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In vivo ultrasound visualization of non-occlusive blood clots with thrombin-sensitive contrast agents

Matthew A. Nakatsuka [a], Christopher V. Barback [b], Kirsten R. Fitch [c], Alexander R. Farwell [c], Sadik C. Esener [a], Robert F. Mattrey [b], Jennifer N. Cha [a,c], Andrew P. Goodwin [c,*]

[a] Department of Nanoengineering, University of California, San Diego, 9500 Gilman Dr. #0448, La Jolla, CA 92093-0448, USA
[b] Department of Radiology, University of California, San Diego, 410 Dickinson Street, San Diego, CA 92121, USA
[c] Department of Chemical and Biological Engineering, University of Colorado, Boulder, 596 UCB, Boulder, CO 80309, USA

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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.

1. 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 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

* Corresponding author. Tel.: +1 303 492 3573; fax: +1 303 492 8425.
E-mail address: andrew.goodwin@colorado.edu (A.P. Goodwin).

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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.

2. Materials and methods

2.1. 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′ H_2N–CCAACCACAAAA, 5′ AAAACAACCCCA–NH_2) were attached to the carboxyl groups of the PAA similarly, with an average yield of 3.3 DNA strands per DSPE-PAA molecule.

2.2. 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 1× 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 GGTTGGTGTGGTTGGTGT TTTTTTTTGTGGTTGGTGTGGTTGG, referred to hereforth as the thrombin aptamer crosslinking sequence (TACS). The symmetrical strand contains two 15-base aptamer sequences (GGTTGGTGTGGTTGG) on either end which possess a half-maximal inhibitory concentration (IC_{50}) 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, GTGTGTGTGGGTTGGTGTTTTTTTTTGTGGTTGGGTG TGTGTG, 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.

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

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.

2.3. 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 500 μL of 1 M aqueous calcium chloride in 1× 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.

2.4. 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 μL of a 1 M aqueous calcium chloride solution in 1 × 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 Acuson Sequoia™ 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.

2.5. 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 5 mg/kg Xylazine and the inferior vena cava (IVC) was exposed. The IVC was tied with a silk suture together