# Towards Effective and Safe Thrombolysis and Thromboprophylaxis: Preclinical Testing of a Novel Antibody-Targeted Recombinant Plasminogen Activator Directed Against Activated Platelets

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## ABSTRACT

*Rationale*: Fibrinolysis is a valuable alternative for the treatment of myocardial infarction when percutaneous coronary intervention is not available in a timely fashion. For acute ischemic stroke, fibrinolysis is the only treatment option with a very narrow therapeutic window. Clinically approved thrombolytics have significant drawbacks, including bleeding complications. Thus their use is highly restricted leaving many patients untreated.

*Objective*: We developed a novel targeted fibrinolytic drug that is directed against activated platelets.

*Methods and Results*: We fused single-chain urokinase plasminogen activator (scuPA) to a small recombinant antibody (scFv$_{SCE5}$), which targets the activated form of the platelet-integrin GPIIb/IIIa. Antibody binding and scuPA activity of this recombinant fusion protein were on par with the parent molecules. Prophylactic *in vivo* administration of scFv$_{SCE5}$-scuPA (75U/g BW) prevented carotid artery occlusion after ferric chloride injury in a plasminogen-dependent process compared to saline (p<0.001) and blood flow recovery was similar to high dose non-targeted urokinase (500U/g BW). Tail bleeding time was significantly prolonged with this high dose of non-targeted urokinase, but not with equally effective targeted scFv$_{SCE5}$-scuPA at 75U/g BW. Real-time *in vivo* molecular ultrasound imaging demonstrates significant therapeutic reduction of thrombus size after administration of 75U/g BW scFv$_{SCE5}$-scuPA as compared to same dose of a mutated, non-targeting scFv-scuPA or vehicle. The ability of scFv$_{SCE5}$-scuPA to lyse thrombi was lost in plasminogen-deficient mice, but could be restored by intravenous injection of plasminogen.

*Conclusions*: Targeting of scuPA to activated GPIIb/IIIa allows effective thrombolysis and the potential novel use as a fibrinolytic agent for thromboprophylaxis without bleeding complications.

**Keywords:**
Platelet, thrombosis, thrombolysis, glycoprotein IIb/IIIa platelet inhibitor, urokinase

**Nonstandard Abbreviations and Acronyms:**

| | |
|---|---|
| ADP | Adenosine diphosphate |
| BW | Body weight |
| CHO | Chinese hamster ovary |
| CRP | Collagen-related peptide |
| GPIIb/IIIa | Glycoprotein IIb/IIIa |
| LIBS | Ligand-induced binding sides |
| LIBS-MBs | Ligand-induced binding sides targeted microbubbles |
| PA | Plasminogen activators |
| PECAM-1 | Platelet endothelial cell adhesion molecule 1 |
| PPP | Platelet poor plasma |
| PRP | Platelet rich plasma |
| scFv | Single-chain antibody fragments |
| scuPA | Single-chain urokinase plasminogen activator |
| tPA | Tissue plasminogen activator |
| TRAP | Thrombin receptor activating peptide |
| uPA | Urokinase plasminogen activator |

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# INTRODUCTION

Acute thrombosis causing vessel occlusion and resulting in ischemic complications such as myocardial infarction and stroke is a major cause of death and disability[1]. Atherosclerosis, as the main underlying disease, is a progressive inflammatory process caused by accumulation of lipids and lipid-loaded macrophages in the artery wall along with the adherence of activated platelets and monocytes[2,3]. Upon rupture of atherosclerotic plaques, thrombogenic material is exposed, which leads to platelet activation, aggregation and vessel occlusion.

Restoring and maintaining perfusion to the affected tissue is crucial and treatment of acute thrombosis using plasminogen activators (PAs) such as streptokinase, tissue plasminogen activator (tPA) and urokinase (uPA) have been proven to be beneficial[4]. However, the clinical utility of these agents has been limited by side effects such as bleeding complications through lysis of hemostatic clots and neurotoxicity in the case of stroke treatment with tPA[5,6]. In addition, thrombolytics are rapidly neutralized and their large size hampers clot penetration causing a delay in restoring perfusion[7].

Further research is warranted to improve the safety and efficacy of fibrinolytic therapy. Antibodies as tools to deliver thrombolytic drugs specifically to developing and existing clots are a promising alternative for locally enhanced potency and reduced side effects.

Platelets play a key role in thrombus formation[3] and are therefore a target of interest for the combination with thrombolytic therapy. Targeting the fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa) on platelets has been extensively studied for the prevention of platelet aggregation and has lead to the reduction of ischemic complications[8-10], especially in patients undergoing percutaneous coronary intervention. However, in large clinical trials, contradictory to its anticipated therapeutic effects, administration of GPIIb/IIIa receptor blockers in combination with fibrinolytic agents has shown little improvement in mortality, mainly due to excess bleeding[11,12], limiting the broader utilization of the combination of GPIIb/IIIa inhibition and fibrinolysis. This can be in part attributed to the fact that all currently available GPIIb/IIIa inhibitors target the receptor regardless of the activation status thereby causing complete systemic inhibition of platelet aggregation and firm adhesion. In addition, the ligand-mimetic properties of clinically used GPIIb/IIIa inhibitors also can lead to paradoxical platelet activation imitating ligand-induced outside-in signaling[13,14]. We have previously addressed these issues by generating conformation-specific small recombinant single-chain antibody fragments (scFv) that only target the activated GPIIb/IIIa receptor on platelets avoiding outside-in signaling and allowing effective platelet inhibition without bleeding time prolongation[15,16]. The potential of these scFv to be used as targeting tools has been demonstrated by their use for non-invasive diagnostic molecular imaging of thrombosis and inflammation[17-19].

Here we use the targeting potential of a specific single-chain antibody SCE5, which selectively binds activated GPIIb/IIIa, to enrich the plasminogen activator scuPA at the site of platelet activation. We describe the recombinant fusion of this scFv to scuPA and the in vitro and in vivo testing of this novel targeted drug. This therapeutic approach facilitates highly effective thrombolysis without bleeding time prolongation. In addition, the described ability to inhibit thrombus formation together with the effective targeting at low systemic concentration could potentially allow the unique application of a fibrinolytic drug in a thromboprophylactic approach.

# METHODS

A detailed description of the methods is provided in the online-only Data Supplement.

*Generation, expression and purification of single-chain antibodies and single-chain urokinase plasminogen activators.*

Two different scFvs (activated GPIIb/IIIa-targeted scFv$_{SCE5}$ and non-targeted scFv$_{mut}$) were fused with active scuPA and cloned into the pSectag2A vector system. Briefly, both scFv-scuPA plasmid constructs were produced using the human embryonic kidney cells (H293F) suspension culture. All scFv-scuPAs contain a 6x His-tag, which was used for purification with nickel-based metal affinity chromatography (Invitrogen, USA).

*Evaluation of the scFv-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 scFv-scuPA constructs. The scuPA in the fusion protein was converted to the active form using plasmin to cleave the Lys-158 to Ile-159 bond.

*Static adhesion assay.*

The specificity of the scFv$_{SCE5}$ targeting activated GPIIb/IIIa was demonstrated using Chinese Hamster Ovary (CHO) cells that were either expressing activated GPIIb/IIIa integrin, non-activated GPIIb/IIIa or not expressing the GPIIb/IIIa integrin. Cells were grown to confluency in 6-well plates (BD Bioscience, USA), incubated with purified scFv-scuPAs, followed by anti-Penta-His AlexaFluor 488-conjugated monoclonal antibody (anti-His-488; Qiagen, Germany). The cells were visualized with the IX81 Olympus microscope (Olympus, Japan) and Cell^P 1692 (ANALysis Image Processing) software.

*Flow cytometry.*

Platelet rich plasma (PRP) was obtained from healthy volunteers. Diluted PRP was either not activated or activated with 20µM adenosine diphosphate (ADP), 5µg/ml collagen-related peptide (CRP) or 30µM thrombin receptor activating peptide (TRAP) before incubation with the purified scFv constructs, followed by anti-His-488 for detection. Activity of platelets was determined by FITC-labeled fibrinogen, PAC1-FITC and CD62P-PE. The specificity of scFv$_{SCE5}$ targeting activated platelets was analyzed using FITC-labeled fibrinogen and PAC1-FITC. Competitive assays were performed using abciximab (ReoPro®) and FITC-labeled fibrinogen. Samples were fixed with 1x Cellfix (BD Bioscience, USA) and analyzed by FACS Calibur (BD Bioscience, USA). In addition, GPIIb/IIIa expressing CHO cells were also used for flow cytometry.

