Novel Single-Chain Antibody-Targeted Microbubbles for Molecular Ultrasound Imaging of Thrombosis: Validation of a Unique Non-Invasive Method for Rapid and Sensitive Detection of Thrombi and Monitoring of Success or Failure of Thrombolysis in Mice

Running title: Wang et al.; Targeted molecular ultrasound imaging of thrombi

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Abstract:

**Background** - Molecular imaging is a fast emerging technology allowing non-invasive detection of vascular pathologies. However, imaging modalities offering high resolution currently do not allow real-time imaging. We hypothesized that contrast enhanced ultrasound with microbubbles selectively targeted to activated platelets would offer high-resolution, real-time molecular imaging of evolving and dissolving arterial thrombi.

**Methods and Results** – Lipid-shell based gas-filled microbubbles (MB) were conjugated to either a single-chain antibody (scFv) specific for activated GPIIb/IIIa via binding to a Ligand-Induced Binding Site (LIBS-MB), or a non-specific scFv (control-MB). Successful conjugation was assessed in flow cytometry and immunofluorescence double-staining. LIBS-MB, but not control-MB, strongly adhered to both immobilized activated platelets and microthrombi under flow. Thrombi, induced in carotid arteries of C57Bl6-mice *in vivo* by ferric chloride injury, were then assessed with ultrasound before and 20 minutes after MB injection, using grayscale area intensity measurement. Grayscale units converted to decibels demonstrated a significant increase after LIBS-MB but not after control-MB injection (9.55 ± 1.7 versus 1.46 ± 1.3 dB; p<0.01). Further-more after thrombolysis with urokinase, LIBS-MB ultrasound-imaging allows monitoring of the reduction of thrombus size (p<0.001).

**Conclusions** - We demonstrate that GPIIb/IIIa-targeted microbubbles specifically bind to activated platelets *in vitro* and allow real-time molecular imaging of acute arterial thrombosis as well as monitoring success or failure of pharmacological thrombolysis *in vivo*.

**Key words:** antibodies; imaging; platelets; targeted-microbubbles; thrombosis
Introduction

Atherosclerosis is a progressive disease, driven by the adherence of activated platelets and monocytes to the site of vascular inflammation and the accumulation of lipids and lipid-loaded macrophages in the artery wall\(^1-3\). Atherosclerosis is often silent for decades until it becomes clinically evident\(^3\). Even though chronic atherosclerotic lesions can significantly reduce blood flow, most acute cases of myocardial infarction and stroke are caused by atherothrombosis, triggered by the exposure of thrombogenic material at sites of plaque ruptures, inducing the activation of the coagulation cascade, as well as platelet adhesion, activation and aggregation\(^4-7\). This finally leads to thrombus formation and vessel occlusion.

Rapid and reliable detection of thrombosis is a highly attractive aim in medical imaging with potential major benefits for patients. One promising strategy is the development of targeted molecular imaging probes. The glycoprotein (GP) IIb/IIIa complex, also known as integrin \(\alpha\)IIb\(\beta\)3 (CD41/CD61), is the main receptor mediating platelet aggregation and the most abundant receptor expressed on the platelet surface\(^8,9\). GPIIb/IIIa undergoes a conformational change from a low to a high-affinity state upon platelet activation. This allows fibrinogen binding, platelet aggregation and thrombus formation\(^8,9\). These features make GPIIb/IIIa an ideal target for molecular imaging of thrombosis. We have developed conformation-specific anti GPIIb/IIIa single-chain antibodies (scFv)\(^9-11\), some of which bind specifically to a Ligand-Induced Binding Site (LIBS) on activated GPIIb/IIIa\(^9-11\) and have previously demonstrated the ability of scFv\(_{\text{anti-LIBS}}\) to detect activated platelets in MRI in the context of inflammation and thrombosis\(^12-15\).

Ultrasound imaging has several advantages over other imaging modalities. It is non-invasive, inherently real-time, free of radiation-associated risk, with no known side effects, and only rarely causes discomfort in patients. Furthermore, the equipment is generally less expensive
than for example MRI equipment, highly portable and already present in most hospitals.

Here we present a non-invasive approach for the specific detection of activated platelets, platelet aggregates and thrombi, which could be used for early and sensitive detection of thrombi, and also monitoring of success or failure of thrombolysis. By conjugation of scFv\textsubscript{anti-LIBS} to microbubbles (LIBS-MB), we demonstrate that targeted molecular imaging of LIBS-MB with contrast-enhanced ultrasound is well suited to detect and visualize thrombi \textit{in vivo} in the carotid artery of mice. Pharmacological thrombolysis was performed to further demonstrate that LIBS-MB and molecular ultrasound imaging is able to reliably detect a reduction in thrombus size. Overall, the presented targeted molecular imaging approach holds potential to be translated after further extensive experimental testing to clinical application in humans.

