

Supplement Material

Supplemental Methods

Generation, expression, and purification of single-chain antibodies and single-chain urokinase plasminogen activator

The scFV_{anti-LIBS} construct was generated, expressed, and purified as previously described¹. The recombinant scuPA with an LPETG peptide motif at the C-terminus was cloned into the pSecTag2A vector system. Polymerase chain reaction (PCR) was performed with a sense primer (5'– CTG AGA ATT CTC CTG AAG TTC CAG TGC GGC CAG A –3') that annealed at the beginning of the scuPA sequence, and an anti-sense primer (5'– TCA TCA CCA CCA CTG ATG AGA GGC GGC CGC CTC G –3') that annealed directly to the 6x His-tag region at the end of the scuPA. The sense strand includes the *EcoRI* restriction site and the anti-sense strand includes the *NotI* restriction site.

After amplification by PCR, the construct and vector were digested with the restriction enzymes *EcoRI* and *NotI* (both NEB, USA). Electrophoresis on a 0.8% agarose gel with SYBR[®] Safe DNA gel stain (Invitrogen, USA) was utilized to analyze DNA amplified by PCR and restriction digests. Ligation of the plasmids was performed with T4 ligase (NEB, USA) at 16°C overnight. The resulting plasmid constructs were then transformed into BL21 Star *E. coli* cells (Invitrogen, USA). The protein construct was produced using mammalian cells via human embryonic kidney cells (HEK293F) suspension culture with polyethylenimine (Polyscience Inc., Germany). 1µg/ml of DNA plasmid and 3µg/ml polyethylenimine (PEI) were used for transfection at cell density of 2 x 10⁶ cells/ml. The cells were supplemented with 6g/L glucose and 5g/L lupin, and kept for 7 days at 37°C, 8% CO₂ and shaking at 110 rpm. The glucose level was maintained at a final concentration of 5–6g/L. The cells were then centrifuged at 3000 xg for 15 min at 4° C and supernatant was collected for protein purification. Our constructs included a 6x His-tag[®], which was used for purification with nickel-based metal affinity chromatography (Invitrogen, USA) according to the manufacturer's instructions.

Evaluation and biotinylation of scuPA constructs

The purity of the proteins was analyzed using SDS-PAGE and Western blotting. Anti-6x His-tag[®] antibody horseradish peroxidase was used to detect the purified scuPA constructs. The addition of the LPETG motif allowed addition of a GGG-biotin peptide onto the scuPA construct using recombinantly produced *Staphylococcus aureus* sortase A enzyme. The reaction was performed as previously described^{2,3}, using a 1:3:3 molar ratio of scuPA:GGG-biotin:sortase A enzyme. The reaction was performed in sortase reaction buffer (0.5mM calcium chloride, 50mM Tris, 150mM sodium chloride, pH8) for 5 hours at 37°C, shaking at 750rpm. The sortase transpeptidase reaction cleaved off the His-tag motif, which was situated behind the LPETG motif. Therefore, the biotinylated scuPA constructs did not contain a His-tag. The non-biotinylated scuPA and recombinant sortase A, which were also tagged with a His-tag, were removed using TALON[®] metal affinity resin (Clontech, USA) columns, and the excess GGG-biotin was removed via dialysis using a 10 K MWCO membrane (Thermo Fisher Scientific, USA).

Flow cytometry

Platelet-rich plasma (PRP) was obtained by centrifugation of blood which was collected from healthy volunteers. Diluted PRP was either non-activated or activated with 20μM adenosine diphosphate (ADP) before incubation with the purified scFv_{anti-LIBS} constructs, followed by an AlexaFluor 488-coupled labeled anti-His-tag antibody for detection. The activation status of platelets was determined either by a FITC-labeled monoclonal PAC1 antibody (BD Bioscience, USA) or by a PE-labeled monoclonal antibody directed against CD62P (BD Bioscience, USA). Samples were fixed with 1x CellFix (BD Bioscience, USA) and analyzed by FACS Calibur (BD Bioscience, USA).

The platelet population was distinguished using the forward and sideward light scatter profile. A gate was set around the platelets and 10,000 cells were analyzed. For single staining, AlexaFluor 488-coupled fluorescence was used to quantify the amount of platelet-bound scFvs. The scFv binding was expressed as mean fluorescence intensity.

