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Stimulus-Responsive Ultrasound Contrast Agents for Clinical Imaging: Motivations, Demonstrations, and Future Directions

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Abstract

Microbubble ultrasound contrast agents allow imaging of the vasculature with excellent resolution and signal-to-noise ratios. Contrast in microbubbles derives from their interaction with an ultrasound wave to generate signal at harmonic frequencies of the stimulating pulse; subtracting the elastic echo caused by the surrounding tissue can enhance the specificity of these harmonic signals significantly. The nonlinear acoustic emission is caused by pressure-driven microbubble size fluctuations, which in both theoretical descriptions and empirical measurements was found to depend on the mechanical properties of the shell that encapsulates the microbubble as well as stabilizes it against the surrounding aqueous environment. Thus biochemically-induced switching between a rigid “off” state and a flexible “on” state provides a mechanism for sensing chemical markers for disease. In our research, we coupled DNA oligonucleotides to a stabilizing lipid monolayer to modulate stiffness of the shell and thereby induce stimulus-responsive behavior. In initial proof-of-principle studies, it was found that signal modulation came primarily from DNA crosslinks preventing the microbubble size oscillations rather than merely damping the signal. Next, these microbubbles were redesigned to include an aptamer sequence in the crosslinking strand, which not only allowed the sensing of the clotting enzyme thrombin but also provided a general strategy for sensing other soluble biomarkers in the bloodstream. Finally, the thrombin-sensitive microbubbles were validated in a rabbit model, presenting the first example of an ultrasound contrast agent that could differentiate between active and inactive clots for the diagnosis of Deep Venous Thrombosis.

Introduction

Gas-filled 1-3 μm microbubbles surrounded by a thin elastic shell -- typically a lipid monolayer -- are highly effective agents for contrast-enhanced ultrasound imaging. Although the large difference in acoustic impedance of gas compared with the surrounding aqueous medium enhances acoustic backscatter, the dominant enhancement derives from the fact that these elastic gas-filled bodies oscillate in an ultrasound field to actually become transmitters of acoustic waves even at very low transmit power. At resonance and optimal

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ultrasound pressure, microbubbles oscillate nonlinearly creating harmonic frequencies that can be detected selectively by creative transmit pulses and filtering of the received signal, allowing ultrasound systems to generate microbubble-specific images that may be displayed side-by-side or overlaid with a standard B-mode image for anatomic orientation. As a result, even a single microbubble can be detected in tissues, allowing the use of relatively small numbers (10^8) of microbubbles as a full diagnostic dose despite the expectation of dilution within the bloodstream. This extreme sensitivity has allowed the imaging of the heart and blood vessels, and while ultrasound cannot resolve individual capillaries themselves, filling of capillaries in a given area produces diffuse tissue enhancement of perfused tissues in real-time. These powerful properties of ultrasound contrast have promoted the success of microbubbles in the clinic worldwide. The complete filling of the vascular space has been used to better delineate the endocardial border in echocardiography and image atherosclerotic plaque to assess stenosis and ulceration as well as their internal vascular channels.¹⁻³ Similarly, tumor enhancement and vessels perfusing tumors can be characterized as well.⁴⁻⁶

The extreme sensitivity to microbubbles has prompted investigators to functionalize the lipid surface to target receptors expressed on the vascular surface of endothelial cells. Their success has made ultrasound a viable option for molecular imaging. In recent years, advances have been made in expanding targeting mechanisms for microbubbles. This can both bias the accumulation of microbubbles to diseased areas and increase microbubble survival by not only preventing the adherent bubbles from recirculating through the heart and lungs – which tends to cause their dissolution – but also because adherent microbubbles are more resilient to ultrasound destruction. This strategy has been described and reviewed elsewhere,⁶ but some highlights include the use of RGD-containing peptides to target integrins associated with angiogenesis, as well as advances in lipid monolayer functionalization to impart useful targeting groups without disrupting the overall self-assembly properties of the lipid monolayer for stabilizing microbubbles. Strategies to incorporate targeting groups have included ligand-biotin association with streptavidin on the microbubble surface,⁷ or conjugation of antibodies to the microbubble shell.⁸ As a cheaper alternative, DNA strands that specifically bind to certain proteins, blood cells, or features within the body have also shown great promise.⁹⁻¹²

While this approach has been successful in targeting membrane-bound receptors, these imaging agents lack the ability to sense soluble biomarkers such as enzymes. Soluble biomarkers of interest include the Vascular Endothelial Growth Factor (VEGF) family, and in particular VEGF-A, which is involved with the growth of new blood vessels during tumor angiogenesis and is thus secreted by cancer cells in malignant tumors. A critical requirement for microbubble detection of biomarkers is that these molecules must enter or be present within the blood stream in sufficient quantity to effectively interact with microbubbles. Since the highest concentration of biomarkers is expected to be where they are produced or activated, interaction with microbubbles will allow detection and localization of biomarker production. An ideal application for enzyme detection is the opportunity to recognize active thrombosis as in acute Deep Venous Thrombosis (DVT), which would allow its distinction from older, organized, less dangerous clots by sensing the elevated levels of enzymes associated with the clotting cascade. Some “smart” fluorescent dyes and MR agents that can

reach the interstitial space are able to change their contrast activity in the presence of soluble biomarkers such as Matrix Metalloproteinases, but fluorescent dyes are limited by photon penetration depth and scattering in living tissue, while MR agents typically require higher concentration of the reporter and show modest ON-OFF signal ratios.^{13, 14} Many of these strategies sense for endogenous enzymes that cleave specific peptide sequences by utilizing the catalytic nature of the enzyme to enhance signal. They are therefore specific for that biomarker enzyme activity and different design strategy will be needed for the detection of these specific enzymes.

In this review, we present our approach for designing stimulus-responsive ultrasound contrast agents based on manipulation of the mechanical properties of the encapsulating shell. This approach derives from the natural response of microbubbles to an ultrasound wave. As stated above, a compressible microbubble responds to an ultrasound wave near the bubble's resonance frequency by expanding and contracting in response to the fluctuating local pressure. The difference in resistance between the pushing and pulling against the surrounding aqueous medium causes the generation of harmonic ultrasound waves that are highly specific to microbubbles. The response of microbubble contrast agents to ultrasound as a function of their shell mechanical properties has been studied for a number of different microbubble formulations. In accordance with both theory and empirical observation, as the bulk modulus of the shell increases, the acoustic cross-section of the microbubble decreases dramatically, presumably due to the increased resistance to expansion and contraction added by the shell. We aim to take advantage of the effect of shell stiffness on harmonic signal generation to produce an ON-OFF signal when microbubbles encounter a molecule of interest, in our case thrombin.