*Urokinase activity assay.*

Urokinase activity was determined with a chromogenic substrate assay. Comparison between clinically used uPA (Medac GmbH, Germany) and scFv-scuPA was made on the basis of equal urokinase activity. 100nmol/L of scFv-scuPA was monitored against urokinase standards (0–100U/mL) used as positive controls. Plasmin was added to activate the scuPA. S2444 (Chromogenix, Italy) was added and samples were measured on a Victor3V Multi-label counter (PerkinElmer, USA) at a wavelength of 405nm.

*Plasmin activity assay.*

The conversion of plasminogen to plasmin using commercial uPA or the two scFv-scuPA versions was determined in microtiter plates using a chromogenic substrate. 10nmol/L of commercial uPA and scFv-scuPAs were incubated with 400nmol/L of human glu-plasminogen (Sigma-Aldrich, USA) 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 seconds over a period of 60 min.

*Fibrin zymography.*

SDS-PAGE-based fibrin zymography[20] was performed to evaluate plasminogen dependent fibrinolytic activity of the targeted and non-targeted scFv-scuPA. Briefly, the commercial uPA and scuPA were subjected to SDS-PAGE. After electrophoresis, gels were washed in 2.5% Triton X-100 for 1.5 hour, then

placed on top of a fibrin/agarose: plasminogen matrix. The washed SDS-PAGE gel was then overlaid onto the exposed agarose gel and incubated in a humidified 37°C oven until lytic zones were evident. Images were captured at various incubation times using a flatbed document scanner.

*Light transmission aggregometry.*
96-well plate light transmission aggregometry was performed using 100μl of PRP. PRP were incubated with abciximab, scFv-scuPA, scFv$_{SCE5}$ alone or commercial uPA, then activated with 10μM ADP. Platelet poor plasma (PPP) was obtained by centrifugation of blood at 1000x$g$ for 10min at room temperature. Light transmission was adjusted to 0% with PRP and 100% with PPP. In order to differentiate the effects of scFv from those of urokinase, 200μM of the urokinase blocker amiloride[21] was added. Light transmission aggregometry was measured using the Bio-Rad Benchmark Plus at wavelength 595nm. Samples were measured every 30 seconds for 60 min.

*Flow chamber adhesion assay.*
Flow chamber in vitro adhesion assays were performed with glass capillaries or microfluidics flow channels, which were coated overnight with collagen. Whole blood was perfused through the capillaries or channels to form microthrombi. Binding of scFv-scuPAs was observed via staining with anti-His-488. Fibrin degradation was demonstrated using Oregon-Green Fibrinogen (Invitrogen, USA). The microthrombi were visualized with the IX81 Olympus microscope and Cell^P 1692 software.

*In vivo mouse experiments.*
Male C57BL/6 mice and plasminogen knockout mice (plg$^{-/-}$ mice, Jackson Laboratories, USA) were maintained at the Alfred Medical Research and Education Precinct Animal Services and assigned randomly to the different groups. The amount of the targeted and non-targeted scFv-scuPA for injection was calculated according to units per gram (U/g) body weight (BW) of the animals. The animals were anaesthetized, shaved and 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/1160/2011/B).

*Femoral vein catheterization and ferric chloride injury model for Doppler flow velocity measurement.*
A catheter was placed into the femoral vein to facilitate injection. A small filter paper saturated with 10% ferric chloride was placed under the carotid artery of the animal for 3 min to induce an occlusive thrombus[22]. Animals were injected with either constructs or controls 1 min before the injury. The nano-Doppler flow-probe (0.5VB, Transonic, Japan) was placed under the carotid artery post injury to measure the thrombotic occlusion.

*Intravital microscopy of the mesenteric arterioles in mice.*
Intravital microscopy was performed as previously described[23]. Briefly, the mesentery was exteriorized through a midline abdominal incision. 6% ferric chloride was used to induce thrombus formation on the mesenteric arterioles. Binding of the scFv-scuPAs conjugated with Cy3 fluorescence dye (Lumiprobe, USA) was monitored using the fluorescence channel on the Nikon A1r confocal microscope (Nikon, Japan).

*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 commercial uPA, scFv-scuPAs or vehicle, 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 visible blood stream, for 1 min.

***In vivo ultrasound molecular imaging of carotid artery 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 imaging station after 6% ferric chloride injury was performed to the left carotid artery. Videos and images were acquired before, during and at several time points after injecting $1.5x10^7$ targeted microbubbles (LIBS-MBs) specific for activated platelets (targeting the ligand induced binding site on activated GPIIb/IIIa) in a total volume of 100μl. We have recently established this ultrasound imaging methodology for the assessment of thrombosis and thrombolysis[19]. We injected 500U/g of urokinase plasminogen activator (uPA) (Medac, Germany), 75U/g of scFv-scuPA or saline as vehicle control. Repetitive ultrasound imaging sequences were performed every 5 min for an hour after thrombolysis. Analysis was performed using a linear contrast agent imaging software (VisualSonics Inc.).

***In vivo ultrasound molecular imaging of carotid artery thrombolysis using plasminogen-deficient (plg$^{-/-}$) mice.***
Plg$^{-/-}$ mice were placed on the VisualSonics imaging station after 6% ferric chloride injury was performed to the left carotid artery. Images were acquired before the injection of LIBS-MBs. Thereafter, we injected 75U/g of scFv$_{SCE5}$-scuPA or scFv$_{mut}$-scuPA. Repetitive ultrasound imaging sequences were performed every 5 min for 30 min. A 150μl bolus of 100μg/ml human plasminogen (Sigma-Aldrich, USA) was injected at the 30 min time-point and repetitive imaging sequences continued for another 30 min. Analysis was performed using a linear contrast agent imaging software.

***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. 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 and purification of scFv-scuPA constructs.*

The success of DNA amplification and restriction digest of scFv-scuPA fragments was evaluated by electrophoresis (Supplemental Figure I). Both constructs were visualized between the 1.5kbp and 2kbp marker after amplification with PCR and restriction digest. The pSectag2A plasmid was visualized at around 5kbps after single cut restriction digest. After the respective constructs were cloned into the pSectag2A plasmid, transformed and purified, they were analyzed by gel electrophoresis. The sequences of both fusion constructs were confirmed via DNA sequencing. After production of the scFvs, SDS-PAGE and Western blot were used to prove successful purification (Supplemental Figure IIa and b). Western blot was also used to demonstrate digestion of scuPA after the addition of plasmin (Supplemental Figure IIb).

*Binding of targeted scuPA to CHO cells in vitro.*

The specificity of scFv$_{SCE5}$-scuPA was also observed under static adhesion conditions. Direct fluorescence staining using anti-His-488 demonstrated binding of scFv$_{SCE5}$-scuPA to the activated GPIIb/IIIa expressing CHO cells but not on either non-expressing or non-activated GPIIb/IIIa expressing CHO cells. No fluorescence staining was observed for all three cell types with scFv$_{mut}$-scuPA (Figure 1).

In flow cytometry, incubation of scFv$_{SCE5}$-scuPA with the CHO cells resulted in an increase in fluorescence intensity for samples with activated GPIIb/IIIa expressing CHO cells but not on either non-expressing or non-activated GPIIb/IIIa expressing CHO cells (p<0.01; Supplemental Figure III).

