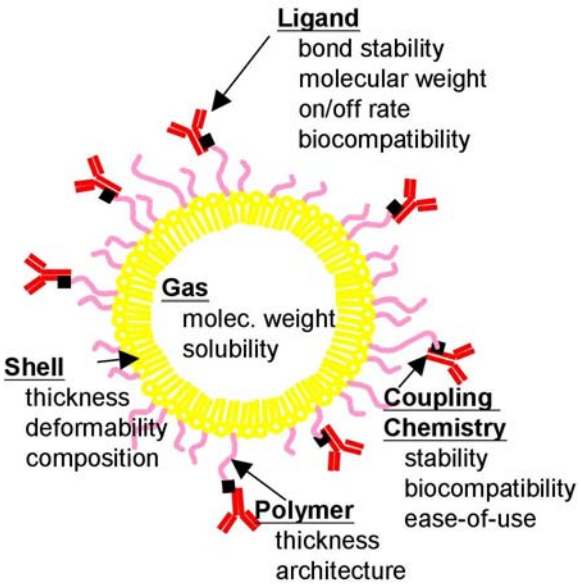

Figure 7. Structural Determinants of Microbubble Targeting Efficiency. The mechanical and physiochemical properties of the microbubble shell, polymer layer, targeting ligand, and ligand coupling chemistry may be tuned individually or in concert to engineer microbubbles able to undergo enhanced targeted retention with low non-specific adhesion.

expression. Microbubble retention has been shown to be proportional to the target site density *in vitro*, although factors such as blood flow rate and vessel geometry are also relevant determinants of microbubble adhesion (106, 108, 119). Thus, interpretation of an ultrasound contrast signal requires at least some knowledge of the vascular characteristic of the target tissue.

Scaling a targeted signal by blood flow has been suggested as a means for eliminating the influence of variable blood flow in different tissues (98). For molecular imaging applications in which the microcirculation is investigated, the influence of the diversity of hemodynamic and anatomical conditions may conceivably be negated by the relatively large (several cubic millimeters) volume imaged. For heterogeneous structures, such as skeletal muscle or solid organs, imaging multiple fields of view within a target tissue may result in increased diagnostic accuracy.

Due to the potential dependence of contrast agent adhesion on vascular characteristics, it appears unlikely that the targeted ultrasound signal can be related to a direct measure of molecular expression (such as molecules per endothelial surface area). Moreover, most published studies have examined the efficacy of ultrasound molecular imaging in fulminant models of disease, and the ability of this technique to detect fine changes in molecular expression corresponding to slight changes in disease severity has not been rigorously demonstrated. Developments in microbubble detection schemes and enhanced targeting strategies are rapidly advancing the sensitivity of ultrasound molecular imaging, and the promise of this technique is considerable.

8. RECENT ADVANCES

8.1. Targeting ligands

Successful molecular imaging requires a microbubble shell and associated targeting ligands capable of mediating efficient and specific adhesion of the contrast agent to the molecular target (Figure 7).

An ideal targeting ligand for ultrasound contrast would possess a rapid on-rate and slow off rate, high mechanical stability, high target specificity, would be non-immunogenic, and would not elicit a substantial physiological response upon ligation of its molecular target. Additionally, the ligand should be exhibit excellent stability upon storage and *in vivo*, be amenable to appropriate coupling chemistries, and, for preclinical imaging, be functional in a wide range of relevant research animal species. Ligands must be coupled to the surface of the contrast agent, and the coupling chemistry must be both stable and non-immunogenic. Biotin-avidin coupling systems have frequently been used in targeted microbubble systems (20), although the strength and flexibility of this coupling chemistry may be counterbalanced by the potential immunogenicity in some settings. Covalent coupling chemistries, such as those based on thioether or amide bonding (83, 114) offer an attractive solution, and have been widely utilized in other targeted microparticle systems (115). Finally, although monoclonal antibodies have been frequently used as targeting ligands in many studies, the species-dependence, immunogenicity, cost, adhesive kinetics, and size of these ligands are potential drawbacks. Peptides, glycoconjugates, and other small molecule ligands are attractive ligand possibilities, and the development of appropriate ligands is crucial to development of ultrasound molecular imaging.