The Effect on Encapsulating Shell on Microbubble Behavior: a Pathway Towards Stimulus-Responsive Behavior

With the advent of imaging protocols such as phase inversion and cadence contrast pulse sequence, the primary mode of ultrasound contrast derives from nonlinear oscillation of microbubbles in response to the incident pressure wave. Bubble dynamics in a pressure wave have been extensively studied for the better part of a century,¹⁵⁻¹⁸ and more extensive reviews of this work have been reported elsewhere.¹⁹⁻²² Briefly, when the ultrasound pulse exerts a lower external pressure on the gas, a gas bubble will naturally expand, and when the ultrasound pulse exerts a higher external pressure, the bubble will contract. Since the times required for the gas sphere to expand against a lower external pressure and compress against a higher external pressure are different, non-linear oscillations are produced, which lead to generation of harmonic frequencies. Thus the total backscattered power can be estimated as a function of resonance frequency and the power of the incident wave.

While these models are fairly accurate for microbubbles with a thin encapsulating shell such as a phospholipid monolayer, the addition of a stiffer encapsulating shell to the microbubble changes the microbubble's behavior considerably. In initial treatments developed by de Jong, the encapsulating shell was considered multiple layers of an elastic material. It was theorized and confirmed by experimentation that the addition of encapsulation results in an increase in resonance frequency and a decrease in scattering. This could be represented

within the Rayleigh-Plesset model through the introduction of parameters for shell friction and elasticity, adding a new component to the damping coefficient.^{23, 24} Church would provide a further derivation of encapsulated bubble motion, showing the dependence of scattering cross-section on the shell viscosity and elastic modulus, among other factors.²⁵ This model in turn was further refined by Hoff,²⁶ who showed that the magnitude and frequency of the microbubble-generated signal could be changed dramatically with the addition of a stiff polymer coat. De Jong would go on to compare bubbles with different shell properties to further confirm the behavior of encapsulated bubbles.²⁷ In the comparison of a thick-shelled bubble (Optison), a flexible shell (Sonazoid), and a thin, flexible shell (Definity), as the shell thickness decreased, the scattered signal increased substantially, and non-linearities within the scattered signal began to appear. Other studies on the effect of shell elasticity, viscosity and thickness have reported similar findings.²⁸ The resulting equation of motion demonstrates that a bubble within a sound field can be described as a second order, non-linear differential equation as a function of the radius over time (Equations 1-4).^{26, 27}

$$\sigma_s = 4\pi R_e^2 \frac{\Omega^4}{(\Omega^2 - 1)^2 + \Omega^2 \delta^2}$$

$$\Omega = \frac{\omega}{\omega_0}$$

$$\omega_0^2 = \frac{1}{\rho_L R_e^2} \left(3\kappa p_0 + 12G_s \frac{d_{se}}{R_e} \right)$$

$$\delta = \frac{4\mu_L + 12\mu_s \frac{d_{se}}{R_e}}{\omega_0 \rho_L R_e^2}$$

Equations 1-4: σ_s = scattering coefficient of a shelled microbubble, R_e = equilibrium radius of the microbubble, ω = angular frequency, ω_0 = angular resonance frequency, ρ_L = density of the surrounding liquid, κ = polytropic gas constant, p_0 = external pressure from the liquid, G_s = Shear modulus of the shell, d_{se} = shell thickness, μ_L = viscosity of the surrounding liquid, μ_s = viscosity of the shell material.

Control over Microbubble Ultrasound Contrast through Manipulation of the Mechanical Properties of the Encapsulating Shell

To create contrast agents that could take advantage of the effect of shell properties on the modulation of ultrasound harmonic signal, we sought to utilize reversible shell crosslinks that could be formed or broken without affecting the microbubbles themselves (Figure 1).

DNA oligonucleotides were utilized for this purpose because the non-covalent hybridization between complementary oligonucleotides can be switched at ambient conditions, while the relative affinity between oligonucleotides and the target molecule of interest can be tuned easily through modification of the different sequences. In this design, the DNA is attached to polymer chains, which are in turn conjugated to phospholipids. This anchors the DNA in the encapsulating shell through the lipid-lipid interactions. In our proof-of-concept design, conjugates of amine-terminated phospholipid, poly(acrylic acid), and an amine-terminated sequence for a fifteen-mer of adenine, or A₁₅, were conjugated using sequential carbodiimide couplings.²⁹ After melting the conjugates with a mixture of commercial phospholipids, the mixtures were probe sonicated under perfluorobutane gas to produce bubbles of 1-5 μm in diameter.

The first testable hypothesis was that the addition of DNA crosslinks would create a measurable change in the microbubbles' response to ultrasound. Hybridization of the oligonucleotides by their complements creates a network of crosslinks around the entire bubble shell, stiffening the shell. This is made possible by the conjugation of at least 2 DNA groups per polymer chain, which in turn is made possible through the use of poly(acrylic acid) as the platform for conjugation. Complementary DNA strands (T₃₀) were added to the A₁₅ microbubbles as confirmed by confocal microscopy. The response of the microbubbles to ultrasound was measured using a flow setup that could measure the time-dependent light scattering from the bubbles during their size oscillation under ultrasound. Briefly, the microbubbles were flowed into a water tank, where they were interrogated by both a continuous wave laser and ultrasound pulse simultaneously.³⁰ The time-resolved light scattering profile was then recorded via a photodetector, and numerous single-bubble data points were acquired at varying incident ultrasound pressures (intensities).

