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In Vivo Ultrasound Visualization of Non-Occlusive Blood Clots with Thrombin-Sensitive Contrast Agents

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Abstract

The use of microbubbles as ultrasound contrast agents is one of the primary methods to diagnose deep venous thrombosis. However, current microbubble imaging strategies require either a clot sufficiently large to produce a circulation filling defect or a clot with sufficient vascularization to allow for targeted accumulation of contrast agents. Previously, we reported the design of a microbubble formulation that modulated its ability to generate ultrasound contrast from interaction with thrombin through incorporation of aptamer-containing DNA crosslinks in the encapsulating shell, enabling the measurement of a local chemical environment by changes in acoustic activity. However, this contrast agent lacked sufficient stability and lifetime in blood to be used as a diagnostic tool. Here we describe a PEG-stabilized, thrombin-activated microbubble (PSTA-MB) with sufficient stability to be used in vivo in circulation with no change in biomarker sensitivity. In the presence of actively clotting blood, PSTA-MBs showed a 5-fold increase in acoustic activity. Specificity for the presence of thrombin and stability under constant shear flow were demonstrated in a home-built in vitro model. Finally, PSTA-MBs were able to detect the presence of an active clot within the vena cava of a rabbit sufficiently small as to not be visible by current non-specific contrast agents. By activating in non-occlusive environments, these contrast agents will be able to detect clots not diagnosable by current contrast agents.

Keywords

Thrombosis; In vitro test; In vivo test; Clotting; DNA

Introduction

While ultrasound is widely used as an imaging tool, its ability to distinguish between normal and diseased soft tissues with similar acoustic impedances is limited [1]. Introduction of contrast agents produces far greater ultrasound reflection, allowing imaging of smaller

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regions of interest with improved resolution and signal-to-noise ratios. For this, gas-filled microbubbles have been shown to be the most effective ultrasound contrast agents primarily because their nonlinear response to ultrasound creates echoes at frequencies that can be detected specifically using specialized pulse sequences [2–4]. However, microbubbles are inherently unstable in the circulation due to gas exchange in the lung, their sensitivity to significant changes in blood pressure as they circulate and when exposed to ultrasound, and shell disruption caused by adhesion of opsonization agents [5]. Significant progress has been made in prolonging the in vivo lifetime of microbubbles through optimization of bubble shell formulation. Bubbles can be stabilized by the introduction of thick, albumin protein shells such as with Optison® [6], polymer coatings such as poly(L-lactide-co-glycolide) (PLGA) [7], or the incorporation of poly(ethylene glycol) (PEG) lipid conjugates into phospholipid monolayers [8, 9]. Many such formulations are currently in trial or in clinical use.[2–4] However, due to their average diameters of 1–5 μm , microbubbles are limited to imaging and detecting abnormalities in the intravascular space. As such, the main targets of microbubble detection are cardiovascular disease [10], tissue perfusion, and tumor angiogenesis [11, 12].

The microbubbles described in this work are designed to detect active clotting that can be used to distinguish acute deep vein thrombosis (DVT) from chronic DVT. Acute DVT affects patients particularly in the hospital setting following surgery, trauma, or childbirth, and it is potentially fatal if the clot embolizes to the lungs. Patients with acute DVT are treated aggressively with anticoagulants to prevent clot progression and promote lysis before the clot can organize and become adherent to the wall of the vein. Anticoagulation therapy, however, not only provides little benefit for chronic DVT but also increases risk for internal bleeding. Since the clot is invisible to ultrasound, acute DVT is currently diagnosed by compression real-time ultrasound imaging, in which the inability to compress the vein along with the absence of Doppler flow signal provides indirect evidence of the physical presence of a clot. However, ultrasound is unable to consistently distinguish acute from chronic DVT, and in particular acute superimposed on chronic DVT, making treatment decisions difficult.

We propose here a new microbubble molecular imaging approach to not only recognize the presence of acute DVT but also distinguish active from chronic thrombosis. Ultrasound angiography with microbubbles has been proposed as a method to detect clots as filling defects [13, 14], but since chronic thrombi will also appear as filling defects it is likely that this approach will not aid in treatment management. Microbubbles have been proposed as molecular imaging agents to target receptors expressed on endothelial surfaces and blood elements [15–19]. However, immobilized bubbles can be dislodged or dissolved by shear stress caused by blood flow, and initial adhesion is limited by the total surface area and porosity of the clot [20]. If instead the microbubbles produced a signal only in response to elevated levels of soluble biomarkers, abnormalities could be diagnosed based on their local chemical environment even if they were otherwise too physically small to distinguish from background noise.