8.1.1. Enhanced capture efficiency

The use of ligands able to mediate enhanced microbubble adhesion has been hypothesized as a means of increasing the signal-to-noise ratio of ultrasound molecular imaging. As described above, most ultrasound molecular imaging studies have utilized monoclonal antibodies as targeting ligands. Antibodies possess the advantages of high specificity and availability, although the kinetic properties may be less than ideal for targeted microparticle adhesion (116). In the context of imaging inflammation, ligands derived from leukocyte adhesion molecules are attractive candidates due to their superior adhesive properties. The selective adhesion of leukocytes to the endothelial surface is a complex biophysical process partially mediated by a specialized group of glycosylated adhesion molecules (80). The unique kinetic and mechanical characteristics of these ligands enables rapid bond formation in shear flow (117), qualities that are desirable in ultrasound contrast agent ligands.

The ability of glycosulfopeptides derived from the leukocyte adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1) (118) to mediate microbubble adhesion to P-selectin has been explored in the flow chamber (119). In this system, the glycosulfopeptide ligands mediated microbubble adhesion to P-selectin 3-5 times more

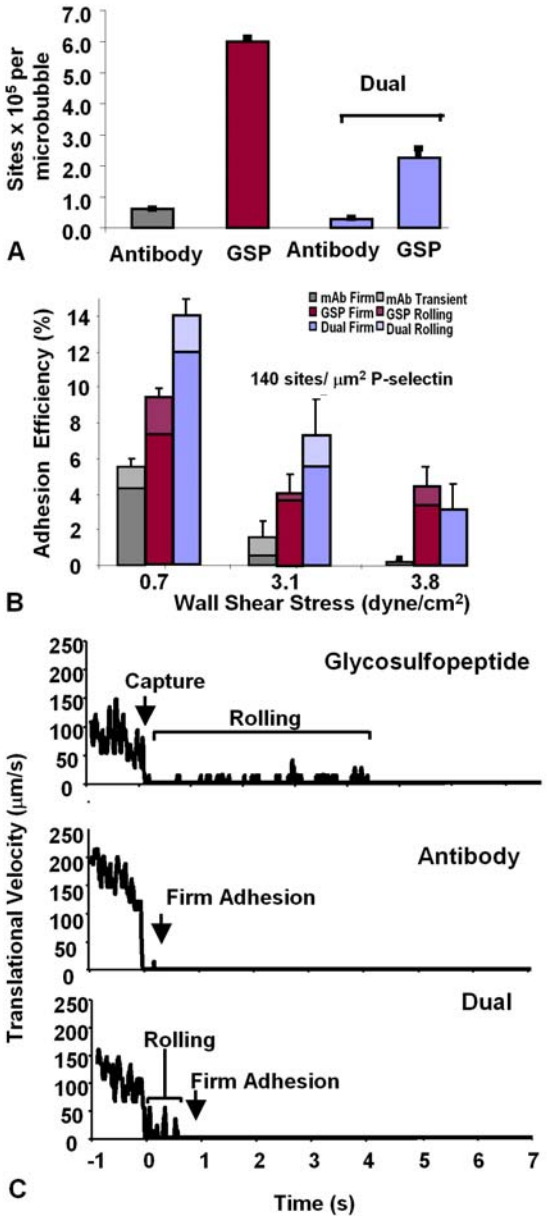

Figure 8. Adhesion of Dual Targeted Microbubbles to P-selectin *In vitro*. Lipid encapsulated microbubbles were targeted to P-selectin using an anti-P-selectin antibody, a selectin-binding glycosulfopeptide (GSP), or both the antibody and GSP (Dual). Ligand density on the microbubble surface was assessed using fluorescence spectroscopy (A), and microbubble adhesion efficiency was assessed in a flow chamber assay using automated particle tracking. Dual-targeting enabled enhanced firm adhesion relative to microbubbles bearing an antibody alone, especially at elevated wall shear stress. (B). On a low target density (7 sites/μm^2 P-selectin), microbubble rolling was observed for GSP-targeted, but not antibody-targeted, microbubbles. For dual-targeted microbubbles, rolling may serve to reduce the microbubble translational velocity at high flow rates, enabling antibody-mediated firm adhesion (C). Rychak *et al*, unpublished results.

efficiently than an anti-P-selectin antibody, and this enhancement increased with fluid shear stress. The adhesive stability of glycosulfopeptide-bearing microbubbles was shown to be modulated by target site density, with firm adhesion occurring only at elevated P-selectin densities. In a similar study, Klibanov and colleagues (120) examined the adhesion of microbubbles targeted to P-selectin with a polymeric form of the oligosaccharide sialyl Lewis^x (sLe^x). Polymeric sLe^x was shown to mediate enhanced microbubble adhesion relative to an anti-P-selectin antibody *in vitro*, and superior adhesion of sLe^x-bearing microbubbles was demonstrated in the mouse femoral artery and vein by intravital microscopy (120). Monomeric sLe^x has also been used as a ligand by Villanueva and colleagues to image ischemic memory in rat myocardium (81).