Two trends emerged from this data. First, the overall oscillation depth of the microbubbles decreased significantly with the addition of crosslinking strands.²⁹ More strikingly, the number of bubbles that underwent size oscillations in a given sample changed dramatically after addition of the crosslinking strand. Since nonlinear bubble oscillation was dependent on the softness of the shell, stiffening the shell resulted in not only dampening the size oscillations but in many cases removing them altogether. To measure this effect, we sampled the microbubble population by counting the number of oscillating light scattering events at a given ultrasound pressure and normalized this to the number of counts obtained at high ultrasound pressures.²⁹ The minimum ultrasound pressure required to initiate size oscillations ("onset pressure") was found to vary linearly with inverse radius. By controlling for microbubble radius it was found that approximately an additional 300 kPa of pressure was required to activate bubbles that had been crosslinked with DNA oligonucleotides (Figure 2). Thus optimal imaging intensities should fall between 100-300 kPa,³¹ which is consistent with protocols for the Siemens-developed contrast pulse sequences (CPS), or Cadence™ mode, which requires similar ultrasound power to obtain an optimal image.^{32, 33} Interestingly, it would seem that factors affecting an isotropic expansion against a resistive medium would vary with R^{-2} (inverse area) dependence rather than R^{-1} . However, our findings agreed with studies from de Jong and coworkers, who found that small microbubbles require a minimum ultrasound intensity to initiate oscillation, and this threshold has an inverse relationship with microbubble radius.^{34, 35} The effect of the

crosslinking could then be reversed through addition of A₃₀, which competes for the crosslinked T₃₀ to soften the encapsulating shell. In CPS images in a phantom at 257 kPa, this activation resulted in a 20 dB, or 100-fold, increase in signal. Thus this method of changing shell properties through addition of DNA was found to be quite successful for modulating the microbubbles' ultrasound response.

Design of Thrombin-Sensitive Microbubbles using Competitive Aptamer Hybridization Schemes

In adapting DNA-crosslinkable microbubbles to sense clinically-relevant biomarkers, a natural bridge is the utilization of aptamers, or ssDNA or RNA sequences that preferentially bind to non-nucleic acid targets.^{36, 37} While often compared in functionality to antibodies, aptamers retain several advantages over their protein-based counterparts that make them ideal for molecular imaging. Aptamers are active at a wide range of pHs and temperatures, and they are far easier and less expensive to produce than antibodies. They also exhibit greater specificity, less immunogenicity, and can be loaded at higher densities onto particle surfaces owing to their lower molecular weights.³⁸ Because they are artificially generated based on affinity to their selected target, aptamers with specific dissociation constants (K_d) can be designed, allowing determination of a “threshold” concentration of activation.³⁹

Aptamer sequences are carefully chosen by systematic evolution of ligands by exponential enrichment (SELEX).^{36, 37} In this method, an extremely large nucleotide library ($\sim 10^{14}$ sequences) of fixed length sequences is generated flanked by polymerase chain reaction (PCR) primers at the 5' and 3' ends. The sequence library is then exposed to the target protein or biomolecule and unbound sequences are washed away. Bound sequences then undergo elution and PCR amplification, and the process is repeated under increasingly rigorous elution conditions to isolate the most strongly binding sequences. The sequence can be tuned through aptamer-target interactions to bind to a number of different soluble targets, including thrombin,⁴⁰ VEGF,⁴¹ and Prostate-Specific Membrane Antigen,⁴² and many others. In biomedical applications, aptamers have found the greatest utility as either surface capture or detection agents, or as targeting groups for imaging contrast or drug delivery.⁴³ However, the ability to easily incorporate competitive binding into aptamer design has allowed their implementation as molecular switches, in which the binding of an aptamer to its target causes its dehybridization from a complement strand and a change in detectable properties.⁴⁴

We chose to adapt the DNA-crosslinkable microbubbles to sense for thrombin, an enzyme in the clotting cascade that is elevated during the clotting process.^{45, 46} Bock, *et al* isolated a thrombin aptamer with the sequence GGTTGGTGTGGTTGG, boasting an affinity on the order of 25 nM.⁴⁰ This sequence was incorporated onto either end of the crosslinking strand, using partial hybridization to leave a “dangling end” with a partial aptamer sequence (Fig. 3).⁴⁷ In the presence of the target molecule, this dangling end acts much like a zipper, first interacting with the target biomolecule and then de-hybridizing from the remainder of the strand. After formulating microbubbles with these DNA sequences using procedures similar to those described above, the thrombin-sensitive microbubbles were introduced to freshly-clotting rabbit blood. Using CPS imaging, the thrombin-sensitive bubbles gave signal

similar to a positive control (PEG-lipid bubbles, similar to Definity®), while a negative control showed little to no signal activation (Figure 3).

To study the specific ultrasound response of microbubbles, bubbles were subjected to a series of benchtop ultrasound studies through interrogation of bubble suspensions at 2.25 MHz and 290 kPa, which are conditions similar to those used for CPS imaging. The scattered signal was recorded on a 5.0 MHz-centered transducer to specifically track generation of second harmonic signal. It was found that while microbubbles without addition of thrombin showed very little signal in this region, increasing amounts of thrombin showed a corresponding increase in signal. A frequency spectrum of this response showed that at high amounts of thrombin addition a peak appeared at 4.5 MHz, which corresponds to the second harmonic of the 2.25 MHz fundamental pulse. At more moderate but clinically relevant loadings of thrombin (25-100 nM), the ultrasound response appeared more broadband, with little peak sharpness at 4.5 MHz (Figure 3). However, integration of these same spectra showed that the response had more of an “S-curve” shape as one would expect for a dissociation curve. This indicates that at moderate loadings, the shell was most likely becoming uncrosslinked only in specific sections rather than uniformly. However, studies of bubble dynamics in non-uniform shells has shown that under sufficient negative pressure the bubble will force its way out of the shell through a weak point, then undergo inertial cavitation and collapse, providing a broadband signal. This property allowed the thrombin-sensitive bubbles to outperform their expected sensitivity, and provide almost full contrast at elevated levels.⁴⁸ This strategy may lead to the opportunity to create new types of encapsulating shells that enhance signal outside the fundamental frequency based on deliberately inhomogeneous structures. For example, studies by Borden and Ferrara have definitively shown that domains in the lipid monolayer can be obtained through the use of longer-chain (and higher T_m) lipids that resist mixing and can be frozen in place; such strategies may be used for creating deliberately non-uniform shells for increased broadband signal generation.⁴⁹

Clinical Applications: Detection of Water Soluble Markers

Because the relatively large size of microbubbles (μm), soluble markers of interest must be within the vascular space or leak into the vascular space with sufficient concentration to be detected. One of the ideal applications that would have a significant clinical impact is the detection of acute thrombosis. Deep venous thrombosis (DVT) occurs in patients when a blood clot or thrombus forms within a deep vein, most often within the lower extremity. First described in 1856 by the physician Richard Virchow, the risk factors for thrombosis can be quantified by “Virchow's Triad”: alterations in blood flow, endothelial injuries, and hypercoagulability.⁵⁰ The early and accurate diagnosis of acute DVT is critical, since untreated DVT can lead to embolization of the clot to the lung causing ventilation-perfusion mismatch, arterial blood desaturation, and death. DVT that is aggressively treated when diagnosed still accounts for 60,000 deaths in the United States each year.⁵¹