Here, we report the in vitro and in vivo validation of a PEG-stabilized, thrombin-activated microbubble (PSTA-MB) contrast agent capable of signaling the presence of active, non-occlusive blood clots. We previously designed microbubbles whose outer lipid monolayer shells contained a crosslinked network of hybridized DNA strands. These microbubbles became acoustically active at thrombin levels greater than 25 nM in static trials when diluted into sterile buffers, but they were insufficiently stable to survive circulation.[21, 22] If such contrast agents could be designed with both stabilizing (PEG-lipids) and stimulus-responsive agents in the same shell, they might be able to circulate for extended periods without losing responsiveness to their target biomarker. In this study, both the stability of the bubbles and their responsiveness to elevated thrombin levels were first validated in a

static clot model, followed by evaluation of performance in a blood clot flow model. Finally, the efficacy of these PSTA-MBs were tested in a rabbit DVT model to test their ability to image actively-growing but non-occlusive blood clots, which would potentially allow for early detection of malignant blood clot formation before the onset of vessel occlusion.

Materials and Methods

Synthesis of DSPE-PAA-DNA

The synthesis of DSPE-PAA-DNA conjugate was described previously [21, 22]. Briefly, poly(acrylic acid) (PAA) was coupled to 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) via carbodiimide-mediated amidation, and two amine terminated DNA strands (5 H₂N-CCAACCACAAAA, 5 AAAACAACCCCA-NH₂) were attached to the carboxyl groups of the PAA similarly, with an average yield of 3.3 DNA strands per DSPE-PAA molecule.

Microbubble Formulation and Lifetime

18 mg 1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 2 mg 1,2-palmitoyl-*sn*-glycero-3-phosphatic acid (DPPA) were dissolved in 2 mL of chloroform and the solvent was subsequently removed by rotary evaporator (Büchi R215). The film was then rehydrated in 1X phosphate buffered saline (PBS, Gibco) to a final concentration of 4 mg/mL and stored at 4°C. This phospholipid mixture was subsequently mixed with varying amounts of DSPE-PEG-5000 (Avanti Polar Lipids) and the DSPE-PAA-DNA conjugate, heated and stirred at 75°C for 30 min, and allowed to cool slowly to RT. The various phospholipid formulations are shown in Table 1. The phospholipid mixture was then probe sonicated (Branson) for 10 s under perfluorobutane gas (PFB), as described previously[23]. Bubbles were crosslinked via the addition of the complementary DNA strand GGTGGTGTGGTGGTGTGTTTTTTTTGTGGTTGGTGTGGTTGG, referred to hereforth as the thrombin aptamer crosslinking sequence (TACS). The symmetrical strand contains two 15-base aptamer sequences (GGTGGTGTGGTTGG) on either end which possess a half-maximal inhibitory concentration (IC₅₀) of 25 nM for thrombin.[24] Nine of the fifteen bases of the aptamer are left unhybridized such that interaction with thrombin results in the displacement of the entire TACS strand. A control strand with the same crosslinking base pair sequences, but possessing scrambled unpaired ends with no affinity to thrombin, GTGTGTGTGGGTGGTGTGTTTTTTTTGTGGTTGGGTGTGTGTG, is referred to here as the scrambled aptamer crosslinking sequence (SACS). Either TACS or SACS was added to the prepared microbubble sample at a molar ratio of 1.5:1 relative to the available number of DNA sites, and allowed to hybridize at RT for 30 min. Microbubbles formed by this method had an average diameter of 1.6±0.1 µm regardless of the amount of DSPE-PEG or DSPE-PAA-DNA.

A Multisizer 3 Coulter Counter (Beckman Coulter) with aperture size 1–30 µm was used to quantify the size and concentration of the microbubbles. 3 µL of microbubble solution was diluted into 15 mL of filtered isoton, and 100 µL was drawn per reading.