*Evaluation of the functionality of scFv-scuPA by flow cytometry.*

To confirm retained binding capacity of the scFv to ADP-activated platelets, the functionality of scFv-scuPA was evaluated with anti-His-488 (Figure 2). No binding was observed for control non-targeted scFv$_{mut}$ to neither activated nor non-activated platelets, (2.69±0.18 vs 2.84±0.25 arbitrary units [AU]; mean ± SD; ns). Incubation of activated platelets with scFv$_{SCE5}$ resulted in an increase in fluorescence intensity as compared to non-activated platelets (2.69±0.16 vs 23.88±8.22 AU; mean±SD; p<0.01). Competitive assays were performed using FITC-labeled fibrinogen to demonstrate the binding of scFv$_{SCE5}$-scuPA. After incubation with scFv$_{SCE5}$-scuPA fibrinogen was not able to bind to activated platelets anymore. However after incubation with scFv$_{mut}$-scuPA, fibrinogen binding was not inhibited (57.11±5.82 vs 75.54±23.2 vs 4.67±2.92 AU; p<0.001). Competitive assays using PAC1 showed similar results as with FITC-labeled fibrinogen (39.03±6.5 vs 41.74±6.45 vs 3.03±0.21 AU; p<0.01). Binding of scFv$_{SCE5}$ was also confirmed with CRP and TRAP activated platelets (Supplemental Figure IVa). Platelet activation by these platelet agonists was demonstrated using PAC1 and anti-CD62P fluorescence staining (Supplemental Figure IVb and c). Specificity of scFv$_{SCE5}$-scuPA binding to activated GPIIb/IIIa on activated platelets was demonstrated via competition with abciximab (Supplemental Figure Va). Specificity of scFv$_{SCE5}$-scuPA towards the fibrinogen binding sites on activated GPIIb/IIIa was demonstrated by decreased binding of fibrinogen upon increased concentrations of scFv$_{SCE5}$-scuPA in flow cytometry (Supplemental Figure Vb).

*In vitro evaluation of scFv-scuPA activity in platelet aggregation.*

Light transmission aggregometry in a 96-well plate assay was performed to determine the ability of the recombinant fusion proteins to inhibit platelet aggregation. High concentrations of the scFv$_{SCE5}$ alone (5µg/ml and 10µg/ml) and the equimolar amounts of scFv$_{SCE5}$-scuPA (10µg/ml and 20µg/ml) demonstrated a strong inhibition of ADP-induced platelet aggregation as opposed to scFv$_{mut}$-scuPA, which showed no inhibitory effect (Figure 3A; n=4; p<0.001). At lower concentrations, scFv$_{SCE5}$ alone (0.1µg/ml and 1µg/ml) and the equimolar amounts of scFv$_{SCE5}$-scuPA (0.2µg/ml and 2µg/ml) would not inhibit platelet aggregation. Platelet aggregation also was not inhibited with 100U or 200U of commercial uPA (p<0.001). Similar results were obtained when 200µM of amiloride was used to block the function of scuPA (Figure 3B), demonstrating that urokinase has no effect on thrombus formation in this assay.

*In vitro evaluation of the urokinase activity of scFv-scuPA.*

Urokinase activity was monitored by incubating scFv-scuPA with urokinase substrate S2444L (Supplemental Figure VIa) in comparison to commercial uPA. Both scFv-scuPAs and standards using commercial uPA at different concentrations resulted in linear enzymatic activity over 60 min.

*In vitro evaluation for the conversion of plasminogen to plasmin using scFv-scuPA*

Conversion of plasminogen to plasmin was monitored using the S2251 amidolytic assay (Supplemental Figure VIb). Both scFv-scuPA versions and the commercial uPA at 10nmol/L generated plasmin activity. uPA-dependent plasmin generation was blocked in the presence of 200µM of the urokinase inhibitor amiloride. SDS-PAGE fibrin zymography was also performed to demonstrate the direct digestion of fibrin (Supplemental Figure VIc). Commercial uPA produced a lytic zone as expected at around 55kD. ScFv$_{SCE5}$-scuPA and ScFv$_{mut}$-scuPA produced a lytic zone at around 70kD.

*Binding to activated platelets and fibrin degradation with targeted scuPA to microthrombi in vitro.*

Targeting of the scFv$_{SCE5}$-scuPA was determined by binding performance in vitro in a flow chamber adhesion experiment with microthrombi. Fluorescence staining using anti-His-488 demonstrated binding of scFv$_{SCE5}$-scuPA but not with scFv$_{mut}$-scuPA (Figure 4A). Fibrin degradation was observed when scFv$_{SCE5}$-scuPA and a high dose of commercial uPA was used but not with scFv$_{mut}$-scuPA (Figure 4B). Using 2µg/ml of scFv$_{SCE5}$-scuPA, fibrin degradation was observed specifically around the platelet aggregates (Supplemental Figure VII).

*Evaluation of scFv-scuPA binding to thrombi in vivo.*

Binding of scFv-scuPA was determined by intravital microscopy in a ferric chloride-induced thrombosis model in the mesenteric arterioles of mice. Binding of scFv$_{SCE5}$-scuPA conjugated with the fluorescent dye Cy3 to developing thrombi could be demonstrated, while no fluorescence was observed with scFv$_{mut}$-scuPA (Figure 4C).

*In vivo evaluation of scFv-scuPA for prophylactic fibrinolysis.*

Thrombi were induced in the carotid artery of mice using 10% ferric chloride for 3 min. Blood flow was measured by a nano Doppler-flow probe and was used as an indicator of an occlusive thrombus (Figure 5). Saline was injected as negative control and 500U/g of commercial uPA was used as a positive control. The baseline Doppler velocity was set to 100%. At 20 min, the Doppler flow velocities obtained from mice treated with 75U/g targeted scFv$_{SCE5}$-scuPA was significantly higher than those treated with saline, the equimolar concentration of scFv$_{SCE5}$ alone, 75U/g of non-targeted scFv$_{mut}$-scuPA, the combination of scFv$_{SCE5}$ and 75U/g of non-targeted scFv$_{mut}$-scuPA, or 75U/g of commercial uPA ($84.0\pm9.4$ vs $5.4\pm2.7$ vs $23.8\pm11.8$ vs $45.3\pm13.9$ vs $38.5\pm11.8$ vs $21.6\pm11.4$, respectively, mean %±SEM, $p<0.05$, n=6). There was no difference observed in groups treated with 75U/g BW of non-targeted scFv$_{mut}$-scuPA, the equimolar concentration of scFv$_{SCE5}$ alone or the combination of both scFv$_{SCE5}$ and 75U/g BW of non-targeted scFv$_{mut}$-scuPA. The Doppler flow velocities obtained from mice treated with 75U/g targeted scFv$_{SCE5}$-scuPA were similar to those treated with 500U/g of commercial uPA throughout the observation period. Similar results were obtained at 30 min.

*In vivo assessment of bleeding time of scFv-scuPA.*

Bleeding times were evaluated by surgical tail transection (Figure 6). Commercial uPA at 500U/g considerably prolonged bleeding compared to vehicle control (saline). In contrast, a lower dose of 75U/g of scFv$_{SCE5}$-scuPA, scFv$_{mut}$-scuPA and commercial uPA minimized bleeding time. The lower dose of scFv$_{SCE5}$-scuPA (75U/g) had an anti-thrombolytic effect without prolonging bleeding time.

*In vivo molecular ultrasound imaging of thrombolysis.*

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. The thrombus was visualized as a white and bright signal after injection with platelet targeted ultrasound contrast (LIBS-MB) on real time ultrasound imaging. The baseline area before injection of uPA was set to 100% and the area was calculated every 5 min for 60 min (Figure 7). The targeting ability of 75U/g of scFv$_{SCE5}$-scuPA is demonstrated by ultrasound imaging as such a reduction in thrombus size was observed following its administration. Treatment with scFv$_{SCE5}$-scuPA (75U/g) significantly reduced thrombus size after 60 min, while no significant difference was observed in the scFv$_{mut}$-scuPA (75U/g) treatment group ($36.8\pm4.6$ vs $81.1\pm2.6$, mean %±SEM, $p<0.001$, n=3) (Figure 7, Video 1 and 2). Thrombolysis was observed via ultrasound imaging using 500U/g of commercial uPA. The ability of scFv$_{SCE5}$-scuPA to target and

dissolve the thrombus was compared against mice injected with a higher dose of commercial uPA. There were no significant differences between groups of mice treated with 500U/g commercial uPA and those treated with 75U/g of scFv$_{SCE5}$-scuPA over a period of 60 min (40.05±9.2 vs 36.8±4.6, ns, n=3). The thrombolytic ability of scFv$_{SCE5}$-scuPA was also compared with the control group where saline was administrated over a period of 60 min. ScFv$_{SCE5}$-scuPA caused a reduction in thrombus size at 60 min post administration, compared to control (36.8±4.6 vs 99.2±1.3, p<0.001, n=3). This control group was also compared against the non-targeted treatment using 75U/g of scFv$_{mut}$-scuPA. Although post administration of scFv$_{mut}$-scuPA showed some reduction in the thrombus area, it was not significantly smaller than those injected with saline (81.1±2.6 vs 99.2±1.3, ns, n=3).