Thrombosis is the body's natural response to endothelial damage and is designed to slow and prevent blood loss, preserving homeostasis. The thrombus is the end result of a cascade of reactions that ultimately lead to the polymerization of fibrin that fills the vascular lumen to

arrest blood flow. Fibrin is formed from fibrinogen through the action of thrombin, a protein enzyme that is converted from its inert form prothrombin through a series of tissue factor/cofactor interactions.^{45, 46} Thrombin has long been seen as an excellent target for not only the early detection of thrombosis but also to arrest thrombus formation, as it is one of the most powerful platelet activators. The production of thrombin during an acute clotting event has been extensively studied.^{52, 53} Under normal conditions, the level of activated thrombin contained within the blood vessels is minimal, as it is primarily contained within the zymogen (non-active) form prothrombin and its production is inhibited through the presence of antithrombin III. However, patients with prior DVT and those in a hypercoagulable state stemming from extensive surgery, trauma, pregnancy and delivery, certain cancers, obesity etc. may either have higher levels of factors that initiate the clotting cascade, or lack antithrombin III increasing their risk of developing DVT.

The gold standard for diagnosis of DVT is venous angiography,⁵⁴ in which veins are filled with x-ray contrast media and then imaged. In this way, filling defects that represent the clot can be detected. However, it is invasive and because it cannot be performed at the bedside, it has been essentially replaced by compression real time ultrasound imaging.⁵⁵ Since acute clots are a homogeneous gelatinous substance, they produce no backscatter and are invisible on standard sonography. The ultrasound study evaluates venous patency with Doppler imaging that is somewhat inconclusive since thrombosis may not be totally occlusive. Because an acute clot is solid, it prevents the vein from collapsing when compressed with the ultrasound transducer under real-time observation. As clots age and organize, they become histologically heterogeneous creating backscatter to become visible on ultrasound imaging. Since patients with prior DVT are at risk for recurrence, the presence of an old clot, cannot exclude the presence of a new DVT, since the acute component is invisible and the old clot prevents the vein from completely collapsing. As such, patient with suspected DVT that have detectable old clots, are typically treated empirically. Unfortunately, because anti-coagulation carries significant risk of bleeding that by itself can lead to severe complications and even death, recognizing patients with only old clots would be of great benefit. Unfortunately, there are currently no imaging techniques that can accurately distinguish chronic DVT from superimposed acute DVT. We used standard harmonically active microbubbles that completely fill the vascular space to detect clots as filling defects;⁵⁶ however, although not tested we suspect that this approach may also not conclusively recognize acute from chronic thrombosis. Others have imaged clots by either targeting microbubbles to the activated platelets,^{57, 58} or the fibrin itself; however, the ability to conclusively recognize acute from chronic thrombosis is also not known. In addition, targeting clot with already harmonically active microbubbles requires time to recognize trapped from circulating microbubbles. Since old clots do not have active thrombosis, thrombin levels within the clot are at baseline levels to allow our proposed technique an opportunity to aid in the management of these difficult patients.

Based on our promising in vitro validation of thrombin-sensitive microbubbles, it seemed straightforward to validate our microbubble formulations to sense for acute DVT in vivo. However, the addition of significant amounts of synthetic polymers to the microbubble surface significantly reduced microbubbles' blood half-life in vivo, likely through the binding of plasma molecules to the bubble surface disrupting the lipid monolayer packing.

Indeed, significant efforts have been devoted to stabilizing microbubbles against these natural bubble destruction mechanisms. Most commonly, lipid monolayers are doped with lipid conjugates of poly(ethylene glycol) (PEG), a common component for passivation of particles for in vivo use. In attempting to adapt the stimulus-responsive mechanisms described earlier to in vivo studies, we found that simply doping in 1 mole percent of DSPE-PEG was sufficient to greatly increase their survival both in static buffers and in whole blood, with 95% of ultrasound signal retained after 5 min in clotting whole blood (Figure 4).⁵⁹ Using a flow phantom in which a blood clot was induced through mixing of calcium and citrate-stabilized whole blood, microbubble stabilization with PEG not only allowed microbubbles to survive the circulation but the active clot caused microbubble activation at the clotting site.

As stated above, working with microbubbles for in vivo biosensing is challenging because of their inability to leave the bloodstream and their short blood half-life. Fortunately with acute DVT, thrombus formation that is life threatening occurs in large veins providing the microbubbles access to thrombin. Further because thrombin is present in sufficient concentration, its rate of activation of our aptamer cross-linked system is fast, and blood flow is slow enough, activated microbubbles concentrated at the thrombus interface rather than rapidly washed away downstream. Using a rabbit model of acute DVT, harmonic signal increased at 25s and reached its maximum at 40s after microbubble administration into the ear vein (Figure 5). This was similar to activation rates measured in the flow phantom shown in Figure 4. Activation within 25-40s after intravenous administration was possible because thrombin dislodged a crosslinking DNA aptamer rather than hydrolyzed a thrombin-specific peptide sequence that typically requires 10-20 minutes.¹³

Future Directions for Clinical Imaging

We showed above that the ability to manipulate microbubble shell stiffness induces a marked increase in harmonic signal that is detectable by clinical ultrasound systems capable of contrast specific imaging. The requirement that the molecule to be detected in vivo must come in direct contact with the microbubble surface limits the potential applications of this approach. We have not tested whether stationary cell surface receptors could extract a sufficient number of cross-linking aptamers to destabilize the shell easing its activation when exposed to ultrasound. Should that be possible as suggested by the in vitro data, it would provide an advantage over targeting already active microbubbles since this approach provides low or no background signal allowing immediate recognition of the region of interest. Should targeting fixed receptors be inefficient, a cascade-type interaction as suggested by Bhatia and coworkers could be employed.⁶⁰ In their study thrombin was generated using RGD targeted nanostructures that provided the tissue factor necessary to initiate the thrombosis cascade. Thus the tissue factor that promotes the production of thrombin can then allow microbubble activation.