Static Contrast Enhanced-Ultrasound Imaging

Freely oscillating PEG microbubbles containing 0.3 mg/mL DSPE-PEG and 0 mg/mL DSPE-PAA-DNA were diluted to various concentrations between 250 mL⁻¹ and 250,000 mL⁻¹ in either 2 mL of citrate-treated, non-clotting bovine blood (Lampire, Inc.), or 2 mL of blood which has been clotted through the addition of 500µL of 1 M aqueous calcium chloride in 1x PBS. The blood-bubble mixture was mixed and allowed to equilibrate for 3 min at RT, open to the atmosphere, within the bulb of a plastic transfer pipet submerged underwater. Using a Siemens Acuson Sequoia C512, cadence mode images were taken at

7.0 MHz and an MI = 0.18. To quantify the signal generated by the microbubbles, the mean pixel brightness within the pipet bulb phantom was measured using ImageJ, and normalized to the mean brightness of the surrounding background. The area of the phantom was determined from first tracing the area of the image taken in B-Mode (not shown). Similar imaging and analysis was applied to PSTA-MB formulations which contained 0 mg/mL, 0.075 mg/mL or 0.225 mg/mL of DSPE-PEG, both in non-clotting and clotting conditions.

Additionally, the acoustic activity of bubbles with 0 mg/mL, 0.075 mg/mL and 0.225 mg/mL of DSPE-PEG were compared to that of freely oscillating microbubbles over a time period of 40 min in non-clotting citrate treated bovine blood as above. Bubble/blood mixtures were imaged every 5 min, and the normalized mean pixel brightness was measured and compared to the initial signal generated immediately after addition of bubble to the blood mixture.

In Vitro Flow Model Imaging

Two 1 mL syringes (BD) were cut down to 1 , keeping the sections with the Luer-slip. Between these two cut pieces, 4 of cellulose dialysis tubing (Fisher Scientific, MWCO = 3500, diameter = 1.1 cm) was affixed and sealed. To form a clot within this cellulose section, it was filled with 2 mL of citrate-treated blood, to which 500 μ L of a 1 M aqueous calcium chloride solution in 1x PBS was added and allowed to rest at RT undisturbed for 40 min.

Tygon tubing (internal diameter = 0.25 , Fisher Scientific) was used to form a closed loop between the syringe ends and a systolic pump (Fisher Scientific). The loop was filled with 25 mL of citrated blood, and blood was circulated at a speed of approximately 30 mL/min. The cellulose pouch containing the clot was imaged using a Sequoia Acuson C512 with and without the presence of a clot at a total system bubble concentration of 10,000 mL⁻¹ in cadence mode. Activity of the PSTA-MBs crosslinked with the thrombin-aptamer crosslinking sequence (TACS) was compared to the activity of PSTA-MBs crosslinked with a scrambled-aptamer crosslinking sequence (SACS) to determine specificity towards thrombin. Again the mean pixel brightness within the cellulose walls was determined and normalized to the background signal over time as bubbles were allowed to circulate.

In vivo Rabbit Deep Venous Thrombosis Model

This procedure was adapted from Coley et. al. [14], and all procedures were conducted with prior approval from the UC San Diego Institutional Animal Care and Use Committee. Female New Zealand white rabbits with an average weight of 3 kg were anesthetized with a cocktail of 35 mg/kg Ketamine and 5mg/kg Xylazine and the inferior vena cava (IVC) was exposed. The IVC was tied with a silk suture together with a blunted 18G needle just inferior to the renal veins. The distal IVC was clamped just above the IVC bifurcation. 5000U of bovine thrombin was injected directly into the occluded IVC segment, and the clot allowed to form for five minutes. The distal clamp was removed allowing the blood to bathe the formed clot. The blunt needle was then removed leaving a tight infrarenal stenosis to prevent the formed clot to migrate to the lungs. The body cavity was filled with saline to eliminate trapped peritoneal air from interfering with imaging, and the incision closed. The rabbit was allowed to recover and the formed clot was imaged 6 h later.

For imaging, the rabbit was anesthetized with isoflurane and medical air, and 10 μ L of Definity (Bristol-Meyers-Squibb) was injected intravenously into the ear vein in order to find and determine the shape of the clot. 10⁷ microbubbles crosslinked with either TACS or SACS were then injected intravenously and microbubble response was charted at the clot site immediately after injection. Images were acquired of the clot using the CPS mode at 7.0

MHz and an MI = 0.18, and the increase in mean brightness contained within the vena cava was measured over time using ImageJ. The signal was not normalized to background as signal from background tissues is nearly totally suppressed by CPS imaging.