8.1.2. Dual- and multi-targeting

The varying adhesive abilities of antibodies and glycoconjugates to the selectins suggests a novel dual targeting strategy. Ligands, such as sLe^x and some glycoconjugates, exhibit a superior ability to bind selectins under rapid blood flow. However, on low target site densities, this adhesion is frequently of an unstable variety called rolling (121), in which the microbubble translates across the target surface at a sub-hydrodynamic velocity but does not achieve firm adhesion. In contrast, many antibodies that have been explored as targeting ligands exhibit primarily firm adhesion, although adhesion is diminished under moderate to high flow conditions when the microbubble is translating rapidly across the target surface (84, 108, 119, 120). In the dual targeting paradigm, a glycoconjugate may be utilized to enable high-efficiency capture of microbubbles to the target surface. Subsequent microbubble rolling may reduce the translational velocity of the microbubble sufficiently to enable antibody-mediated firm adhesion. This dual-targeted strategy has been explored *in vitro* and *in vivo*, and dual-targeted microbubbles bearing a selectin-binding glycosulfopeptide and an anti-P-selectin antibody exhibit enhanced adhesion to P-selectin under elevated flow and low target site density (Figure 8).

A similar strategy, commonly referred to as multi-targeting, has been investigated by Weller and colleagues (125). Multi-targeting entails utilizing two or more distinct ligands targeted to different molecular markers of the targeted disease. It has been hypothesized that synergistic adhesion of the multiple ligands can increase overall microbubble adhesion efficiency, and enable greater specificity. A similar strategy is found in the leukocyte adhesion cascade (121), where the presence of both a selectin and integrin ligand (such as ICAM-1) are necessary to mediate firm leukocyte arrest (122, 123). Multi-targeting of lipid encapsulated microbubbles using sLe^x and an anti-ICAM-1 antibody exhibited more robust adhesion *in vitro* than microbubbles targeted with either of the two ligand moieties alone (125). Recently, the multi-targeting strategy was used to image atherosclerotic plaque in the innominate artery and aortic arch in mice (89). In this study, microbubbles were targeted via polymeric sLe^x and an anti-VCAM-1 antibody, and exhibited appreciable