Although DVT is an ideal application since the amount of soluble thrombin within a clot can efficiently extract the cross-linking aptamers to activate microbubbles within 10's of seconds, there are potentially other soluble molecules that could be targeted. Many molecules excreted by non-endothelial cells at high local extracellular concentration can

leak across capillaries to reach sufficient local blood concentration to activate microbubbles. This could also be aided by the slower blood flow in capillary networks and the extreme sensitivity of contrast specific imaging to microbubbles, which requires only a few microbubbles to be activated for detection. One such example is Vascular Endothelial Growth Factor (VEGF), or more specifically VEGF-A, which is a soluble protein that is secreted by tumor cells to help promote tumor angiogenesis and can be detected in peripheral blood.⁶¹⁻⁶³ Elevated VEGF levels in the tumor microenvironment are associated with poor patient prognosis, but these levels must be found by analyzing biopsied or resected tumor tissues in vitro by using assays such as ELISA.^{61, 63} To improve the accessibility of a VEGF-based screening process, efforts have been made to relate VEGF levels measured in peripheral blood to level of tumor burden for detection and progression. Indeed, VEGF is known to be elevated within nascent blood vessels both through diffusion from the tumor cells and the presence of VEGF receptors.⁶⁴⁻⁶⁹ While median levels in circulation are known to be elevated in breast cancer patients, the large variability of systemic VEGF levels within a given patient reduces its screening power,⁷⁰⁻⁷⁴ as VEGF concentration may depend on other factors such as platelet count, vascular injury, age, menstrual phase, or menopause.⁷⁵⁻⁸¹ Thus the development of a tool that could reliably and inexpensively quantify overexpressed VEGF in the growing tumor vasculature would help reduce the number of unnecessary biopsies for breast cancer patients and the physical, financial, and psychological costs associated with biopsy.⁸² One challenge with such an approach is the possibility that microbubbles may activate in the systemic circulation rather than only the abnormal region. While activation can be controlled by design, formulation optimization would be needed to assess the added benefit of our approach to the targeting of VEGF by already active microbubbles.

Conclusions

We have presented here a novel approach for the ultrasound detection of soluble molecular targets using microbubbles, along with in vitro and in vivo data describing the use of this approach to detect thrombin as an example of an intravascular soluble biomarker. Since the presence of thrombin implies active clot formation, our approach not only aids in the detection of acute thrombosis, but also allows the distinction of acute from chronic thrombi to identify patients in need of treatment. We have also showed that by using aptamers specific to a molecular target of interest to crosslink the microbubble shell and deactivate its harmonic generation, the microbubbles will activate rapidly when the molecular target binds and extracts the aptamer. This also allows the detection of any biomarker, whether or not it is an enzyme, or regardless of enzyme activity. Since the administered microbubbles provide very limited or no signal prior to activation, our proposed approach provides a high target to background ratio immediately after administration. The in vitro data also suggest that signal generation may be related to local biomarker concentration, providing an opportunity for quantification in vivo.

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References

1. Wei K, Jayaweera AR, Firoozan S, Linka A, Skyba DM, Kaul S. Quantification of myocardial blood flow with ultrasound-induced destruction of microbubbles administered as a constant venous infusion. *Circulation*. 1998; 97:473–83. [PubMed: 9490243]
2. Clevert DA, Stickel M, Johnson T, Glaser C, Clevert DA, Steitz HO, Kopp R, Jauch KW, Reiser M. Imaging of aortic abnormalities with contrast-enhanced ultrasound. A pictorial comparison with CT. *Eur Radiol*. 2007; 17:2991–3000. [PubMed: 17219146]
3. Kono Y, Pinnell SP, Sirlin CB, Sparks SR, Georgy B, Wong W, Mattrey RF. Carotid Arteries: Contrast-enhanced US Angiography--Preliminary Clinical Experience. *Radiol*. 2004; 230:561–568.
4. Wilson SR, Burns PN. Liver mass evaluation with ultrasound: the impact of microbubble contrast agents and pulse inversion imaging. *Semin Liver Dis*. 2001; 21:147–159. [PubMed: 11436569]
5. Chomas JE, Pollard RE, Sadlowski AR, Griffey SM, Wisner ER, Ferrara KW. Contrast-enhanced US of microcirculation of superficially implanted tumors in rats. *Radiology*. 2003; 229:439–446. [PubMed: 14526091]
6. Unnikrishnan S, Klibanov AL. Microbubbles as ultrasound contrast agents for molecular imaging: preparation and application. *AJR Am J Roentgenol*. 2012; 199:292–299. [PubMed: 22826389]
7. Klibanov AL, Rychak JJ, Yang WC, Alikhani S, Li B, Acton S, Lindner JR, Ley K, Kaul S. Targeted ultrasound contrast agent for molecular imaging of inflammation in high-shear flow. *Contrast Media Mol Imaging*. 2006; 1:259–266. [PubMed: 17191766]
8. Villanueva FS, Jankowski RJ, Klibanov S, Pina ML, Alber SM, Watkins SC, Brandenburger GH, Wagner WR. Microbubbles targeted to intercellular adhesion molecule-1 bind to activated coronary artery endothelial cells. *Circulation*. 1998; 98:1–5. [PubMed: 9665051]
9. Wang CH, Kang ST, Lee YH, Luo YL, Huang YF, Yeh CK. Aptamer-conjugated and drug-loaded acoustic droplets for ultrasound theranosis. *Biomaterials*. 2012; 33:1939–1947. [PubMed: 22142768]
10. Maul TM, Dudgeon DD, Beste MT, Hammer DA, Lazo JS, Villanueva FS, Wagner WR. Optimization of ultrasound contrast agents with computational models to improve selection of ligands and binding strength. *Biotechnol Bioeng*. 2010; 107:854–864. [PubMed: 20665479]
11. Simberg D, Mattrey R. Targeting of perfluorocarbon microbubbles to selective populations of circulating blood cells. *J Drug Target*. 2009; 17:392–398. [PubMed: 19505207]
12. Shi G, Cui W, Benchimol M, Liu YT, Mattrey RF, Mukthavaram R, Kesari S, Esener SC, Simberg D. Isolation of rare tumor cells from blood cells with buoyant immuno-microbubbles. *PLoS One*. 2013; 8:e58017. [PubMed: 23516425]
13. Weissleder R, Tung CH, Mahmood U, Bogdanov A. In vivo imaging of tumors with protease-activated near-infrared fluorescent probes. *Nat Biotechnol*. 1999; 17:375–378. [PubMed: 10207887]
14. Olson ES, Jiang T, Aguilera TA, Nguyen QT, Ellies LG, Scadeng M, Tsien RY. Activatable cell penetrating peptides linked to nanoparticles as dual probes for in vivo fluorescence and MR imaging of proteases. *Proc Natl Acad Sci U S A*. 2010; 107:4311–4316. [PubMed: 20160077]
15. Rayleigh. On the pressure developed in a liquid during the collapse of a spherical cavity. *Phil Mag*. 1917; 34:94–98.
16. Minnaert M. On musical air bubbles and the sounds of running water. *Phil Mag*. 1933; 16:235–248.
17. Vokurka K. On Rayleigh Model of a Freely Oscillating Bubble .2. Results. *Czechoslovak Journal of Physics*. 1985; 35:110–120.
18. Medwin H. Counting Bubbles Acoustically - Review. *Ultrasonics*. 1977; 15:7–13.
19. Sboros V. Response of contrast agents to ultrasound. *Adv Drug Deliv Rev*. 2008; 60:1117–1136. [PubMed: 18486270]
20. Schutt EG, Klein DH, Mattrey RM, Riess JG. Injectable microbubbles as contrast agents for diagnostic ultrasound imaging: the key role of perfluorochemicals. *Angew Chem Int Ed Engl*. 2003; 42:3218–3235. [PubMed: 12876730]