Methods

A more detailed description of the methods is provided in the supplemental material.

\textbf{Generation, expression and purification of single-chain antibodies}

The generation of the two different scFvs (activated GPIIb/IIIa targeted scFv\textsubscript{anti-LIBS} and non-targeted mut-scFv) has been described previously\textsuperscript{9,10,12-15}. Briefly, both scFvs were sub-cloned into the AviTag\textsuperscript{TM} containing pAC6 vector system, transformed into electrocompetent EVB101 (all Avidity, USA). \textit{In vivo} biotinylation was performed according to the manufacturer’s instruction. All scFvs carry a 6x His-tag, which was used for purification with nickel-based metal affinity chromatography (Invitrogen, USA).

\textbf{Evaluation of purity and efficiency of in vivo biotinylation of scFv-AviTag\textsuperscript{TM} constructs}

Purity of the proteins was analyzed using SDS-PAGE, while the efficiency of \textit{in vivo} biotinylation was determined by Western blot. An anti-6x His-tag antibody HRP was used to
detect the protein construct, and streptavidin-HRP (BD Bioscience, USA) was used to determine
the success of \textit{in vivo} biotinylation.

\textbf{Flow cytometry}

Platelet rich plasma (PRP) was obtained by centrifugation of blood collected from healthy
volunteers. Diluted PRP was either not activated or activated with ADP, before incubation with
purified scFv, followed by incubation with an anti-Penta-His AlexaFluor 488 conjugated mono-
clonal antibody (Qiagen, Germany) or R-phycoerythrin streptavidin (Jackson ImmunoResearch,
USA). Samples were fixed and analyzed by FACS Calibur (BD bioscience, USA).

\textbf{Conjugation and purification of biotinylated scFv- AviTag\textsuperscript{TM} constructs to streptavidin-
coated microbubbles}

Conjugation of 200\( \mu \)g of scFv-AviTag to 1.5 \( \times 10^9 \)/ml Targestar-SA microbubbles (Targeson,
Inc, USA) was performed at room temperature for 20 minutes. This reflects an excess of scFv
aiming for saturation of all streptavidin binding sites on the microbubbles. To remove unbound
scFv, purification of conjugated microbubbles was performed with a gradient centrifugation.

Microbubbles were incubated with anti-Penta-His AlexaFluor 488-conjugated monoclonal
antibody or R-phycoerythrin biotin (Sigma, Germany) and analyzed by FACS Calibur.

\textbf{Inverted flow chamber adhesion assay}

Inverted flow chamber \textit{in vitro} adhesion assays were performed with a glass capillary, which was
either coated with washed, ADP-activated platelets, or with 100\( \mu \)g/ml of collagen. To
accommodate the buoyancy of the microbubbles, the capillary was used in an inverted manner. A
syringe pump (PhD 2000, Harvard Apparatus, USA) was used in the aspiration mode to
withdraw the microbubble dispersion through the flow chamber at defined shear rates.

\textbf{In vivo mouse experiments}
Animals were injected with either non-coupled microbubbles (MB) or those coupled to scFv_{anti-LIBS} (LIBS-MB) or mut-scFv alone (control-MB) after ferric chloride-induced carotid artery injury. All experiments involving animals were approved by the AMREP Animal Ethics Committee (E/0910/2010/E).

Animals were anaesthetized with Ketamine (50mg/Kg, Parnell Laboratories, NSW, Australia) and Xylazine (10mg/kg, Troy Laboratories, NSW, Australia) and placed on a 37°C heater mat to prevent hypothermia.

**Femoral vein catheterization and ferric chloride injury to carotid artery of mice**

A catheter was placed into the femoral vein to facilitate injection. The left common carotid artery was dissected from circumferential connective tissues and a small filter paper saturated with 6% ferric chloride was placed under the carotid artery of the animal for 3 minutes, to induce injury, which results in the formation of a non-occlusive thrombus. Presence of non-occlusive thrombi was previously confirmed by histology^{15}.

**In vivo ultrasound molecular imaging of carotid artery thrombosis and thrombolysis**

Ultrasound of animals was performed with a Vevo770 high-resolution imaging system (VisualSonics Inc. Canada) using a 40 MHz RMV704 transducer. Animals were placed on the VisualSonics imaging station after ferric chloride injury was performed to the left carotid artery. Videos and images were acquired before, during and at several time points after injection of $1.5 \times 10^7$ microbubbles in 100 μl, as counted in a Neubauer hemocytometer. Videos and images were analyzed using a linear contrast agent imaging software (VisualSonics Inc.) or Image Pro Plus. Frames obtained before the injection of microbubbles were digitally subtracted from the frames obtained 20 minutes after injection.