*In vivo ultrasound molecular imaging of carotid artery thrombolysis on plasminogen-deficient mice.*

Plg$^{-/-}$ mice were subjected to ferric chloride induced thrombosis. Mice were then administered either scFv$_{SCE5}$-scuPA (75U/g) or scFv$_{mut}$-scuPA (75U/g). Over a 30 min period, there was no change in thrombus size in both groups of animals (96.0±0.1 vs 98.55±0.1, mean %±SEM, ns, n=3). However, when plg$^{-/-}$ mice were then reconstituted with human plasminogen (100μg/ml, 150μl bolus at 30 min time-point) mice treated with scFv$_{SCE5}$-scuPA developed a significant thrombus size reduction, while no significant difference was observed in plg$^{-/-}$ mice treated with scFv$_{mut}$-scuPA (23.1±1.5 vs 92.1±2.2, mean %±SEM, p<0.001, n=3) (Figure 8).

## DISCUSSION

Thrombolysis has been widely used as therapy in acute myocardial infarction, and ischemic stroke as well as pulmonary embolism[4,24]. However, limitations in thrombolytic efficacy, neurotoxicity of tPA[25] and bleeding complications have restricted overall use[6,26]. Administration of anticoagulants in combination with fibrinolytic agents in particular has shown little improvement in mortality, safety and efficacy[27]. Therefore, research into more effective treatments is required which could overcome the challenges associated with current fibrinolytic therapy. Targeting activated platelets with antibody-mediated delivery of thrombolytic drugs can provide an effective and safe alternative. This ideal agent would: 1) allow a low concentration in the systemic circulation thereby eliminating bleeding complications; 2) enrich by targeting at the site of the thrombus thereby being present in localized high concentration to lyse the clot.

In this study we fused a highly specific targeting scFv that binds to the activated platelet integrin receptor GPIIb/IIIa to recombinant scuPA. Our data demonstrates that scuPA delivery to activated platelets allows local enrichment of the fibrinolytic agents at the site of the developing or existing thrombus, thereby increasing fibrinolytic potency without increasing side effects. Through in vitro assays we provide evidence that both the scFv and the scuPA retain their individual function in the fusion molecule. *In vivo* evaluation of these targeted fibrinolytics both as a prophylactic and therapeutic agent showed plasminogen-dependent inhibition of thrombus growth as well as reduction in thrombus size via molecular ultrasound imaging. A low dose of 75U/g of platelet-targeted scuPA was sufficient for localized thrombolysis, which was not achieved using non-targeted scuPA or commercial uPA at the same dose. The same effects were only achieved using urokinase at 500U/g. We did not observe increased bleeding at the effective dose of the novel targeted scuPA, compared to an equally effective higher dose of non-targeted scuPA, which resulted in a significantly prolonged bleeding time. This antithrombotic effect of the low dose of 75U/g of platelet-targeted scuPA was more potent than the combination of equimolar non-targeted scuPA and the activation-specific anti-GPIIb/IIIa scFv$_{SCE5}$. This indicates that the superior anti-thrombotic effect of the fusion protein scFv$_{SCE5}$-scuPA can be attributed to the antibody-targeting of scuPA to activated GPIIb/IIIa on activated platelets and the resulting local enrichment of

scuPA at the thrombus. If a stronger anti-platelet effect is required, a dual effect composed of effective platelet inhibition and low dose targeted fibrinolysis would be attractive and could be achieved with the co-administration of activation-specific anti-GPIIb/IIIa scFv alone. For a potential human therapy, the optimal dosing regiment would have to be determined in clinical trials. Notably, the combination of low dose targeted fibrinolytic and activation-specific platelet inhibition would not be expected to have similar bleeding problems as seen in clinical trials with non-targeted fibrinolytics and non-activation specific GPIIb/IIIa inhibitors[27].

Early work mainly focused on the use of full monoclonal antibodies targeting specific components of the clot such as fibrin or platelets. Indeed, *in vivo* studies have shown greater antithrombotic therapy with the use of fibrin targeting thrombolytics[28,29]. It has also been shown that the potent fibrin targeting during thrombolysis has resulted in a high plasminogen concentration on the surface of the clot[30].

Our study focuses on platelets, as activated platelets are an obvious target for the enrichment of thrombolytics at the clot. To explore the advantages of targeting PAs to platelets, monoclonal antibodies directed against both GPIIb/IIIa and the ligand binding epitope of GPIIb/IIIa have been reported[31]. GPIIb/IIIa is the most abundant platelet receptor with 50,000 – 80,000 glycoproteins per platelet and thus constituting up to 2% of the amount of total platelet protein[32]. Despite increasing the potency by fusing PAs to monoclonal antibodies or Fab fragments, chemical conjugation has a number of disadvantages, including inactivation of enzymatic sites by the harsh chemical conditions for conjugating, which can reduce efficacy[31,33]. In addition, the large size of the antibodies fusion constructs hampered tissue penetration.

In order to overcome these problems, recombinant fusion technology was utilized to avoid chemical conjugation thereby preserving activity of the construct. Antibody fragments such as scFvs are preferred moieties for the construction of recombinant fusion constructs because they are small, minimally immunogenic and the cost of production is generally low[34,35].

Antibody targeted delivery also eliminates the reliance on the specificity of certain PA such as tPA for components of clots like fibrin. Still the gold standard therapy for acute ischemic stroke[36], tPA is neurotoxic[25] and the use of urokinase or desmoteplase avoids these complications inherent to tPA[37,38]. This has the potential to widen the currently very narrow therapeutic window for stroke thrombolysis of 3-4h[6]. The truncated, low molecular weight form scuPA is the preferred entity over full-length urokinase as it provides a smaller size with improved thrombus accessibility, no immunogenicity and similar fibrinolytic potency[39].

In addition to fibrin and platelet targeting approaches, there are several other components of the vasculature and blood that have been targeted, such as platelet endothelial cell adhesion molecule 1 (PECAM-1), glycophorin A on red blood cells and thrombomodulin to anchor PAs to the arterial or venous lumen[40-43]. PECAM-1 fused with urokinase in a model of cerebrovascular thromboembolism mediated almost complete clot lysis without exacerbating the characteristic side effects such as intracerebral hemorrhage[44]. Targeting red blood cells was shown to have effective prophylaxis against arterial and venous thrombosis for up to 24 hours[42].

Although targeting PECAM1 or red blood cells has shown successful thrombolysis, they do not serve as exclusive targets due to ubiquitous expression. We have previously reported that scFv against fibrin conjugated to scuPA[45] or against platelets conjugated to an anticoagulant[22] are predominantly active only at the site of the clot allowing increased potency. In the present study we have expanded our work to a scFv fusion molecule which delivers scuPA activation specific to the GPIIb/IIIa platelet receptor. Upon activation, GPIIb/IIIa undergoes a conformation change, which allows a high-affinity state for fibrinogen

binding, resulting in platelet aggregation and thrombus formation[9,15]. This property is ideal for specifically targeting activated platelets. The scFv[SCE5] used in this work is exclusive in its specificity for the activated conformation of GPIIb/IIIa thereby representing a unique targeting tool[15,16,23].

In addition to our well established Doppler flow measurements[15,22] allowing assessment of vessel patency, we also applied a recently developed real-time ultrasound method with scFv-targeted microbubbles used as echo enhancers[19]. The small size of the scFv has advantages for molecular imaging as it provides better access to the targeted epitopes. In contrast to the anti-GPIIb/IIIa scFv used in the scuPA fusion construct, the scFv on the microbubbles binds to ligand-induced binding sides (LIBS) on GPIIb/IIIa preserving ligand binding function. This new molecular imaging approach allows the unique real-time and direct monitoring of the success and the extent of thrombolysis and it holds promise to substantially facilitate further research in thrombogenesis and especially thrombolysis. Furthermore, this technique is also highly promising for clinical use. It is non-invasive, inexpensive, and most importantly provides real-time analysis of vessel occlusion and reopening in an emergency setting. Direct ultrasound imaging of changes in thrombus size is a technique, which could have broad clinical application in detecting success or failure of the therapeutic interventions in thrombotic disease and thus provide major benefits for patients.