Results and Discussion

Synthesis and Characterization of PEGylated-DNA Microbubbles

The stimulus-responsive mechanism of the microbubbles shown here derives from a DNA-aptamer switch that we employed successfully in previous studies. [22, 25] Microbubbles formed from a mixture of DPPC and DPPA phospholipids were stabilized by a DSPE-PAA-oligonucleotide conjugate (Figure 1). Neighboring single-strand oligonucleotides were crosslinked by a complementary, aptamer-containing strand, which provided the encapsulating bubble shell with enhanced rigidity on the order of 25 pN per crosslink and greatly reduced the generation of nonlinear echoes. However, in the presence of clinically-relevant, elevated amounts of thrombin, the crosslinking aptamer strands preferentially bound to free thrombin, removing the crosslinks from the shell. This process caused the microbubble shells to become flexible, thus increasing their non-linear response when exposed to ultrasound, which can be distinguished from tissue signals using contrast specific imaging such as cadence contrast pulse sequencing.

In previous attempts to perform in vivo validation, these microbubbles as designed did not survive circulation at sufficient concentration to show a positive signal in a rabbit DVT model. We hypothesized that adding poly(ethylene glycol) (PEG) to our microbubble formulations would improve their stability without sacrificing their ability to interact with thrombin, as addition of DSPE-PEG as an emulsifying agent to gas-filled microbubbles has been applied previously for improving microbubble yield, homogeneity, and stability compared to lipid-only formulations [9]. To add DSPE-PEG to the microbubbles, films of DPPC and DPPA phospholipids were melted in 1X PBS with varying amounts of the stabilizing agent DSPE-PEG5000 and crosslinking attachment site molecule DSPE-PAA5000-DNA, probe sonicated under perfluorobutane (PFB) to form bubbles, and mixed with the crosslinking strand. Four formulations were considered, all containing 1.0 mg/mL DPPC/DPPA: “Pure PEG”, “0 mg/mL PEG”, “0.075 mg/mL PEG” and “0.225 mg/mL PEG”. The exact formulations can be found in Table 1. After bubble preparation, bubble samples were measured by a Multisizer 3 Coulter Counter at varying timepoints to determine concentration (Figure 2a). As expected, microbubbles without DSPE-PEG showed the lowest initial concentration of about $1.2 \times 10^8 \text{ mL}^{-1}$, while microbubbles with 0.3 mg/mL DSPE-PEG and no DSPE-PAA yielded about twice that. To our surprise, however, addition of 0.075 mg/mL DSPE-PAA to the mixture improved the yield by two-fold over bubbles with 0 mg/mL PEG, and 4-fold over DSPE-PAA only, indicating that this was an ideal formulation for future studies with PEG-stabilized, thrombin-activated microbubbles (PSTA-MBs).

Microbubble stability was also determined in citrate treated whole blood. The four formulations described previously were diluted to a concentration of 2500 mL^{-1} in 2 mL of citrate-treated non-clotting whole blood within the bulb of a transfer pipette and submerged underwater. The bubbles in the pipette were imaged by a Sequoia Acuson 512 in cadence mode, and the mean pixel brightness, normalized to the background, was measured every five minutes to determine bubble viability (Figure 2b). When comparing the signal generated after 5 minutes of blood exposure to that signal generated immediately after bubble addition, we found that PSTA-MBs containing 0.075 mg/mL of DSPE-PEG were able to retain 95% of their acoustic activity while other formulations retained approximately 70% of their acoustic ability. While all microbubble formulations showed exponential decay in retained activity over time, PSTA-MB formulations with 0.075 mg/mL DSPE-PEG

loadings were consistently 10% more active throughout the 40 min observation period, validating that the incorporation of small amounts of DSPE-PEG provide a boost in bubble lifetime, even in non-ideal environments such as in whole blood and open to air.