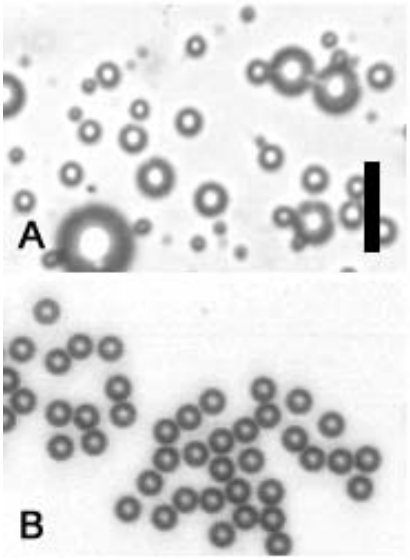

Figure 9. Microscopy images of polydisperse contrast agents made by mechanical agitation (A) and monodisperse contrast agents made with a microfluidic technique (B). Scale bar represents approximately 10 microns. Talu *et al.* and Hettiarachchi *et al.*, unpublished results.

accumulation to plaques found in large vessels with rapid blood flow.

8.2. Acoustic radiation force enhanced adhesion

An interesting method that has been shown to enhance the retention of targeted contrast agents is the use of acoustic radiation force to increase ligand-receptor interaction (106, 124, 126,). Radiation force is produced on objects in an acoustic field, and is orders of magnitude greater on highly compressible microbubbles than surrounding tissue or blood components (113, 127). Primary radiation force exerts a force on contrast agents in the vasculature away from the transducer. The result is that microbubbles moving through the ultrasound beam are pushed to the opposite vessel wall, and ligand-target interaction frequency is greatly increased. In an imaging setting, radiation force would be applied by the imaging transducer during the microbubble accumulation phase before imaging the desired area. This technique has the potential to increase targeted contrast agent retention over an order of magnitude, however, improvements are likely limited to large vessels where microbubble-endothelial interaction is small under normal conditions.

8.3. New contrast agent design

The contrast-enhanced ultrasound field has recently experienced an explosion of innovative formulations aimed at enhancing specificity, signal-to-noise, and expanding the use of ultrasound into therapeutic applications. Several strategies have been investigated, including modulation of the microbubble surface architecture, modification of the microbubble's mechanical properties, and the development of new techniques for microbubble synthesis. The use of microbubbles and other ultrasound contrast agents as vehicles for gene and drug delivery and other therapeutic applications is an ongoing topic of exploration, and has been reviewed extensively elsewhere (128-132).

8.3.1. Tiered polymer brush architecture

Kim and colleagues (133) demonstrated a tiered PEG surface architecture, in which the targeting ligand was immobilized to the distal tip of the longer PEG layer, while a shorter layer grafted to the surface provides microbubble stability. This arrangement was shown to result in enhanced microbubble adhesion in a model target:ligand setting, reportedly due to the enhanced mobility of the projected ligand. Borden and colleagues recently developed a novel variation on the tiered PEG surface architecture (134). In this scheme, the ligand bound to a short PEG layer and is shielded by a PEG "overbrush" layer, serving to shield the ligand from immune components. Acoustic radiation force, applied selectively at the target site, forces the particle against the target surface and causes the overbrush layer to part, thus exposing the ligand to the target molecules.

8.3.2. Increasing microbubble surface area

Manipulation of the mechanical properties of the microbubble shell has also been investigated as a mechanism of enhancing targeting efficiency. Slight pressurization of the microbubble dispersion can induce an outward migration of the encapsulated gas, leaving excess shell surface area. This excess surface area imparts an enhanced ability to deform, potentially enabling a greater number of ligand:target bonds to form. Microbubbles prepared using this method have been shown by intravital microscopy to undergo up to two-fold greater targeted retention than spherical, non-deformable microbubbles (135).