To monitor thrombolysis, we injected 500 international unit/gram of urokinase
plasminogen activator (UPA) (Medac, Germany). Repetitive ultrasound imaging sequences were performed. The animals in the vehicle group were treated with saline. In addition, a 2\textsuperscript{nd} bolus of LIBS-MB was administrated 30 minutes after treatment with UPA.

\textbf{Conversion of grayscale area intensity value to decibels}

The Vevo770 ultrasound scanner uses a linear contrast mode. In order to convert grayscale values to decibel (dB) values, a “tissue-phantom” (12\% gelatine and 6\% Metamucil) was imaged to obtain grayscale values at 2 dB intervals from 10 dB to 30 dB. System dB and associated grayscale values were plotted on a X-Y graph to obtain a linear formula that enables a conversion from grayscale data points to dB values (Supplemental Figure 1). Equation:

\[
\text{Grayscale value} = 4.4673(\text{dB}) - 4.8626
\]

In addition, grayscale values were obtained from measurements of various microbubble concentrations imaged on a gel phantom (Supplemental Figure 2). The linear relationship was similar to the equation obtained using the “tissue-phantom” conversion and has a slope of 4.461 grayscale intensity values/dB.

\textbf{Statistical analysis}

Unless otherwise specified, data are expressed as mean ± standard error of the mean (SEM). Flow cytometry, flow chamber and data for thrombolysis were analyzed with two-way ANOVA repeated measures analysis using Bonferroni’s multiple-comparison post test. Decibel values for thrombi imaging were analyzed using a one-way ANOVA with Bonferroni correction for multiple testing. Differences were considered significant when p<0.05. In order to determine inter-observer reproducibility, the ultrasound images were analyzed by five separate investigators using Image-Pro Plus 6.0 software for the determination of grayscale area intensity units. For determination of intra-observer reproducibility, the ultrasound images were analyzed three times. The differences obtained, before and after the injection of the respective
microbubbles, were analyzed using the intra-class correlation coefficient analysis. All analyses containing more than two groups were corrected by post hoc analysis and the corrected p values are given. Statistical analyses were performed using Graphpad Prism 5.0.

Results

Cloning, in vivo biotinylation, and purification of scFv-AviTag™ constructs

The generation of scFv antibodies suitable for microbubble conjugation was achieved by adding a C-terminal AviTag™, an avidin tag, which enables the scFv to bind biotin. The success of DNA amplification and restriction digest was evaluated by electrophoresis (Figure 1A). Both constructs were visualized at the 1kB mark after amplification with PCR and restriction digest. The pAC6 plasmid was visualized at the 3kB mark after restriction digest. After the respective constructs were cloned into the pAC6 plasmid, transformed and purified, they were analyzed by gel electrophoresis. After production of the scFvs, SDS-PAGE and Western blot were used to prove successful purification and in vivo biotinylation of the recombinant protein (Figure 1B).

Evaluation of the functionality of the scFv-AviTag™ constructs by flow cytometry

To confirm retained binding capacity of the scFv to activated platelets, the functionality of the scFv-AviTag™ constructs was evaluated with anti-Penta-His AlexaFluor 488-conjugate (Figure 2A). No binding was observed for the isotype control to activated or non-activated platelets (1.69 ± 0.2 vs 1.47 ± 0.07, mean ± SD, AU, respectively, p=0.88), nor for the control mut-scFv construct (1.48 ± 0.02 vs 1.5 ± 0.09 AU, p=0.99). Incubation of activated platelets with the scFvanti-LIBS construct resulted in an increase in fluorescence intensity as compared to non-activated platelets (24.96 ± 3.47 vs 2.69 ± 0.16 AU, p<0.001). Similar results were obtained when efficiency of in vivo biotinylation and scFv functionality were determined using R-
phycoerythrin streptavidin on flow cytometry (Figure 2B). The specificity of scFv<sub>anti-LIBS</sub> targeting activated platelets was demonstrated in a whole blood flow cytometry assay, using dual staining with CD41-PE for platelet identification and AlexaFluor 488-coupled anti-Penta His antibody for scFv<sub>anti-LIBS</sub> staining (Supplemental Figure 3). No binding was observed in the non-platelet population of cells in both, the activated and non-activated group (6.55 ± 4.5 vs 1.61 ± 0.6 AU, respectively, p=0.97). An increase in fluorescence intensity was observed in the platelet population when the scFv<sub>anti-LIBS</sub> construct was incubated with activated whole blood samples as compared to non-activated whole blood samples (108.87 ± 38.8 vs 2.17 ± 0.2 AU, p<0.01). This demonstrates strong and specific binding of scFv<sub>anti-LIBS</sub> to activated GPIIb/IIIa.