Activation of DNA-Microbubbles in Actively Clotting Blood

Since PEG has been shown to retard the association of buried ligands with their protein targets, [26] addition of PEG to the microbubble shell may prevent thrombin from interacting with the crosslinking aptamer strands. It has also been shown that the addition of large amounts of PEGylated lipids to a microbubble shell can decrease the viscosity of the bubble, changing both its resonance frequency and corresponding acoustic behavior [27]. To ensure that the addition of DSPE-PEG to thrombin-activated microbubbles (PSTA-MBs) did not prevent silencing of signal, PSTA-MBs formulated with 0 mg/mL, 0.075 mg/mL and 0.225 mg/mL DSPE-PEG were evaluated in a static in vitro model in whole blood. First, we established an “acoustic concentration curve” by measuring the acoustic response of freely oscillating DSPE-PEG microbubbles without DSPE-PAA-DNA, diluting the bubbles to various concentrations in 2 mL in citrate-stabilized (non-clotting) whole blood within the bulb of a transfer pipette submerged underwater. Plotting mean pixel brightness after background subtraction against bubble concentration revealed a clear logarithmic relationship that enabled us to determine the number, or “effective concentration,” of freely-oscillating microbubbles associated with a given acoustic signal (Fig. S1). Next, PSTA-MBs were diluted to a concentration of 2500 mL⁻¹ and measured as above. In non-clotting blood, the acoustic activity of DNA crosslinked microbubbles with no PEG incorporation at a concentration of 2500 mL⁻¹ were found to be equivalent to an effective bubble concentration of approximately 215 mL⁻¹, indicating that crosslinking has prevented roughly 90% of these bubbles from generating acoustic signal (Table 2). The addition of DSPE-PEG appears to further reduce the signal generated from the crosslinked bubbles, although the small differences between the signals fall within 95% confidence intervals of each other. However, the clear difference between effective and actual concentration indicates that incorporation of DSPE-PEG within the microbubble shell did not prevent the formation of oligomer crosslinks, and the acoustic activity of the microbubbles was silenced accordingly.

To determine the response of bubbles to elevated thrombin levels, 1 M calcium chloride in 1X PBS was first added to the citrate-stabilized whole blood, causing the blood to initiate clotting. Bubbles were added immediately, the thrombin levels were allowed to increase for 3 min, and the acoustic signal was obtained as described above. To control for potential effects of clotting on acoustic signal, an effective concentration curve was obtained for freely-oscillating DSPE-PEG bubbles in clotting blood as well, which showed little change in the relationship between effective bubble concentration and mean pixel brightness (Fig. S1). For DNA-crosslinked microbubbles without PEG stabilization, acoustic signal increased roughly 5-fold, which equates to an effective concentration of 900 mL⁻¹ (Figure 3). Effective microbubble concentrations for PSTA-MBs with 0.075 and 0.225 mg/mL DSPE-PEG in clotting blood were similar to this value, and again fell within their respective 95% confidence intervals. This increase in acoustic signal showed that the presence of DSPE-PEG did not prevent aptamer-thrombin interaction, although the presence of thrombin did not activate the microbubbles to their expected effective concentration. This “partial activation” effect was previously noted in our other DNA crosslinked microbubble systems, and we attributed this to the formation of an equilibrium between free thrombin, bound thrombin-aptamer, and hybridized aptamer that prevented complete dehybridization of the silencing strand.

Contrast Imaging in a Flow Model of an In vitro Blood Clot

While the previous experiment suggested that the acoustic signal of PSTA-MBs increased in a static area of high local thrombin concentration, this idealized case is not representative of the typical journey of a microbubble contrast agent through the bloodstream. To further assess the clinical viability of our microbubbles, we designed an in vitro model of clot formation based on the Wessler model [28] in order to better approximate in vivo conditions. In the Wessler model, an area within the jugular vein is physically isolated to restrict blood flow. Thrombogenic material is injected into this static “pouch,” a clot is allowed to form, and then venous flow is restored. To form the pouch, cellulose dialysis tubing was partially filled with citrate-stabilized whole blood, air bubbles were removed from the tubing through manual compression, and a 1 M solution of calcium chloride in 1X PBS was added to induce thrombus formation. This resulted in a small, non-occlusive blood clot being formed on the walls of the cellulose dialysis tubing. The pouch was then connected within a closed Tygon loop, filled with citrate-stabilized blood, and connected to a peristaltic pump. This procedure preserved the local biochemical environment of the clot by retaining all of the thrombin produced during the formation of the clot.