The potential clinical use of these targeted fusion proteins for the prevention and treatment of stroke, myocardial infarction and venous thrombosis presents a novel strategy for highly effective thrombolysis. The expected reduction of bleeding complications could lead the way out of the current stagnation in the field of fibrinolytic therapy. Our data also has interesting implications for the potential use of a fibrinolytic drug for prophylaxis/prevention of thrombosis. Monoclonal antibodies (e.g. Humira®) are increasingly used as subcutaneous drugs for self administration by patients[46]. This opens the possibility that recombinant fusion proteins, such as scFv[SCE5]-scuPA, could potentially be used for thromboprophylaxis. More preclinical and clinic research is warranted to investigate the feasibility of such an application. Furthermore, the described molecular ultrasound imaging with platelet targeted microbubbles allows precise monitoring of interventional success.

## Conclusion.

This study demonstrates the successful generation of a novel recombinant single-chain antibody-scuPA fusion protein, specifically targeting activated platelets. The function of both components of the fusion protein, thrombolytic activity and antibody binding to activated GPIIb/IIIa were preserved. *In vivo* evaluation of this targeted fibrinolytic agent in comparison to clinically used non-targeted fibrinolytic agents showed that a low systemic concentration of the fusion protein allows enrichment of fibrinolytic activity at the site of the thrombus and thus the potentiation of fibrinolytic activity. Most importantly, this could be achieved without bleeding time prolongation. Therefore, this novel fibrinolytic agent promises to overcome the current limitations in thrombolytic therapy associated with the risk of bleeding complications. It has the potential to break the fatal link between increased fibrinolytic potency and bleeding complications. This targeting strategy also allows considering the application of a fibrinolytic drug for thromboprophylaxis; an approach that warrants further preclinical and potentially clinical testing.

**DISCLOSURES**
A patent has been granted to protect the intellectual property of the described single-chain antibody. A patent has been filed to protect the intellectual property of the described antibody – urokinase fusion contruct.

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**FIGURE LEGENDS**

**Figure 1: Static adhesion assay showing the specificity of scFv$_{SCE5}$-scuPA to CHO cells expressing activated GPIIb/IIIa receptors.** Representative microscopy images showing direct fluorescence staining of scFv-scuPA on CHO cells. Direct fluorescence staining of His-tag on scFv-scuPA by anti–Penta-His AlexaFluor 488–conjugated monoclonal antibody demonstrating binding of scFv$_{SCE5}$-scuPA to activated GPIIb/IIIa expressing CHO cells but neither to non-expressing nor non-activated GPIIb/IIIa expressing CHO cells. No fluorescence staining of scFv$_{mut}$-scuPA was observed on all three cells types.

**Figure 2: Flow cytometry assay demonstrating preserved function of scFv-scuPA after fusion. A.** Binding of scFv was shown with an anti–Penta-His AlexaFluor 488–conjugated monoclonal antibody. Bar graphs depict the median fluorescence intensity values of 3 independent experiments. Representative fluorescence histograms are shown underneath the bar graphs. Activated platelet samples were incubated with 20µM of the platelet agonist ADP. **B.** Competitive assays using fibrinogen-labeled FITC. Fibrinogen-FITC binds to activated GPIIb/IIIa on activated platelets when incubated with the negative control (PBS with 2mM $Ca^{2+}$ and $Mg^{2+}$) or the scFv$_{mut}$-scuPA. However, Fibrinogen-FITC did not bind to activated platelets in the presence of scFv$_{SCE5}$-scuPA. **C.** Competitive assays using PAC1-FITC. PAC1-FITC binds to activated GPIIb/IIIa on activated platelets when incubated with the negative control (PBS with 2mM $Ca^{2+}$ and $Mg^{2+}$) or scFv$_{mut}$-scuPA. However, PAC1-FITC did not bind to activated platelets in the presence of scFv$_{SCE5}$-scuPA (mean±SD; \*\*P<0.01, \*\*\*P<0.001). These assays were analyzed with a 2-way repeated measures ANOVA with the Bonferroni post test.

**Figure 3: 96-well plate light transmission aggregometry demonstrating antithrombotic effects of scFv$_{SCE5}$-scuPA. A.** Bar chart showing % aggregometry after the addition of ADP. A high concentrations of scFv$_{SCE5}$-scuPA (10µg/ml and 20µg/ml) and the equimolar amounts of scFv$_{SCE5}$ alone (5µg/ml and 10µg/ml) demonstrated a strong inhibition of ADP-induced platelet activation as opposed to scFv$_{mut}$-scuPA (n=3, \*\*\*p<0.001). Lower concentrations of scFv$_{SCE5}$-scuPA (0.2µg/ml and 2µg/ml) and the equimolar amounts of scFv$_{SCE5}$ alone did not show inhibition of platelet aggregation. Platelet aggregation was not inhibited with commercial uPA. **B.** Aggregometry after addition of 200µM of the urokinase inhibitor amiloride further demonstrating that urokinase has no effect on thrombus formation in this assay. ScFv$_{SCE5}$-scuPA (10µg/ml and 20µg/ml) and equimolar amounts of scFv$_{SCE5}$ alone (5µg/ml and 10µg/ml) demonstrated inhibition of ADP-induced platelet activation as opposed to scFv$_{mut}$-scuPA (n=3, \*\*\*p<0.001). Lower concentrations of scFv$_{SCE5}$-scuPA (0.2µg/ml and 2µg/ml) and the equimolar amounts of scFv$_{SCE5}$ alone did not show inhibition of platelet aggregation. Platelet aggregation was not inhibited with commercial uPA

**Figure 4: Binding of scFv$_{SCE5}$-scuPA to microthrombi resulting in fibrin degradation in vitro and fluorescence staining of scFv$_{SCE5}$-scuPA *in vivo*. A.** Representative microscopy images of microthrombi with scFv$_{SCE5}$-scuPA. Fluorescence-labeled anti-His-tag antibody demonstrates attachment of scFv$_{SCE5}$-scuPA to the microthrombi. No fluorescence was detected on microthrombi with scFv$_{mut}$-scuPA (n=3 each). **B.** ScFv$_{SCE5}$-scuPA caused fibrin degradation in vitro on microfluidics flow channels. Fibrin degradation was observed at platelet aggregation perfused with scFv$_{SCE5}$-scuPA but not with scFv$_{mut}$-scuPA. Fibrin degradation was also observed in platelet aggregates perfused with a high dose of commercial uPA (n=3 each). Image analysis was done with ImageJ applying a median filter (1.5pixel) and a "fire" false colour look-up table. **C.** Intravital microscopy demonstrating binding of scFv$_{SCE5}$-scuPA conjugated with Cy-3 fluorescence dye to thrombi *in vivo*. Representative images showing the binding of scFv-scuPA fusion proteins to thrombi induced by ferric-chloride injury in the mesenteric arteriole (n=3 each). ScFv-scuPA fusion proteins conjugated with Cy-3 fluorescence dye were injected into mice after the formation of stable thrombi. Increased binding of scFv$_{SCE5}$-scuPA to activated platelets/thrombus was detected on the fluorescence channel. No specific fluorescence/ binding was observed using scFv$_{mut}$-scuPA.

**Figure 5: Doppler flow velocity of carotid arteries of mice for monitoring of thrombolysis showing that scFv$_{SCE5}$-scuPA prevents occlusion.** Thrombi were induced in the carotid artery of mice using 10% ferric chloride for 3 min. The nano Doppler flow meter was used to measure occlusion time and the baseline Doppler velocity was set to 100%. Saline was injected as negative controls and 500U/g BW of clinically used commercial uPA was used as a positive control. Analysis of velocity 10 min post injury showed occlusion for mice treated with saline and 75U/g BW commercial uPA. 20 min post injury, the Doppler velocity from mice treated with 75U/g BW targeted scFv$_{SCE5}$-scuPA was significant higher than those treated with saline, 75U/g BW of non-targeted scFv$_{mut}$-scuPA, 75U/g BW of commercial uPA, the equimolar concentration of scFv$_{SCE5}$ alone or the combination of scFv$_{SCE5}$ with 75U/g BW of non-targeted scFv$_{mut}$-scuPA. No difference was observed in groups treated with 75U/g BW of non-targeted scFv$_{mut}$-scuPA, the equimolar concentration of scFv$_{SCE5}$ alone or the combination of both scFv$_{SCE5}$ and 75U/g BW of non-targeted scFv$_{mut}$-scuPA. Similar results were obtained for 30 min. The Doppler flow velocities obtained from mice treated with 75U/g targeted scFv$_{SCE5}$-scuPA was similar to those treated with 500U/g BW of commercial uPA throughout the observation period. These assays were analyzed with 2-way repeated measures ANOVA with the Bonferroni post test. Data shown as mean % ± SEM, *p<0.05, **p<0.01, ***p<0.001, n=6 each.