Attachment of targeted microbubbles to activated platelets and microthrombi in vitro

Conjugation of scFv to microbubbles was demonstrated in flow cytometry (Figure 3A). Conjugated microbubbles showed a decrease in fluorescence intensity as observed with R-phycoerythrin biotin (p<0.01) and an increase with AlexaFluor 488-coupled anti-Penta His antibody (p<0.01). Binding performance in vitro was investigated by flow chamber experiments (Figure 4). After 5 minutes of continuous low shear rate (50s<sup>-1</sup>) over activated platelets, attachment of LIBS-MB was significantly greater when compared to either MB or control-MB (84 ± 10 vs 15 ± 2 vs 13 ± 2 microbubbles, respectively; adherent microbubbles, p<0.001, Movies 1-3). Competitive inhibition assays via pre-incubation of activated platelet monolayers with 1.5mg/ml of scFv<sub>anti-LIBS</sub> demonstrated a significant reduction of LIBS-MB attachment (p<0.001) to background level, as seen with control-MB and MB. After the initial 5 minutes, the shear rate was increased to 1000s<sup>-1</sup>. With the increase in shear rate, LIBS-MB remained firmly attached to the activated platelet coating, while both control-MB and MB alone dislodged (81 ± 12 vs 8 ± 1 vs 8 ± 1 microbubbles, respectively; p<0.001). The shear rate was further increased
to 6000s\(^{-1}\) where LIBS-MB continued to be firmly bound to the coated activated platelets, whereas the majority of control-MB and MB were dislodged (74 ± 5 vs 2 ± 1 vs 3 ± 1 microbubbles, respectively; p<0.001), demonstrating strong binding of LIBS-MB even under arterial shear conditions.

Similar results were obtained for flow chamber experiments with microthrombi (Figure 5), where attachment of LIBS-MB at 50s\(^{-1}\) was significantly higher compared to control-MB and MB (34 ± 3 vs 7 ± 1 vs 11 ± 1 microbubbles, respectively; p<0.01). When shear rates were increased to 1000s\(^{-1}\) and 6000s\(^{-1}\), LIBS-MB were more firmly attached than control-MB and MB (27 ± 1 vs 3 ± 1 vs 6 ± 1 microbubbles and 16 ± 1 vs 0 ± 0 vs 1 ± 0 microbubbles, respectively; p<0.01). In addition, experiments performed directly at 1500s\(^{-1}\), led to adhesion of LIBS-MB (66 ± 2 microbubbles per 2cm of the flow chamber capillary) to microthrombi, while no adhesion at all was noted in control-MB or MB. Moreover, LIBS-MBs attachment could be specifically localized to activated platelets in microthrombi (Figure 5). Direct dual fluorescence staining of LIBS-MB attached to activated platelets in a flow chamber experiment showed fluorescence labelled double staining of activated platelets and LIBS-MB (Figure 3B).

**In vivo molecular imaging of thrombus-formation**

Imaging of the mouse carotid artery on ultrasound typically shows luminal blood as black or dark color and microbubbles appear as a bright white color in the lumen (Figure 6A). 8 mice were injected with MB, 10 mice with LIBS-MB and 8 mice with Control-MB. The dB values of the thrombi on the carotid artery before injection of microbubbles were analyzed as baseline. A certain amount of background was observed with recirculating microbubbles. Since this blood pool background has clearly returned to baseline levels at 20 minutes (Supplemental Figure 4), we chose this time point for analysis. On real time ultrasound imaging, the thrombus visualized
after injection with LIBS-MB as a white and bright signal whereas the thrombus visualized after injection of control-MB or MB was dull (Figure 6B, Movies 4-6). Figure 6C shows the calculated differences in dB 20 minutes after microbubbles injection versus the preinjection baseline. For the LIBS-MB a significant increase in the dB occurred when compared with MB or control-MB (9.55 ± 1.7 vs 2.0 ± 1.4 vs 1.46 ± 1.3 dB, respectively, p<0.01). Although thrombus identification and quantification using these B-mode images provide the necessary scientific and clinical information, digital subtraction and color highlighting was used for demonstrational purpose and as an additional aid to facilitate immediate recognition of thrombi. Frames obtained before microbubble injection were subtracted from those obtained after microbubble injection. Areas brighter than the reference image are presented in green after analysis with digital subtraction (Figure 6D, Movies 4-6). Movement artifacts can be discriminated by their localization outside of the vessel lumen. Statistical analysis for inter-observer and intra-observer reproducibility were analyzed using the intra-class correlation coefficient analysis between the five different investigators or the three independent analyses by an individual observer. The obtained intra-class correlation coefficients were 0.984 and 0.993, respectively, demonstrating specific and reproducible antibody-targeted ultrasound imaging in vivo.