Based on their performance in the static model and their resistance to degradation in whole blood, PSTA-MBs with 0.075 mg/mL were evaluated in the flow model. To measure the specificity of thrombin activation, PSTA-MBs were prepared with one of two crosslinking strands: either a thrombin-aptamer crosslinking sequence TACS, or a scrambled-aptamer crosslinking sequence (SACS) that dampens acoustic signal but should not interact significantly with thrombin (see Methods for specific sequences). As a point of comparison, microbubbles were first introduced upstream of the pouch at a final dilution of 10,000 mL⁻¹ in citrate-stabilized whole blood without the presence of a clot. Without the clot present, the PSTA-MBs generated little to no acoustic signal in the pouch region, regardless of crosslinking strand sequence (Figure 4). The few points of acoustic signal were most likely caused by either very large diameter bubbles, which will reflect signal very efficiently, or air bubbles generated by the shear flow in the model. Alternatively, the shear stress generated by the flow environment may have caused weakening of some of the bubble shells; upon insonation, the bubble would then leave the rest of the shell, producing a freely-oscillating microbubble that would generate nonlinear echoes quite efficiently, though it would be short-lived and non-specific in its activation [29, 30].

When the bubble samples were circulated with a clot present, signal generated from TACS bubbles, as determined by the mean pixel intensity within the clot site, increased approximately 5-fold as the microbubbles encountered the clot (Figure 4), while bubbles crosslinked with the SACS bubbles showed little overall change. As seen in Figure 4, PSTA-MBs circulate for approximately 15 s before dramatically changing their acoustic activity. Because of the non-occlusive nature of the clot, blood flow within the pouch was not affected, and active microbubbles were allowed to clear the imaging site at the same rate that they were introduced. As such, after activation by exposure to thrombin, signal remained constant within the pouch until bubble loss began to take effect, at about 25 s after administration. Due to the constant circulation of calcium in addition to bubbles, clotting occurred rapidly throughout the system and led to substantial activation of bubbles even outside of the clot site within 30 s of introduction. Interestingly, signal for bubble samples crosslinked with the non-reactive SACS strand also showed a very small increase in signal in the presence of a clot. While the unhybridized ends of the SACS sequence (GTGTGTGTG) should not show any affinity towards thrombin, the hybridized hexamer sequence immediately preceding it (GGTTGG) has some affinity towards thrombin[31], allowing for some interaction and decrosslinking in these samples.

In Vivo Imaging of Rabbit Venous Thrombosis

We further investigated the ability of the PSTA-MBs to act as site-specific contrast agents in a modified Wessler static thrombosis model in a rabbit. Briefly, a segment of the Inferior Vena Cava of the rabbit was temporarily occluded, and bovine thrombin added to generate a clot, followed by re-establishing flow and allowing the rabbit to recover for 6 h. Although this model does not produce a clot under natural conditions because blood flow at the clot site was stopped entirely for several minutes, in our hands, this procedure not only proved simpler and more reliable than other methods such as damaging the vessel wall or chemically inducing vascular damage, but it also ensured that the chemical environment around the clot remained relatively unaffected.

Under cadence mode imaging of the clot region, TACS and SACS bubbles were injected into the rabbits in separate trials. Unlike our in vitro model, blood flow was visibly reduced within the clot region and bubble clearance from the clot site was limited. For TACS bubbles, after the first few bubbles were detected at the clot site approximately 30 s after intravenous injection, signal increased constantly and dramatically over the next 10 s, peaking at a 5-fold increase in mean brightness within the blood vessel, as seen in Figure 5. The increase in signal differed from that observed in vitro because of an imbalance between microbubble activation and clearance allowing for gradual accumulation of active contrast agents within the clot site. SACS bubbles showed no increase in acoustic signal over this same period. Only high concentrations of bubbles associated with full activation visibly changed the acoustic activity. Additionally, despite potential loss of bubbles during circulation, an injected concentration two orders of magnitude less than a typical diagnostic dose still remained viable roughly two minutes after injection, enough to detect and image the clot after several full cycles through the cardiovascular system.

It is noteworthy in these images that, despite the clear increase in acoustic activity and the saturation of the IVC with contrast agents, no specific clot can be distinguished. Current non-targeted contrast agents rely on the exclusion of signal, or filling defect, where the clot is present to detect and diagnose the presence of clots, and thus a clot must cause a significant displacement of blood within the vein to be visible by this method. Targeted contrast agents require bubbles to accumulate at the site of the clot, but because of the reduced flow velocity within the vein, even bubbles without targeting ligands cannot clear easily, reducing the clot-specific contrast. The immediate change in acoustic signal caused by local thrombin activation within an active clot, however, allow detection and diagnosis by the PSTA-MBs. Thus, these PEGylated, thrombin-activated microbubbles show excellent promise for improving early detection of active clot formation within the cardiovascular system and particular acute deep venous thrombosis.