**Figure 6: Bleeding time in mice determined by tail transection shows that there is no bleeding time prolongation at the effective dose of scFv$_{SCE5}$-scuPA.** Commercial uPA at 500 U/g BW demonstrated considerably longer bleeding time as compared to saline vehicle controls (*p<0.05, n=6 each). Low dose scFv$_{SCE5}$-scuPA, scFv$_{mut}$-scuPA and commercial uPA at 75U/g BW did not cause prolong bleeding time. These assays were analyzed with 1-way repeated measures ANOVA with the Bonferroni post test

**Figure 7: Monitoring of thrombolysis via molecular ultrasound imaging showed a reduction of thrombus size post administration of scFv$_{SCE5}$-scuPA. A.** Monitoring of thrombus area post administration of scFv-scuPA. A reduction of thrombus size was observed for animals administered with 500 U/g BW of commercial uPA (black line and **B**) as compared to saline (light blue line and **C**) as vehicle control. A reduction of thrombus size was also observed with 75 U/g BW activated platelets targeting scFv$_{SCE5}$-scuPA (red line and **D**) as compared to 75 U/g BW non-targeted scFv$_{mut}$-scuPA (dark blue line and **E**). Baseline area before injection of uPA was set to 100% and areas were calculated every 5 min for 60 min. Thrombus size was traced and calculated using the VisualSonics software. The groups were compared by use of repeated measures ANOVA over time with Bonferroni post tests at each time point (Mean % ± SEM, (*p<0.05, **p<0.01, ***p<0.001, n=3 each).

**Figure 8: Reduction of thrombus size post administration of scFv$_{SCE5}$-scuPA and bolus of plasminogen. A.** Monitoring of thrombus area post administration of scFv-scuPA. No reduction of thrombus size was observed in mice administered with 75 U/g BW of scFv-scuPA for the first 30 min. A bolus of plasminogen was injected into the animal at the 30 min time-point. A reduction of thrombus size was observed with 75 U/g BW activated GPIIb/IIIa targeting scFv$_{SCE5}$-scuPA (line with circles and **B**) but not with 75 U/g BW non-targeted scFv$_{mut}$-scuPA (line with squares and **C**). Baseline area before injection of uPA was set to 100% and areas were calculated every 5 min for 60 min. Thrombus size was traced and calculated using the VisualSonics software. The groups were compared by use of repeated measures ANOVA over time with Bonferroni post tests at each time point (Mean % ± SEM, ***p<0.001, n=3 each).

**Novelty and Significance**

*What Is Known?*

- The initiating events in acute thrombotic events such as myocardial infarction (MI) and stroke include platelet activation, conversion of fibrinogen to fibrin, and eventual formation of an obstructing/occluding thrombus/clot.

- Thrombolytic drugs like urokinase were developed to lyse clots through the conversion of the endogenous proenzyme plasminogen to the active fibrinolytic protease plasmin.

- Treatment with thrombolytic drugs; however, is often ineffective and associated with bleeding complications due to a lack of site-specificity and hence, disruption of normal haemostasis.

*What New Information Does This Article Contribute?*

- Molecular cloning techniques can be used to create a fusion construct of single-chain urokinase plasminogen activator (scuPA) and a small single-chain antibody (scFv$_{SCE5}$) directed specifically against the activated GPIIb/IIIa receptor of platelets in a developing clot.

- The fusion construct (scFv$_{SCE5}$-scuPA) allows for a small administered dose of urokinase to concentrate at the site of the developing clot, leading to a high local concentration with low systemic concentrations.

- Systemic administration of this targeted scuPA to mice subjected to arterial thrombosis leads to effective thrombolysis without negative effects on haemostasis.