Urokinase activity assay

Urokinase activity was determined with a chromogenic substrate assay. Comparison between clinically used uPA (Medac GmbH, Germany) and scuPA was made on the basis of equal urokinase activity. 100nmol/L of scuPA was monitored against commercial uPA standards (0–2000U/mL) used as positive controls. 0.1U/L plasmin was added to activate the scuPA. 0.5mmol/L of S2444 (Chromogenix, Italy) was added and samples were measured on a Victor3V Multi-label counter (PerkinElmer, USA) at a wavelength of 405nm every 5 min over a period of 60 min. Reactions were performed using 100µl total volume.

Plasmin activity assay

The conversion of plasminogen to plasmin using commercial uPA or scuPA was determined in microtiter plates using a chromogenic substrate. 10nmol/L of scuPA was monitored against commercial uPA (0–15U/ml) of and were incubated with 400nmol/L of human glu-plasminogen (Sigma-Aldrich, Germany) and 1mmol/L of S2251 (Chromogenix, Italy). Samples were measured using the Bio-Rad Benchmark Plus (Bio-Rad, USA) at a wavelength of 405nm every 30 sec over a period of 60 min. Reactions were performed using 100µl total volume.

96-well plate fibrinolysis assay

Fibrinolysis activity was determined in microtiter plates. PRP was mixed with 8 mM calcium chloride, 1:50 thromboplastin (Siemens, USA), and 20µM thrombin receptor activator peptide (Sigma-Aldrich, Germany), leading to platelet activation and clotting. The mixture was then incubated with either commercial uPA or biotinylated scuPA. Samples were measured using the Bio-Rad Benchmark Plus at a wavelength of 405nm every minute over a period of 60 min.

Flow-chamber adhesion assay

Flow-chamber *in vitro* adhesion assays were performed with glass capillaries (Vitrotubes, USA), which were coated overnight with 100µg/ml collagen (Takeda, Austria). Whole blood was collected

from healthy volunteers and was perfused through the capillaries to form microthrombi. 20µg of scFV_{anti-LIBS} and 20µg of scuPA were added to target-ready microbubbles (VisualSonics Inc., Canada), according to the manufacturer's instructions, in order to form targeted theranostic microbubbles (TT-MBs). 1x10⁶ microbubbles/ml were perfused through the capillaries for 5 minutes. Microthrombi and the binding of microbubbles were visualized with bright field microscopy using the IX81 Olympus microscope (Olympus, USA) and Cell[^]P 1692 software.

In vivo mouse experiments

Male C57BL/6 mice, 6 to 7 weeks of age (20 to 25g), were used and maintained at the Alfred Medical Research and Education Precinct Animal Services, and assigned randomly to the different groups. The animals were anesthetized with ketamine (50mg/kg, Parnell Laboratories, NSW, Australia) and xylazine (10mg/kg, Troy Laboratories, NSW, Australia). Fur was removed by shaving with cream (Dove, Australia). Mice were placed on a 37°C heater mat to prevent hypothermia. All experiments involving animals were approved by the Alfred Medical Research and Education Precinct Animal Ethics Committee (E/1406/2013/B).

Femoral vein catheterization and ferric chloride injury model for in vivo ultrasound molecular imaging

A catheter was placed into the femoral vein to facilitate injection. Ultrasound of animals was performed with a Vevo2100 high-resolution imaging system (VisualSonics Inc., Canada) using the 40MHz MS550D transducer. Animals were placed on the imaging station after ferric-chloride-induced injury was performed to induce a thrombus. A small filter paper (2mm x 1mm) saturated with 6% ferric chloride was placed under the left carotid artery of the animal for 3 min. Animals were injected with either LIBS-MBs with commercial uPA, TT-MBs, or LIBS-MBs with saline as the vehicle control.

Videos and images were acquired before, during, and at several time points after injecting 1.5×10^7 microbubbles in a total volume of 100 μ l. We have recently established this ultrasound imaging methodology for the detection of thrombi and the monitoring of thrombolysis^{1,4}. We injected a high dose of 500U/g of commercial uPA (Medac, Germany), a low dose of 75U/g of commercial uPA, or saline as the vehicle control. Repetitive ultrasound imaging sequences were performed every 5 min for 45 min after thrombolysis. Analysis was performed using VisualSonics imaging software (VisualSonics Inc., Canada). The microbubbles used in this study could also be visualized on an ultrasound scanner that is used on patients (iE33 with L15-7io transducer, Philips).