21. Raisinghani A, DeMaria AN. Physical principles of microbubble ultrasound contrast agents. *Am J Cardiol.* 2002; 90:3J–7J.
22. May DJ, Allen JS, Ferrara KW. Dynamics and fragmentation of thick-shelled microbubbles. *IEEE Trans Ultrason Ferroelectr Freq Control.* 2002; 49:1400–1410. [PubMed: 12403141]
23. Dejong N, Hoff L, Skotland T, Bom N. Absorption and Scatter of Encapsulated Gas Filled Microspheres - Theoretical Considerations and Some Measurements. *Ultrasonics.* 1992; 30:95–103. [PubMed: 1557838]
24. Dejong N, Hoff L. Ultrasound Scattering Properties of Albunex Microspheres. *Ultrasonics.* 1993; 31:175–181. [PubMed: 8484195]
25. Church CC. The Effects of an Elastic Solid-Surface Layer on the Radial Pulsations of Gas-Bubbles. *J Acoust Soc Am.* 1995; 97:1510–1521.
26. Hoff L, Sontum PC, Hovem JM. Oscillations of polymeric microbubbles: effect of the encapsulating shell. *J Acoust Soc Am.* 2000; 107:2272–2280. [PubMed: 10790053]
27. Postema M, Vanwamel A, Lancee CT, Dejong N. Ultrasound-induced encapsulated microbubble phenomena. *Ultrasound Med Biol.* 2004; 30:827–840. [PubMed: 15219962]
28. Khismatullin DB, Nadim A. Radial oscillations of encapsulated microbubbles in viscoelastic liquids. *Phys Fluids.* 2002; 14:3534–3557.
29. Nakatsuka MA, Hsu MJ, Esener SC, Cha JN, Goodwin AP. DNA-Coated Microbubbles with Tunable Ultrasound Contrast Activity. *Advanced Materials.* 2011; 23:4908–4912. [PubMed: 21956383]
30. Hsu MJ, Eghtedari M, Goodwin AP, Hall DJ, Mattrey RF, Esener SC. Characterization of Individual Ultrasound Microbubble Dynamics with a Novel Light Scattering System. *J Biomed Opt.* 2011; 16:067002. [PubMed: 21721823]
31. Hilgenfeldt S, Lohse D, Zomack M. Response of bubbles to diagnostic ultrasound: a unifying theoretical approach. *Eur Phys J B.* 1998; 4:247–255.
32. Streeter JE, Gessner R, Miles I, Dayton PA. Improving sensitivity in ultrasound molecular imaging by tailoring contrast agent size distribution: in vivo studies. *Mol Imaging.* 2010; 9:87–95. [PubMed: 20236606]
33. Stieger SM, Dayton PA, Borden MA, Caskey CF, Griffey SM, Wisner ER, Ferrara KW. Imaging of angiogenesis using Cadence contrast pulse sequencing and targeted contrast agents. *Contrast Media Mol Imaging.* 2008; 3:9–18. [PubMed: 18335479]
34. Emmer M, Vos HJ, Goertz DE, van Wamel A, Versluis M, de Jong N. Pressure-dependent attenuation and scattering of phospholipid-coated microbubbles at low acoustic pressures. *Ultrasound Med Biol.* 2009; 35:102–111. [PubMed: 18829153]
35. Tang MX, Eckersley RJ, Noble JA. Pressure-dependent attenuation with microbubbles at low mechanical index. *Ultrasound Med Biol.* 2005; 31:377–384. [PubMed: 15749561]
36. Ellington AD, Szostak JW. In vitro Selection of RNA Molecules That Bind Specific Ligands. *Nature.* 1990; 346:818–822. [PubMed: 1697402]
37. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science.* 1990; 249:505–510. [PubMed: 2200121]
38. Jayasena SD. Aptamers: an emerging class of molecules that rival antibodies in diagnostics. *Clin Chem.* 1999; 45:1628–1650. [PubMed: 10471678]
39. Drabovich AP, Berezovski MV, Musheev MU, Krylov SN. Selection of smart small-molecule ligands: the proof of principle. *Anal Chem.* 2009; 81:490–494. [PubMed: 19055427]
40. Bock LC, Griffin LC, Latham JA, Vermaas EH, Toole JJ. Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature.* 1992; 355:564–566. [PubMed: 1741036]
41. Kaur H, Yung LYL. Probing High Affinity Sequences of DNA Aptamer against VEGF(165). *PLoS One.* 2012; 7
42. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R. Targeted nanoparticle-aptamer bioconjugates for cancer chemotherapy in vivo. *Proc Natl Acad Sci U S A.* 2006; 103:6315–6320. [PubMed: 16606824]
43. Bunka DHJ, Stockley PG. Aptamers come of age - at last. *Nat Rev Microbiol.* 2006; 4:588–596. [PubMed: 16845429]