In vivo molecular imaging of thrombolysis
Pharmacological thrombolysis was used to further determine whether molecular ultrasound imaging with LIBS-MB is able to monitor the thrombus size, particularly any reduction in size. After the administration of urokinase plasminogen activator (UPA), a reduction in thrombus size and/or thrombus dislodgement could be directly observed (Figure 7, Movies 7-10). The baseline area before injection of UPA was set to 100% and the area was calculated every 5 minutes for 60 minutes (Figure 7). There were no significant changes in thrombus size in the vehicle group over
60 minutes, while thrombus size was significantly reduced in the UPA treated group (p<0.001, n = 3 each). Similar trends of results were obtained when comparing the thrombus area reduction to thrombus area grayscale intensity unit reduction (Figure 7B, C). Histology of carotid artery thrombi of vehicle or UPA treated mice confirmed the differences in thrombus size between the two groups (Supplemental Figure 5A, B). Finally, we imaged thrombi and thrombolysis without microbubbles (Supplemental Figure 5C, D) to demonstrate the lack of visual distinction of thrombi in conventional ultrasound without the LIBS-MB molecular imaging approach.

To investigate whether a 2\textsuperscript{nd} dose of microbubbles was required to successfully monitor the stages of thrombolysis, we performed two experiments comparing the time span between the 1\textsuperscript{st} dose injection of microbubbles and UPA administration (Supplemental Figure 6). There were no significant changes in dB observed after a 2\textsuperscript{nd} dose of LIBS-MB when LIBS-MB were recirculating and thus available during thrombolysis (p=0.56). However, if UPA treatment was administrated later than 30 minutes post LIBS-MB injection, a significant decrease in grayscale intensity unit could be seen (p<0.01). This value was subsequently increased, when a 2\textsuperscript{nd} dose of LIBS-MB was administrated (p<0.05).

In addition to longitudinal section imaging cross section ultrasound imaging was also performed. The results in monitoring thrombolysis of cross section analysis are similar in the overall outcome to that of the longitudinal section analysis (n=4, p=0.36, Supplemental Figure 7). However, due to the limited examinable area in the cross section of the mouse carotid artery (diameter: 0.3mm) and the technical challenges that accompany this approach, this variability limits accuracy of cross section imaging in mice.
Discussion

Direct ultrasound imaging of thrombi is a long sought-after technical achievement that could be broadly used in various clinical settings and would provide major benefits for patients. This study shows that single-chain antibodies (scFvs), which are specific for the activated GPIIb/IIIa receptor on platelets, can be conjugated to microbubbles and can be successfully used for selective in vivo ultrasound imaging of thrombi. To the best of our knowledge, this is the first demonstration of ultrasound imaging visualizing the attachment of scFv-targeted microbubbles to a thrombus in vivo. These data raise the possibility of a non-invasive and inexpensive tool to accurately diagnose early stages of thrombus formation and to monitor the success or failure of pharmacological thrombolysis.

Ultrasound has evolved from a crude anatomical imaging to a high-resolution and three-dimensional imaging tool. The recently emerging technique of molecular ultrasound imaging promises to provide a platform for functional characterization of diseased tissue without the need of invasive biopsies or other surgical procedures. Currently clinical molecular imaging is mainly focusing on MRI or PET/SPECT as imaging modality. However, molecular imaging with ultrasound will offer a real-time, inexpensive, radiation-free, and portable alternative. The latter point is of pivotal importance for example in coronary artery thrombosis, where a rapid diagnosis and therapy of myocardial infarction is of major benefit for patients.

Targeting of microbubbles via antibodies directed to cell adhesion molecules has been reported previously, including vascular cellular adhesion molecule-1, intercellular adhesion molecule-1, and P-selectin. Furthermore, recombinant cell adhesion proteins such as GPIbα have been successfully used to target ultrasound microbubbles to activated von Willebrand factor on the vascular endothelium. Single-chain antibodies (scFv) have not yet
been reported as targeting molecules for ultrasound microbubbles and they do bear several advantages: ScFvs are a minimal form of a functional antibody lacking the Fc region, therefore are minimally immunogenic\textsuperscript{29}. They are easy to modify with specific tags for detection and specific groups for bioconjugation, which avoids loss of function due to non-specific coupling. The cost of production in prokaryotic hosts is generally low\textsuperscript{29}.