Histology with hematoxylin and eosin staining

The carotid arteries of mice were embedded in optimal cutting temperature (OCT) compound (Sakura Finetechnical), frozen in liquid nitrogen, and stored at -20°C until sectioning. 6 μ m thick cross-sections of cryosection were prepared using a cryostat (Leica, CM1950) and stained with hematoxylin and eosin.

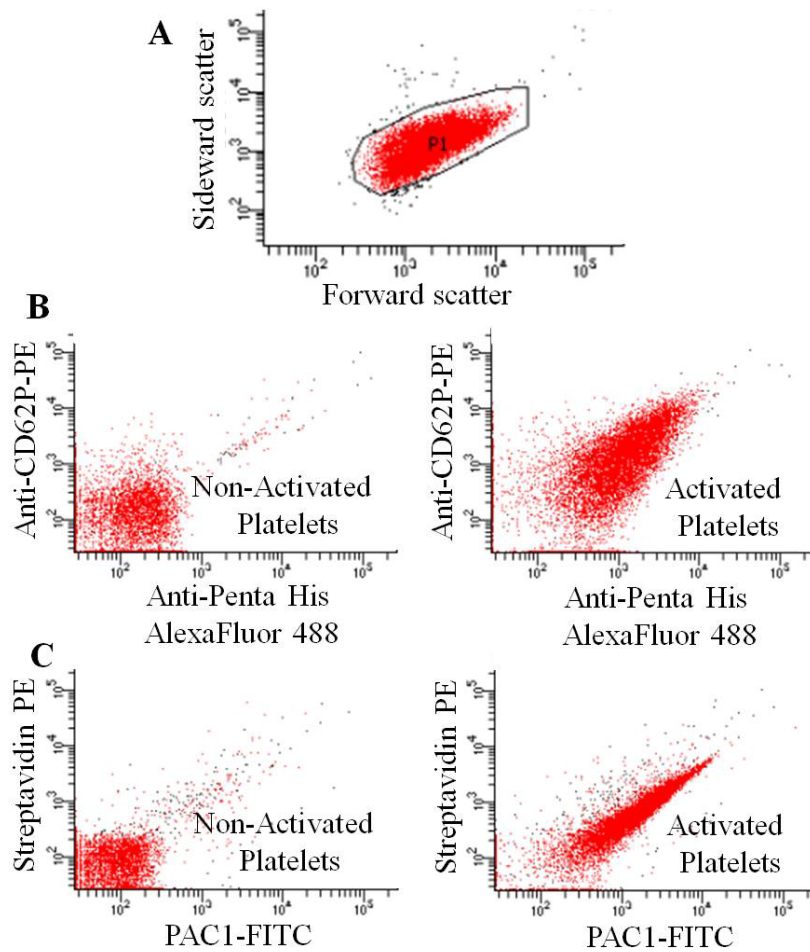
Assessment of tail bleeding time

An incision to reveal the left jugular vein was made in order to insert a catheter to facilitate injections. 1 min after injecting TT-MBs, LIBS-MBs with commercial uPA, or saline as the vehicle control, the tail was transected 5mm from the tip and immediately submersed in saline at 37°C . The bleeding time was monitored and recorded as the time needed for the cessation of a visible blood flow for 1 min.

Statistical analysis

Unless otherwise specified, data is expressed as mean \pm standard error of the mean (SEM). Flow cytometry and data for thrombolysis were analyzed with two-way ANOVA repeated measures analysis using Bonferroni's multiple-comparison post-test. 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 6.0.