44. Xu PF, Noh H, Lee JH, Domaille DW, Nakatsuka MA, Goodwin AP, Cha JN. Imparting the unique properties of DNA into complex material architectures and functions. *Materials Today*. 2013; 16:290–296.
45. Furie B, Furie BC. In vivo thrombus formation. *J Thromb Haemost*. 2007; 5(Suppl 1):12–17. [PubMed: 17635703]
46. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest*. 2005; 115:3355–3362. [PubMed: 16322780]
47. Nutiu R, Li Y. Aptamers with fluorescence-signaling properties. *Methods*. 2005; 37:16–25. [PubMed: 16199173]
48. Nakatsuka MA, Mattrey RF, Esener SC, Cha JN, Goodwin AP. Aptamer-Crosslinked Microbubbles: Smart Contrast Agents for Thrombin-Activated Ultrasound Imaging. *Adv Mater*. 2012; 24:6010–6016. [PubMed: 22941789]
49. Borden MA, Martinez GV, Ricker J, Tsvetkova N, Longo M, Gillies RJ, Dayton PA, Ferrara KW. Lateral phase separation in lipid-coated microbubbles. *Langmuir*. 2006; 22:4291–4297. [PubMed: 16618177]
50. Bagot CN, Arya R. Virchow and his triad: a question of attribution. *Br J Haematol*. 2008; 143:180–190. [PubMed: 18783400]
51. Hirsh J, Hoak J. Management of deep vein thrombosis and pulmonary embolism. A statement for healthcare professionals. Council on Thrombosis (in consultation with the Council on Cardiovascular Radiology), American Heart Association. *Circulation*. 1996; 93:2212–2245. [PubMed: 8925592]
52. Hemker HC, Giesen PL, Ramjee M, Wagenvoort R, Beguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. *Thromb Haemost*. 2000; 83:589–591. [PubMed: 10780322]
53. Ninivaggi M, Apitz-Castro R, Dargaud Y, de Laat B, Hemker HC, Lindhout T. Whole-blood thrombin generation monitored with a calibrated automated thrombogram-based assay. *Clin Chem*. 2012; 58:1252–1259. [PubMed: 22665918]
54. Bates SM, Jaeschke R, Stevens SM, Goodacre S, Wells PS, Stevenson MD, Kearon C, Schunemann HJ, Crowther M, Pauker SG, et al. Diagnosis of DVT: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. *Chest*. 2012; 141:e351S–418S. [PubMed: 22315267]
55. Zierler BK. Ultrasonography and diagnosis of venous thromboembolism. *Circulation*. 2004; 109:I9–I14. [PubMed: 15051663]
56. Coley BD, Trambert MA, Mattrey RF. Perfluorocarbon-enhanced sonography: value in detecting acute venous thrombosis in rabbits. *Am J Roentgenol*. 1994; 163:961–964. [PubMed: 8092043]
57. Della Martina A, Allemann E, Bettinger T, Bussat P, Lassus A, Pochon S, Schneider M. Grafting of abciximab to a microbubble-based ultrasound contrast agent for targeting to platelets expressing GP IIb/IIIa - characterization and in vitro testing. *Eur J Pharm Biopharm*. 2008; 68:555–564. [PubMed: 17884399]
58. Schumann PA, Christiansen JP, Quigley RM, McCreery TP, Sweitzer RH, Unger EC, Lindner JR, Matsunaga TO. Targeted-microbubble binding selectively to GPIIb IIIa receptors of platelet thrombi. *Invest Radiol*. 2002; 37:587–593. [PubMed: 12393970]
59. Nakatsuka MA, Barback CV, Fitch KR, Farwell AR, Esener SC, Mattrey RF, Cha JN, Goodwin AP. In vivo ultrasound visualization of non-occlusive blood clots with thrombin-sensitive contrast agents. *Biomaterials*. 2013; 34:9559–9565. [PubMed: 24034499]
60. von Maltzahn G, Park JH, Lin KY, Singh N, Schwoppe C, Mesters R, Berdel WE, Ruoslahti E, Sailor MJ, Bhatia SN. Nanoparticles that communicate in vivo to amplify tumour targeting. *Nat Mater*. 2011; 10:545–552. [PubMed: 21685903]
61. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. *Nat Med*. 2003; 9:669–676. [PubMed: 12778165]
62. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol*. 2006; 3:24–40. [PubMed: 16407877]
63. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. *FASEB J*. 1999; 13:9–22. [PubMed: 9872925]

64. Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, et al. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology*. 2003; 161:1163–1177. [PubMed: 12810700]
65. Wu FT, Stefanini MO, Mac Gabhann F, Kontos CD, Annex BH, Popel AS. Computational kinetic model of VEGF trapping by soluble VEGF receptor-1: effects of transendothelial and lymphatic macromolecular transport. *Physiol Genomics*. 2009; 38:29–41. [PubMed: 19351908]
66. Stefanini MO, Wu FT, Mac Gabhann F, Popel AS. The presence of VEGF receptors on the luminal surface of endothelial cells affects VEGF distribution and VEGF signaling. *PLoS Comput Biol*. 2009; 5:e1000622. [PubMed: 20041209]
67. Stefanini MO, Wu FT, Mac Gabhann F, Popel AS. A compartment model of VEGF distribution in blood, healthy and diseased tissues. *BMC Syst Biol*. 2008; 2:77. [PubMed: 18713470]
68. Wu FT, Stefanini MO, Mac Gabhann F, Popel AS. A compartment model of VEGF distribution in humans in the presence of soluble VEGF receptor-1 acting as a ligand trap. *PLoS One*. 2009; 4:e5108. [PubMed: 19352513]
69. Stefanini MO, Qutub AA, Mac Gabhann F, Popel AS. Computational models of VEGF-associated angiogenic processes in cancer. *Math Med Biol*. 2012; 29:85–94. [PubMed: 21266494]
70. Foekens JA, Peters HA, Grebenchtchikov N, Look MP, Meijer-van Gelder ME, Geurts-Moespot A, van der Kwast TH, Sweep CG, Klijn JG. High tumor levels of vascular endothelial growth factor predict poor response to systemic therapy in advanced breast cancer. *Cancer Res*. 2001; 61:5407–5414. [PubMed: 11454684]
71. Xu X, Wang B, Ye C, Yao C, Lin Y, Huang X, Zhang Y, Wang S. Overexpression of macrophage migration inhibitory factor induces angiogenesis in human breast cancer. *Cancer Lett*. 2008; 261:147–157. [PubMed: 18171602]
72. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. *Nature*. 2000; 407:249–257. [PubMed: 11001068]
73. Ghosh S, Sullivan CA, Zerkowski MP, Molinaro AM, Rimm DL, Camp RL, Chung GG. High levels of vascular endothelial growth factor and its receptors (VEGFR-1, VEGFR-2, neuropilin-1) are associated with worse outcome in breast cancer. *Hum Pathol*. 2008; 39:1835–1843. [PubMed: 18715621]
74. Weidner N, Semple JP, Welch WR, Folkman J. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med*. 1991; 324:1–8. [PubMed: 1701519]
75. Relf M, LeJeune S, Scott PA, Fox S, Smith K, Leek R, Moghaddam A, Whitehouse R, Bicknell R, Harris AL. Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis. *Cancer Res*. 1997; 57:963–969. [PubMed: 9041202]
76. Salgado R, Vermeulen PB, Benoy I, Weytjens R, Huget P, Van Marck E, Dirix LY. Platelet number and interleukin-6 correlate with VEGF but not with bFGF serum levels of advanced cancer patients. *Br J Cancer*. 1999; 80:892–897. [PubMed: 10360671]
77. Byrne GJ, McDowell G, Agarawal R, Sinha G, Kumar S, Bundred NJ. Serum vascular endothelial growth factor in breast cancer. *Anticancer Res*. 2007; 27:3481–3487. [PubMed: 17972505]
78. Benoy I, Salgado R, Colpaert C, Weytjens R, Vermeulen PB, Dirix LY. Serum Interleukin 6, Plasma VEGF, Serum VEGF, and VEGF Platelet Load in Breast Cancer Patients. *Clin Breast Cancer*. 2002; 2:311–315. [PubMed: 11899364]
79. Lichtenberger BM, Tan PK, Niederleithner H, Ferrara N, Petzelbauer P, Sibilio M. Autocrine VEGF signaling synergizes with EGFR in tumor cells to promote epithelial cancer development. *Cell*. 2010; 140:268–279. [PubMed: 20141840]
80. Ishizuka M, Nagata H, Takagi K, Iwasaki Y, Kubota K. Combination of platelet count and neutrophil to lymphocyte ratio is a useful predictor of postoperative survival in patients with colorectal cancer. *Br J Cancer*. 2013
81. Bachelot T, Ray-Coquard I, Menetrier-Caux C, Rastkha M, Duc A, Blay JY. Prognostic value of serum levels of interleukin 6 and of serum and plasma levels of vascular endothelial growth factor in hormone-refractory metastatic breast cancer patients. *Br J Cancer*. 2003; 88:1721–1726. [PubMed: 12771987]