Typically studies in the past successfully demonstrated molecular imaging in tissues where flow velocities are low or at sites with low wall shear stress\textsuperscript{30-33}. Dual targeted microbubbles, commonly including selectins as targets, have been proposed and tested in vitro as a means to allow targeting under higher wall shear stress\textsuperscript{34,35}. In our study, flow chamber adhesion assays demonstrated that LIBS-MB on its own, as a mono targeting approach, is sufficient to bind fast and firmly to its target, the activated GPIIb/IIIa receptor. Our in vivo data provide proof of concept that our scFv-conjugated microbubbles are suitable for molecular imaging of arterial thrombosis under high wall shear stress and high flow velocity.

Platelet-targeted microbubbles can be used for monitoring success or failure of thrombolysis in ultrasound. The mechanism for this is either based on the recirculation of a sufficient number of microbubbles or on a 2\textsuperscript{nd} injection of microbubbles. In the first scenario, recirculating microbubbles are available to “stain” the disintegrating, remaining thrombus. In the second scenario, after a longer time period (e.g. > 20 minutes), when the number of recirculating microbubbles has dropped substantially, a 2\textsuperscript{nd} injection of microbubbles is needed to visualize the remaining thrombus.

Elegant pioneering studies by others demonstrated the feasibility of targeting the integrin GPIIb/IIIa\textsuperscript{30,32,33}. MRX408, an arginine-glycine-aspartic acid (RGD) analog and the non-activation specific antibody fragment abciximab have been used in particular for this
approach\textsuperscript{30,32,33}. RGD analogs are ligand-mimetics that bind to all circulating platelets but in addition also to other cells, based on their cross-reactivity towards other RGD ligand recognizing integrins. Similarly, abciximab binds to all circulating platelets and it also cross-reacts with other integrin receptors\textsuperscript{36,37}. Furthermore, abciximab is used as a therapeutic drug, preventing platelet aggregation and thrombus formation e.g. in patients undergoing percutaneous coronary interventions. Due to its strong affinity to the GPIIb/IIIa receptor, abciximab inhibits platelet function for several days, which comes at the cost of a potentially increased risk of bleeding\textsuperscript{38,39}. The scFv\textsubscript{anti-LIBS} is exclusive in its specificity for the target molecule GPIIb/IIIa and the activated conformation of this integrin receptor, as well as the preservation of the GPIIb/IIIa ligand binding function; thereby representing a unique targeting approach for molecular imaging\textsuperscript{9-15}.

There are several clinical scenarios in which ultrasound molecular imaging with micro-bubbles targeted to the activated GPIIb/IIIa receptor could be useful. Following the data from the \textit{in vivo} mouse model, it appears to be feasible using ultrasound molecular imaging to detect a thrombus in the carotid artery, providing the ability to identify high risk plaques and thrombi prone to dislodge and to cause strokes. This diagnostic technique might also provide us with the ability to distinguish between arterial platelet rich thrombi and venous thrombi, which is primarily coagulatory factor driven containing less platelets. Furthermore, molecular ultrasound imaging with platelet targeted microbubbles allows monitoring of therapeutic thrombolysis. This could be used to detect success or failure of therapeutic interventions in thrombotic disease.

Another potential area of application that is of high clinical interest is the molecular imaging/detection of thrombi in the cardiac chambers, with a focus on the left atrial appendage. Previous data indicate that scFv\textsubscript{anti-LIBS} can be used for detection of older thrombi\textsuperscript{15}. Therefore, our scFv\textsubscript{anti-LIBS} might be suitable to detect thrombi formed over longer time periods in the left
atrium e.g. during atrial fibrillation. However, the influence of thrombus age on scFv\textsubscript{anti-LIBS} has to be systematically evaluated further.

**Limitations**

The Vevo770 small animal ultrasound system that we employed utilizes a linear contrast agent imaging software, while clinical scanners typically use non-linear software. However, a clinical scanner is not tailored for imaging of small vessels such as the carotid artery of mice due to the requirement of high frequency transducers. Our experiments with the clinical scanner iE33 and the use of phantoms can only provide an indication that results from the proof-of-concept studies in mice are transferrable to the clinical setting (Supplemental Figure 7). Ultimately, the suitability of our novel approach of targeted molecular imaging of thrombi remains to be tested and proven in humans. Also, it should be highlighted that the microbubble dose used in mice was quite large and despite this the signal intensity at the clot was only modest. The relation between microbubble dose and signal intensity also will have to be ultimately established in patients. However, the potential of our targeting approach to provide enrichment of microbubbles at the clot may be of particular benefit in this regard.

Microbubble destruction via ultrasound scanning might be of concern. We imaged at a frame rate of 12Hz and used a transducer with a low mechanical index of 0.14. With these settings, an extensive destruction of microbubbles would not be expected\textsuperscript{40}. If microbubble destruction would occur, it happened at a level that did not prevent contrast enhanced ultrasound imaging in our *in vivo* experiments.