Conclusions

In this work we studied the effect of addition of DSPE-PEG to microbubbles capable of increasing their acoustic response in the presence of thrombin. The microbubbles responded to thrombin through interaction of this biomarker with DNA aptamer crosslinkers that rigidify the bubble shell, decreasing or eliminating its nonlinear response to ultrasound. Adding DSPE-PEG to this formulation did not change the ability of the microbubbles to respond to elevated thrombin, while the addition of PEG resulted in a 2-fold increase in bubble yield, and a 25% increase in stability within the first 5 min of blood exposure, allowing them to be used under shear flow both in vitro and in vivo. The PSTA-MB contrast agent increased its acoustic signal five-fold in actively clotting blood. It also showed a sharp and immediate increase in signal when encountering a mature clot under shear flow when crosslinked with a DNA strand with an affinity towards thrombin, but very little change when crosslinked with a DNA strand with little affinity towards thrombin, demonstrating

specificity of the activation mechanism. Finally, PSTA-MBs proved capable of imaging a non-occlusive clot in vivo, despite the clot being too small to see with traditional contrast enhanced imaging. These studies indicate that microbubbles functionalized with a combination of lipids, DSPE-PEG, and polymer-DNA conjugates represent a simple, non-invasive method for early, efficient diagnosis of deep venous thrombosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Diagram of Aptamer Crosslinked, PEGylated contrast agent. Poly(acrylic acid) (PAA) conjugated to the phospholipid DSPE is functionalized with the complementary DNA strand to a longer thrombin aptamer crosslinking sequence (TACS). The addition of the TACS causes crosslinks between neighboring phospholipid groups, and increasing the rigidity of the shell. In the presence of an acute blood clot, the unhybridized ends of the TACS will bind to the free thrombin and dislodge the strand from the bubble surface, reducing the number of crosslinks on the surface of the bubble and restoring acoustic ability.

Figure 2.

Comparison of bubble lifetime with various microbubble formulations in buffer (a), and amount of bubbles retained over time in citrate-treated non-clotting blood (b). 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.

Figure 3.

Static Contrast Enhanced Ultrasound Imaging: TACS crosslinked microbubbles with 0 mg/mL PEG loading in non-clotting (a) and actively-clotting blood (b), bubbles with .075 mg/mL PEG loading in non-clotting (c) and actively-clotting blood (d), and bubbles with .225 mg/mL PEG loading in non-clotting (e) and actively-clotting blood (f). (g) Average of three brightness analyses within the respective phantoms, error bars are the 95% confidence interval.

Figure 4.

Contrast Enhanced Ultrasound Imaging in In Vitro Flow Model: TACS crosslinked microbubbles when a clot is absent (a) or present (b), SACS crosslinked microbubbles in the absence (c) or presence (d) of an acutely formed clot Mean signal generated by TACS microbubbles (e) and SACS microbubbles (f) 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.

Figure 5.

In Vivo Contrast Enhanced Ultrasound Imaging of Deep Venous Thrombosis: Activation of TACS (a) crosslinked microbubbles and SACS (b) 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 TACS (red) or SACS (blue) 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.

Table 1

Bubble formulation compositions

	DPPC/DPPA	DSPE-PEG	DSPE-PAA-DNA
Pure PEG	1 mg/mL	0.3 mg/mL	0 mg/mL
0 mg/mL PEG	1 mg/mL	0 mg/mL	0.3 mg/mL
0.075 mg/mL PEG	1 mg/mL	0.075 mg/mL	0.3 mg/mL
0.225 mg/mL PEG	1 mg/mL	0.225 mg/mL	0.3 mg/mL

Table 2

Effective bubble concentration calculated for various PEGylations of DNA-crosslinked microbubbles before and after the presence of thrombin as a function of mean pixel brightness.

	0 mg/mL PEG, No Clot	0 mg/mL PEG, Clot	0.075 mg/mL PEG, No Clot	0.075 mg/mL PEG, Clot	0.225 mg/mL PEG, No Clot	0.225 mg/mL PEG, Clot
Mean Pixel Intensity	5.08	12.96	3.95	13.37	3.42	14.04
Effective Concentration (mL^{-1})	215.75	911.23	178.40	961.78	163.35	1048.08
% Bubbles Active	8.63	36.45	7.14	38.47	6.53	41.92