8.3.3. Optimization of microbubble size distribution

Current approaches of producing lipid-encapsulated microbubble contrast agents include sonication and mechanical agitation, methods which result in the production of microbubbles with a broad size distribution (Figure 9A). Since the resonant frequency of a microbubble is directly related to its size, and available ultrasound systems have limited frequency bandwidth, a clinical imaging system operating in an harmonic imaging mode may be optimized for only a small percentage of a polydisperse contrast agent population. For traditional contrast imaging, this is not a problem due to the large number of microbubbles injected into circulation, where many of the microbubbles are within the diameter range optimized for detection. However, for targeted imaging, where only a small number of agents are retained at the site of pathology, it may be beneficial to have all of the contrast agents optimized for detection by the imaging system for maximum sensitivity. Additionally, microbubbles of different diameter exhibit different retention characteristics *in vivo*, and similarly-sized microbubbles may have advantages in imaging studies where the biodistribution is of specific interest. Hettiarachchi *et al* and Talu *et al* (136, 137, 138) have recently demonstrated the manufacture of monodisperse lipid-shelled agents for contrast imaging (Figure 9B). These contrast agents with a reduced size variance can be specifically tailored to the bandwidth of the

imaging system, and have the potential to increase the detection sensitivity by several fold.

8.4. New detection techniques

Currently, there are no imaging modes available on clinical ultrasound systems designed specifically for molecular imaging with ultrasound. One of the major challenges of molecular imaging with ultrasound is that a very small percentage of the total injected dose of targeted contrast is retained at the diseased site. Thus, it is challenging in some applications to isolate the signal from molecularly targeted agents from that of freely-circulating agents. Many previous ultrasound molecular imaging studies utilized a dwell period of several minutes during which time contrast agents accumulate at the target site, followed by signal subtraction before and after a contrast clearing "destructive" pulse. During the dwell period the target-adherent agents may degrade, resulting in decreased signal intensity. Additionally, this method is cumbersome due to the requirement for offline-processing. Zhao *et al* has demonstrated *in vitro* that signal processing methods with take advantage of trends in the scattered echoes from bound contrast agents compared to freely circulating agents can be used to image molecularly targeted microbubbles in near real-time (139, 140). The ability to image targeted agents in this fashion without the need for destruction-subtraction processing will be important for molecular imaging to become a practical clinical technology.

8.4. Hardware improvements

Another limitation of current clinical imaging systems with regards to molecular imaging is transducer bandwidth. High-bandwidth imaging will have the potential to improve molecular imaging with ultrasound because it will be able to take full advantage of imaging the harmonic response from microbubbles. Of particular interest would be a system which can take full advantage of the broadband transient response from microbubbles, which is known to present a high contrast-to-tissue signal (54). One method for enhancing transducer bandwidth is by using multi-frequency arrays. Stephens *et al.,* working with Siemens Medical Systems, have recently demonstrated the capabilities of multi-row transducers, which have both a -6 dB bandwidth of 73% at 5.2 MHz and >50% at 1.5 MHz (141).

Additionally, recent improvements in the development of capacitive micromachined ultrasonic transducers (CMUTs) have demonstrated that these transducers can be made with broad frequency bandwidth (130%) and high transduction efficiency (142). Finally, high-frequency ultrasound biomicroscopy, operating at center frequencies of 20-60 MHz, is emerging as a preclinical imaging modality for small animal research. The increased imaging frequency allows greater spatial resolution (143), and molecular imaging applications are currently being investigated.

9. CONCLUSION

The technology for molecular imaging with ultrasound contrast agents was first demonstrated *in vitro* over ten years ago. Since then, ultrasound molecular imaging has progressed substantially, and there are currently several different strategies available for pre-clinical molecular imaging of angiogenesis, inflammation, thrombosis, and other applications. To date, molecular imaging with ultrasound has yet to be used in clinical applications. However, upcoming advances such as improvements in adhesion mechanisms, contrast agent properties, and imaging system hardware and software promise to increase the sensitivity and convenience of molecular imaging several fold, elevating this exciting new modality to a clinical technique with the potential for assessing disease earlier and more sensitively than currently available methods permit.

10. ACKNOWLEDGEMENTS

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