The use of the biotin/streptavidin binding for scFv coupling offers major advantages such as flexibility and rapid coupling. However, although biotinylated drugs and avidin based reagents have been recently introduced in the clinic, potential problems with immunogenicity may limit
their use in humans. Therefore, we are currently developing novel enzymatic bioconjugation techniques, which can be adapted to be used with our ultrasound molecular imaging approach. One such approach for bioconjugation is the use of the Staphylococcus aureus enzyme Sortase A for coupling of scFvs to sulfhydryl (SH) functionalized microbubbles. This provides highly functional microbubbles as shown in flow chamber adhesion assays (Supplemental Figure 8).

**Perspective**

In this study, we chose to target microbubbles to the activated GPIIb/IIIa receptor, which is an ideal target being highly abundant and uniquely cell-specific. Platelets are major players throughout the development of atherosclerosis, in particular in the final stage of plaque rupture resulting in arterial thrombosis. Atherothrombosis is the major cause of acute myocardial infarction and sudden death, yet definitive detection of the rupture-prone, vulnerable plaques is typically made only post mortem. These pathological studies have shown that thrombi containing activated platelets exist long before the presentation of sudden coronary death indicating that vessel occlusion is often preceded by (micro)rupture associated with non-occlusive events. Our method of diagnostic ultrasound imaging with LIBS-MB, might offer the sensitivity and specificity to identify those microthrombi before rupture. Furthermore, given the ability to specifically target thrombi, anti-thrombotic or fibrinolytic drugs could be incorporated into the microbubbles. Ultrasound bursts can be utilized to destroy the microbubbles and thus release the drug load locally at the thrombus.

**Conclusions**

Our study indicates that LIBS-MB bind specifically to activated platelets in vitro and in vivo thereby facilitating ultrasound molecular imaging of thrombi. Thrombus size and its reduction in
size following pharmacological fibrinolysis can be monitored in real-time, offering early
diagnosis and monitoring of therapeutic thrombolysis. This proof of concept study justifies
further development towards ultrasound diagnosis of thrombotic disease and monitoring of
thrombolytic therapy in humans. To achieve this, further pre-clinical testing, including charac-
terization of targeting of thrombi in different vessel areas and optimizations of biocompatible
conjugation strategies are the next steps towards transition into clinical application.

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property of the described single-chain antibody and its derivatives. A.N is an employee of
VisualSonics.

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Figure Legends:

**Figure 1.** Generation of scFv for microbubble conjugation. **A.** Electrophoresis with 0.8% agarose gel: pAC6 plasmid (4186bp) after double cut restriction digest, mut-scFv (892bp) and scFv<sub>anti-LIBS</sub> (925bp) after PCR amplification. **B.** Western blot analysis: successful protein purification of scFv demonstrated with an HRP-coupled anti-6x His-tag antibody and in vivo biotinylation of scFv demonstrated with streptavidin HRP.

**Figure 2.** Flow cytometry assay demonstrating the functionality of scFvs and efficiency of in vivo biotinylation. **A.** Functionality of scFv was proven with an AlexaFluor 488-coupled anti-Penta-His antibody in flow cytometry. Bar graphs depict the median fluorescence intensity values of three independent experiments. Representative fluorescence histograms are shown underneath the bar graphs. **B.** Functionality of scFvs as well as the efficiency of in vivo biotinylation was evaluated using R-phycocerythrin streptavidin in flow cytometry (mean ± SD, ***p<0.001). These assays were analyzed using a two-way ANOVA repeated measures with Bonferroni post test.

**Figure 3.** Flow cytometry and flow chamber adhesion assay demonstrating the successful conjugation of scFvs to microbubbles. **A.** Microbubbles were identified by flow cytometry. **B.** Detection of scFv<sub>anti-LIBS</sub> by AlexaFluor 488-coupled anti-Penta His antibody on microbubbles (FL1 intensity: **p<0.01) and reduced intensity of Biotin-PE (FL2 intensity: **p<0.01), thereby demonstrating that excess scFv<sub>anti-LIBS</sub> at a saturation concentration of 200µg competitively occupied streptavidin binding sites on the microbubbles. **C.** Direct fluorescence staining of
LIBS-MB attached to activated platelets in a flow chamber experiment, showing fluorescence double staining of activated platelets (P-selectin) and LIBS-MB (AlexaFluor 488-coupled anti-Penta His antibody).