82. Berg WA, Zhang Z, Lehrer D, Jong RA, Pisano ED, Barr RG, Bohm-Velez M, Mahoney MC, Evans WP 3rd, Larsen LH, et al. Detection of breast cancer with addition of annual screening ultrasound or a single screening MRI to mammography in women with elevated breast cancer risk. *J Am Med Assoc.* 2012; 307:1394–1404.

Further Reading/Resources

Contrast Agents: Physical and Biological Basis

Peter Dawson, London, UK

DOI: 10.1038/npg.els.0002337

Ultrasound

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Published online: September 2009

DOI: 10.1002/9780470015902.a0003146

Blood Clotting: General Pathway

Kathleen L Berkner, The Cleveland Clinic Foundation, Cleveland, Ohio, USA

Published online: April 2001

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Figure 1. Microbubble design and synthesis. (a) Schematic of crosslinking and reactivation in a microbubble shell. (b) Synthesis of conjugates of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine, poly(acrylic acid), and A₁₅ oligonucleotides. (c) Bright field optical micrograph of synthesized microbubbles. Scale bar (yellow) = 5 μ m. (d, e) 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoethanolamine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatic acid form the dominant portion of the shell. From Reference 21.

Figure 2.

Ultrasound analysis of DNA-crosslinkable microbubbles. (a) Plotting microbubble onset pressure vs. inverse microbubble radius yields a linear fit for uncrosslinked (green) and crosslinked (red) microbubbles. The intercept corresponds to an activation pressure of ~ 300 kPa. (b) Integrated light scattering signal for oscillating uncrosslinked (green) and crosslinked (red) microbubbles. While the size fluctuations of uncrosslinked bubbles appear to increase steadily with increasing incident pressure, the crosslinked bubbles lose signal as pressure increases. (c) Contrast enhanced ultrasound image of crosslinked microbubbles in phantom. (d) Contrast enhanced ultrasound image of same suspension after addition of A_{30} . From Reference 21.

Figure 3.

Top: Sequences of crosslinking strand (top) and microbubble-bound strand (bottom). The aptamer sequence is denoted in red, while the portion of the bound strand complementary to the aptamer is blue. (a) Freshly drawn rabbit blood clots in a few min. (b-d) Contrast enhanced ultrasound sonograms of (b) aptamer-crosslinked microbubbles, (c) non-aptamer-crosslinked microbubbles, and (d) PEG-coated, freely oscillating microbubbles in freshly-drawn rabbit blood, 5 min after mixing. (e) Frequency response of microbubbles as function of thrombin addition at 2.25 MHz. At higher thrombin loadings the second harmonic at 4.5 MHz appears as a sharp peak, while at moderate amounts the response has a large broadband contribution. (f) Integrations of data from (e) showing that total power output is approximately equal at thrombin concentrations ≥ 50 nM. From Reference 41.

Figure 4.

(a) Ultrasound signal retained over time in citrate-treated non-clotting blood. Purple Circle = 0 mg/mL PAA-DNA, 0.3 mg/mL PEG, Blue Diamond = 0.3 mg/mL PAA-DNA 0 mg/mL PEG, Red Square = 0.3 mg/mL PAA-DNA, .075 mg/mL PEG, Green Triangle = 0.3 mg/mL PAA-DNA, .225 mg/mL PEG. (b, c) Aptamer-crosslinked microbubbles when a clot is absent (b) or present (c). (d) Mean signal generated by thrombin-sensitive microbubbles within the clot site in the presence (red) or absence (blue) of thrombin over time. T = 0 s corresponds to when blood first begins to circulate. From Reference 41.

Figure 5.

In Vivo Imaging of Deep Venous Thrombosis: Activation of (a) thrombin-sensitive and (b) thrombin-insensitive crosslinked microbubbles at clot site in cadence mode. Arrows point at probable clot site as determined by physical tie. Blue outlines indicate vena cava walls. (c) Acoustic signal generated within the clot site over time for thrombin-sensitive (red) or thrombin-insensitive (blue) crosslinked microbubbles within the inferior vena cava. (d) Acoustic signal generated as bubble encounter the clot and become active. This corresponds to area of interest highlighted in blue. From Reference 52.