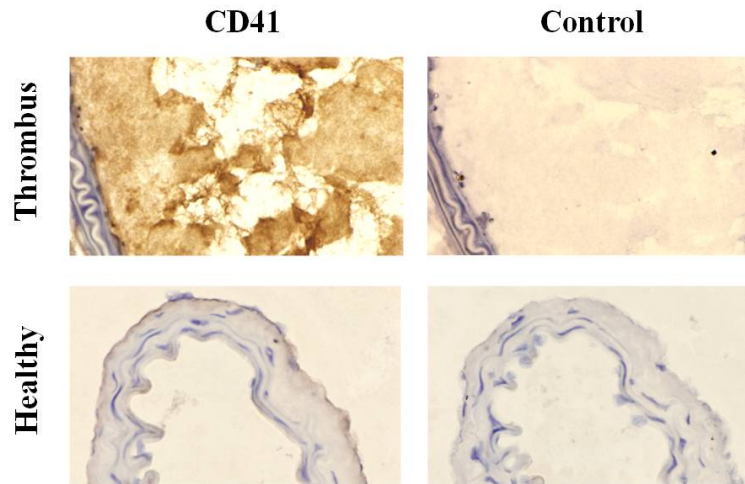
Supplemental Results



Supplemental Figure 1: Flow cytometry assay demonstrating the specific binding of scFv_{anti-LIBS} to activated platelets. Specificity of scFv_{anti-LIBS} targeting to activated platelets was shown in a flow cytometry assay using platelet rich plasma. **A.** Representative dot-plot for gating of platelets using the forward-sideward scatter plot. 10000 events in the drawn gate were used for further analysis. **B.** Representative dot plot showing dual staining with anti-CD62P-PE for the identification of activated platelets and AlexaFluor 488-coupled anti-Penta-His antibody for secondary staining/detection of scFv_{anti-LIBS} binding to activated platelets. There was a rightwards and upwards shift of both fluorescence intensity when platelets were activated with ADP. **C.** Representative dot plot showing dual staining with Streptavidin-PE for scFv_{anti-LIBS} staining and PAC1-FITC for activated platelet identification. Likewise, there was a rightwards and upwards shift of both fluorescence intensities when platelets were activated with ADP.

Thrombus area (cm²)

Supplemental Figure 2: Thrombus area was reduced after treatment with TT-MB. Thrombus size was significantly decreased after the treatment with TT-MB and those injected with LIBS-MB and treated with high dose comm. uPA. Measurement of thrombus area did not show changes after injection of LIBS-MB and given treatment with low dose comm. uPA or saline. Groups were compared by use of repeated measures Two-way ANOVA with Bonferroni post tests (Mean % \pm SEM; *** $p < 0.001$, $n \geq 3$ each).



Supplemental Figure 3: Representative images of immunohistochemistry for platelets in the thrombus using an anti-CD41 antibody. The anti-CD41 antibody is specific for the integrin subunit alpha IIb. The thrombus in the vessel was induced by ferric chloride injury and showed a dark brown staining with the anti-CD41 antibody (upper left). In order to demonstrate the specific CD41 immunohistochemistry staining to the thrombus, we used an isotype control (right panel). A healthy vessel with no thrombus was stained with the anti-CD41 antibody as additional control (lower left).

Video Legends

Video 1: *In vivo* real time molecular ultrasound imaging and post processed digital subtraction allows the identification of a thrombus in the carotid artery of a mouse. LIBS-MB and control (saline) were injected intravenously. Contrast signal depicting areas that are brighter in green is observed via digital subtraction of frames from the baseline image taken before injection of microbubbles. After injection of LIBS-MB and saline, the dark lumen of the carotid artery turns green, identifying a thrombus in the carotid artery. Ultrasound images were acquired every 5 min for a time period of 45 min. Both the size of the thrombus and its contrast intensity remains stable during the 45 min of ultrasound monitoring.

Video 2: *In vivo* real time molecular ultrasound imaging and post processed digital subtraction shows no change in thrombus size in LIBS-MB and low dose urokinase treated mice. Ultrasound images were acquired as described for video 1. The size of the thrombus and the contrast intensity remains stable during the 45 min of ultrasound monitoring.

Video 3: *In vivo* real time molecular ultrasound imaging and post processed digital subtraction shows successful thrombolysis with LIBS-MB and high dose of urokinase treated mice. Ultrasound images were acquired as described in video 1. Reduction in thrombus size and contrast intensity can be seen during the 45 min of ultrasound monitoring.

Video 4: *In vivo* real time molecular ultrasound imaging and post processed digital subtraction showing successful thrombolysis in targeted theranostics microbubbles (TT-MB) treated mice. Ultrasound images were acquired as described in video 1. Reduction in thrombus size and contrast intensity can be seen during the 45 min of ultrasound monitoring, demonstrating the imaging/monitoring potential with the concurrent effective thrombolysis at systemic concentrations that do not cause bleeding time prolongation.