**Figure 4.** Flow chamber adhesion- and detachment assay showing strong binding of LIBS-MB on a monolayer of activated platelets. A. Microbubble attachment under flow at a low shear rate of 50s⁻¹: The number of microbubbles was counted after 5 minutes of continuous flow. Attachment of LIBS-MB was significantly higher than for non-targeted control-MB or non-coupled MB. Attachment of LIBS-MB was also significantly higher in comparison to saturating competitive inhibition of the platelet monolayer by preincubation with scFv_anti-LIBS_ (LIBS-MB blocked). B. After initial 5 minutes of continuous flow, shear rate was increased to 1000s⁻¹. LIBS-MB remained firmly attached to the activated platelet coating, while control-MB and MB were dislodged. Similar results were obtained when the flow was increased to 6000s⁻¹. Mean ± SEM for three independent experiments is given in A and B (***p<0.001). C. Representative microscopy images of flow chamber adhesion assays demonstrating that more LIBS-MBs attaches to the activated platelet monolayer as compared to LIBS-MB (blocked), MB and control-MB. Black arrows point to representative microbubbles. These assays were analyzed using a two-way ANOVA repeated measures with Bonferroni post test.

**Figure 5.** Flow chamber adhesion- and detachment assays showing strong binding of LIBS-MB to microthrombi on collagen fibres. A. Microbubble attachment under flow at a low shear rate of 50s⁻¹: The number of microbubbles was counted after 5 minutes of continuous flow. Attachment of LIBS-MB was significantly higher than for control-MB or MB. After initial 5 minutes of
Demonstration of imaging with microbubbles with continuous flow, the shear rate was increased to 1000s⁻¹. LIBS-MB remained firmly attached to the activated platelet coating, while control-MB and MB were dislodged. Similar results were obtained when the shear rate was increased to 6000s⁻¹. Mean ± SEM for three independent experiments is given (**p<0.01, ***p<0.001). B. Representative microscopy images of flow chamber adhesion assays demonstrating that more LIBS-­MBs attached to the microthrombi on collagen coating of capillaries as compared to MB and control-MB. C. Representative microscopy images of PAC-1-FITC staining for platelets, demonstrating that LIBS-MBs attached specifically to activated platelets in microthrombi. These assays were analyzed using a two-way ANOVA repeated measures with Bonferroni post test.

**Figure 6.** Demonstration of imaging with microbubbles with *in vivo* targeting towards left carotid artery thrombi, which are induced by ferric chloride injury in mice. A. Representative ultrasound images of the carotid artery before and immediately after the injection of non-targeted Targeson SA microbubbles (MB) injection. Ultrasound images of the carotid artery showed a dark lumen area before the injection of microbubbles. When microbubbles were injected via the femoral vein of mice, ultrasound images demonstrated that the dark lumen area of the carotid artery became a bright and white area. B. Representative images of real time ultrasound images of thrombi 20 minutes after injection of microbubbles. Images acquired 20 minutes after the injection of MB or control-MB showed the dark lumen of the carotid artery with an intense gray area where the thrombus sits. Images acquired 20 minutes after injection of LIBS-MB showed the dark lumen of the carotid artery with a white and bright area where LIBS-MBs were attached to the thrombus. Areas of analysis for grayscale intensity are outlined in yellow squares. C. Increased dB values via contrast enhanced ultrasound imaging (mean ± SEM)
of thrombi in the left carotid artery of mice. The baseline dB values of the thrombi before injection of microbubbles were analyzed. Differences in dB values were calculated for values obtained 20 minutes after injection of microbubbles against its baseline. A significant increase in the dB occurred 20 minutes after the injection of LIBS-MB (n=10) when compared MB (n=8, **p<0.01) or control-MB (n=8, **p<0.01). These assays were analyzed using a one-way ANOVA repeated measures with Bonferroni post test. D. Representative images of digital subtraction of frames, where areas, which are brighter than baseline before the injection of microbubbles, are shown in green. Images of thrombi 20 minutes after injection of LIBS-MB are brighter as compared to those of MB or control-MB.

**Figure 7.** Monitoring of thrombus size upon pharmacological thrombolysis via ultrasound imaging. A. Representative images of thrombi from both vehicle (saline) and pharmacological thrombolysis (UPA) treated groups. Ultrasound images were obtained every 5 minutes and thrombus size was calculated. Thrombus size of vehicle group stayed constant while those administrated with UPA fluctuates over 60 minutes of monitoring. B. Baseline areas before injection of UPA and vehicle were set to 100% and areas were calculated every 5 minutes for 60 minutes. Thrombus sizes were traced and calculated using VisualSonics software. C. Baseline areas before injection of UPA and vehicle were set to 100% and grayscale intensity units were calculated every 5 minutes for 60 minutes. Grayscale intensity units were analyzed using Image Pro Plus. Area of interest for this analysis is outlined in red. The two groups were compared using repeated measures ANOVA over time with additional corrected Bonferroni post tests at each time point (mean % ± SEM, comparison of UPA treated mice vs vehicle treated mice: *p<0.01, **p<0.01, ***p<0.001, n=3 each).