Thrombolytic drugs are highly effective when used for the treatment of MI and ischemic stroke. Bleeding complications due to interference with normal hemostasis, however, restrict application to a relatively small group of patients. Strategies to target the thrombolytic activity specifically to the site of thrombosis are attractive as they promise effective thrombolysis without undesired bleeding complications. We genetically fused scFv$_{SCE5}$ with the scuPA to form scFvSCE5-scuPA. The unique affinity of scFvSCE5 for the activated conformation of the GPIIb/IIIa receptor and thus for activated platelets means that once injected systemically, scFvSCE5-scuPA enriches in the region of a developing thrombus and exerts potent thrombolytic activity due to its high local concentration, whilst avoiding bleeding complications thanks to its low systemic concentration. In mice with induced arterial thrombosis, a six-fold lower dose of targeted scuPA had equivalent efficacy to clinically used uPA, but without associated bleeding time prolongation. This demonstration of targeted thrombolysis using an activation-specific platelet antibody holds promise for safer and more effective thrombolysis in patients with MI or stroke, including those that have been excluded until now based on bleeding concerns. Furthermore, the improved safety profile allows consideration of fibrinolytics being given as thromboprophylactic drugs.
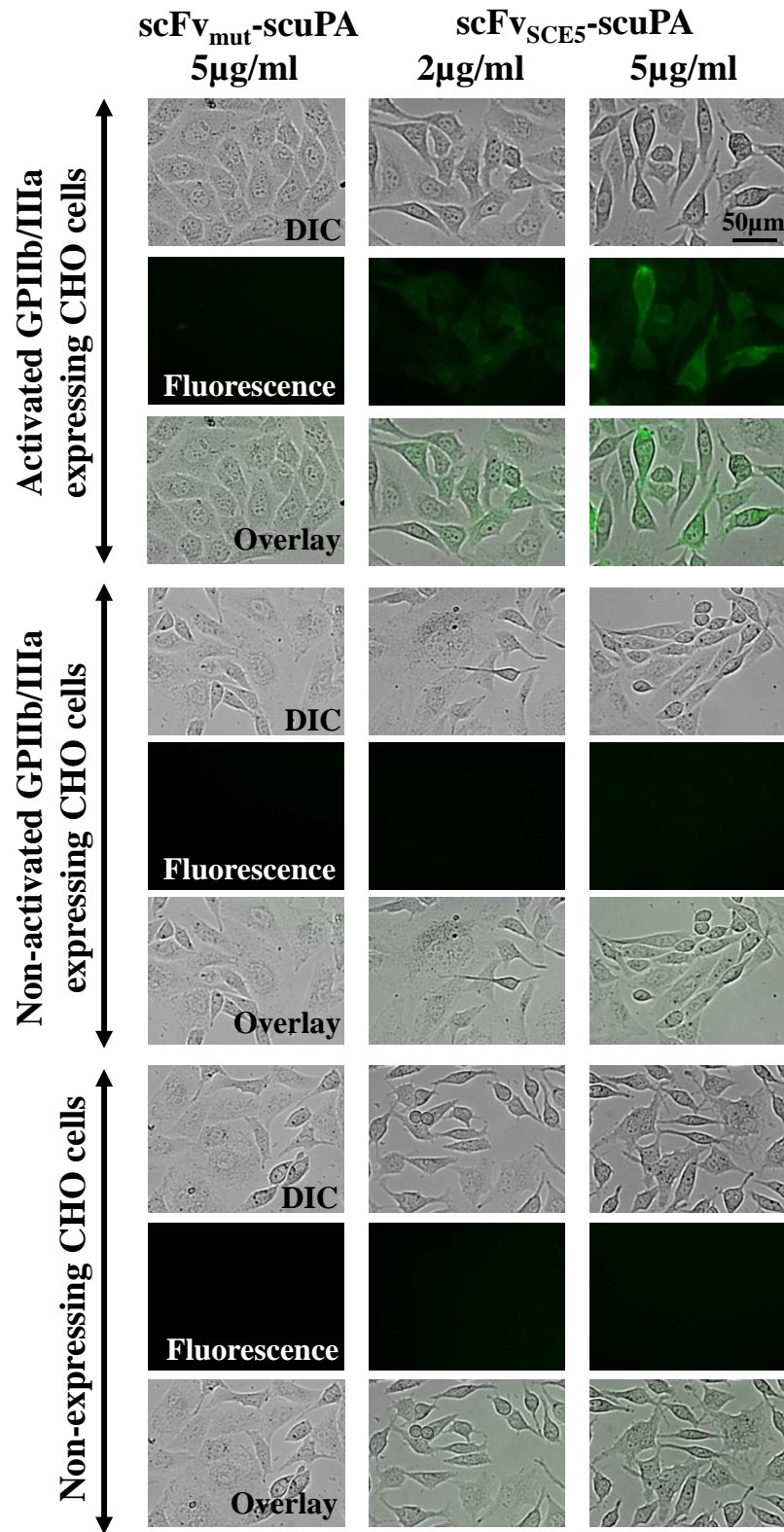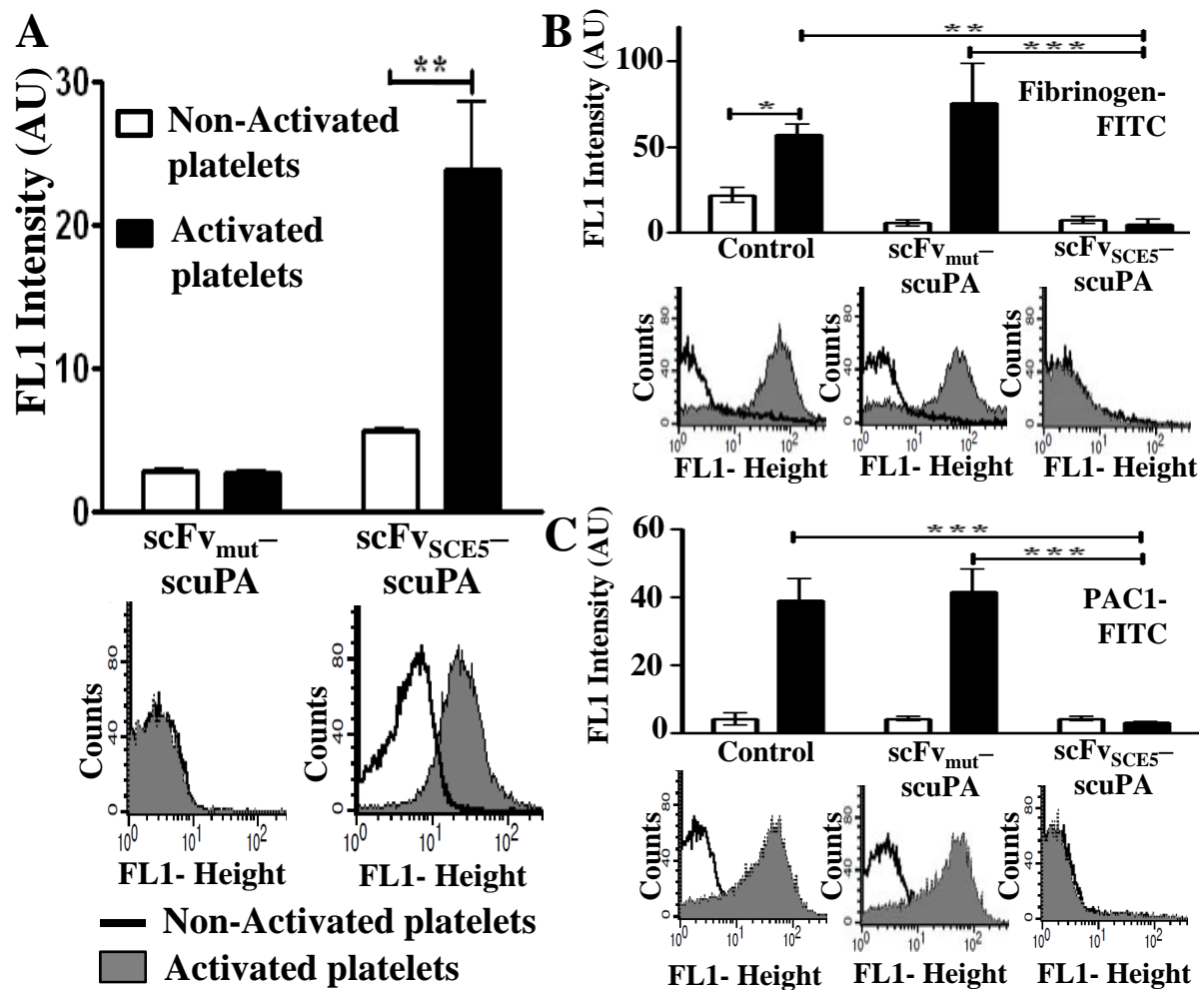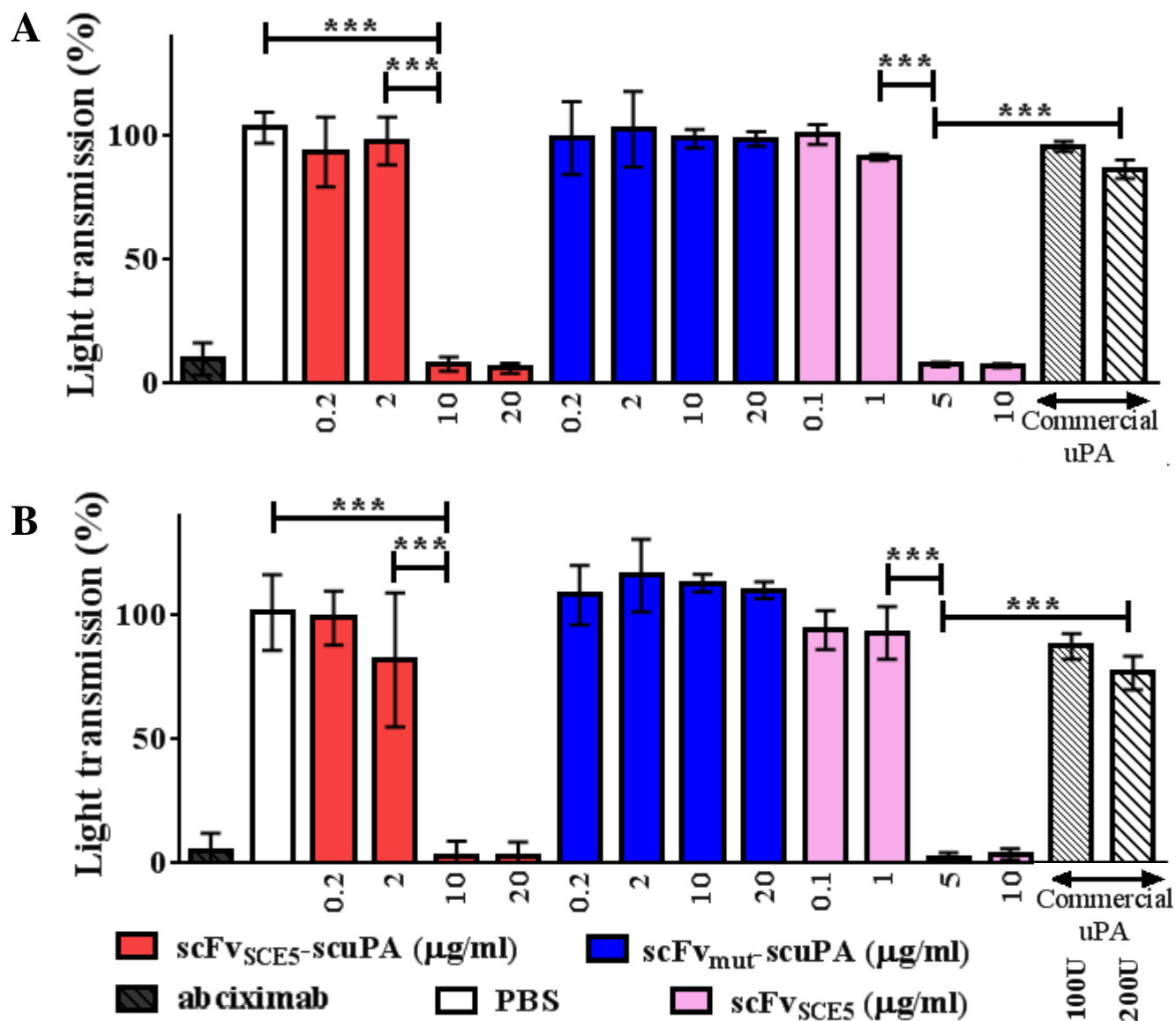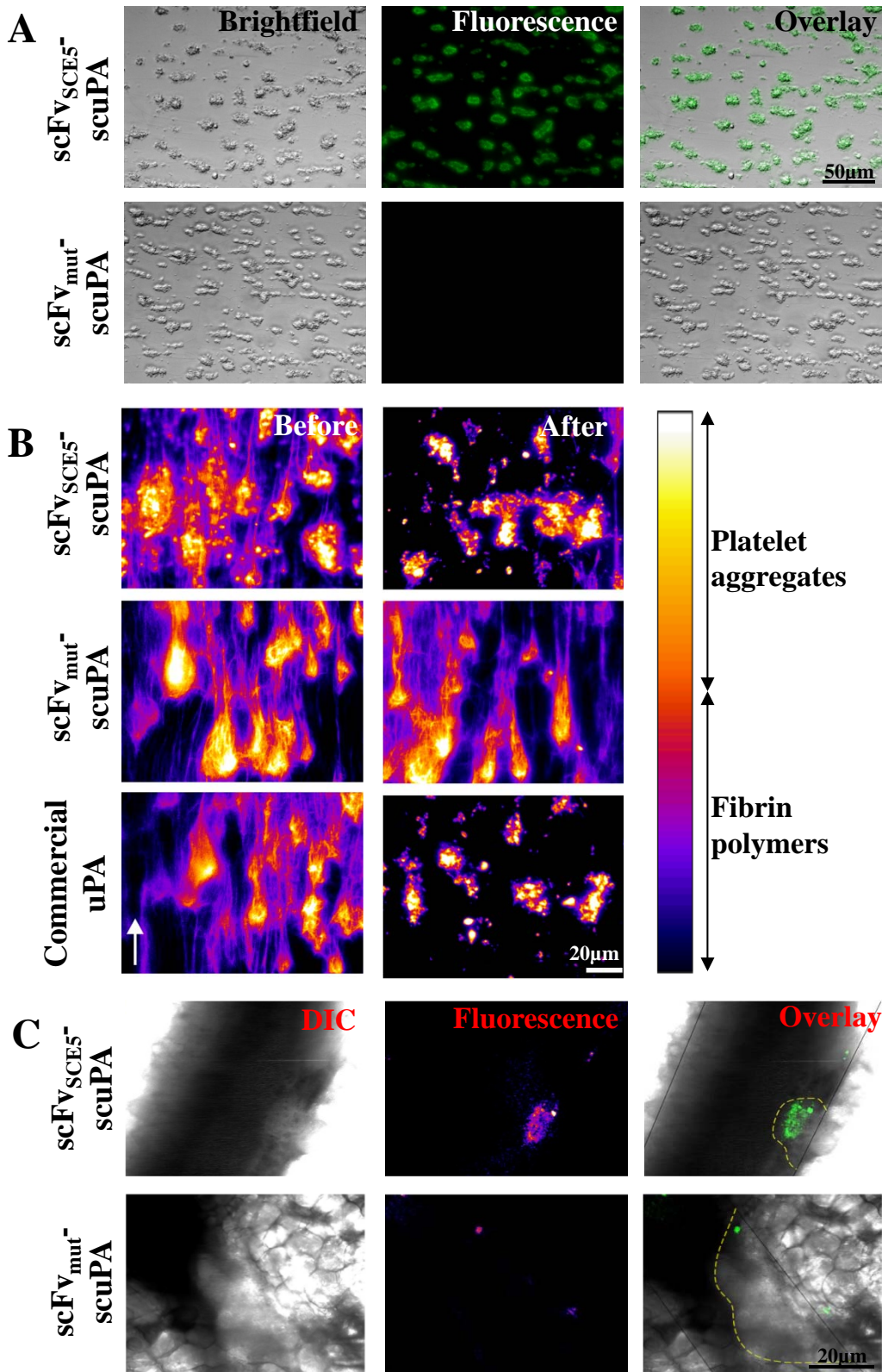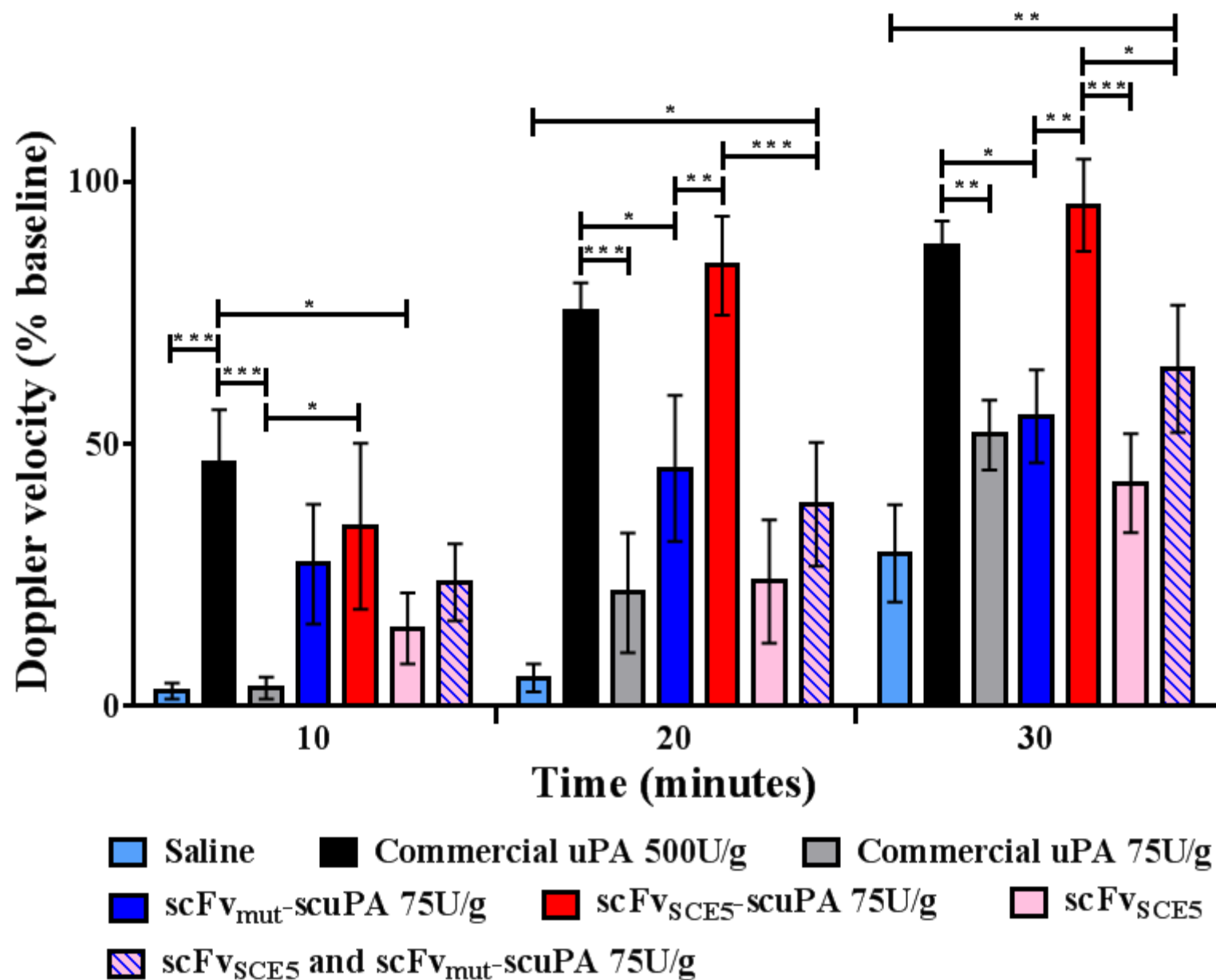
**Figure 1**

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

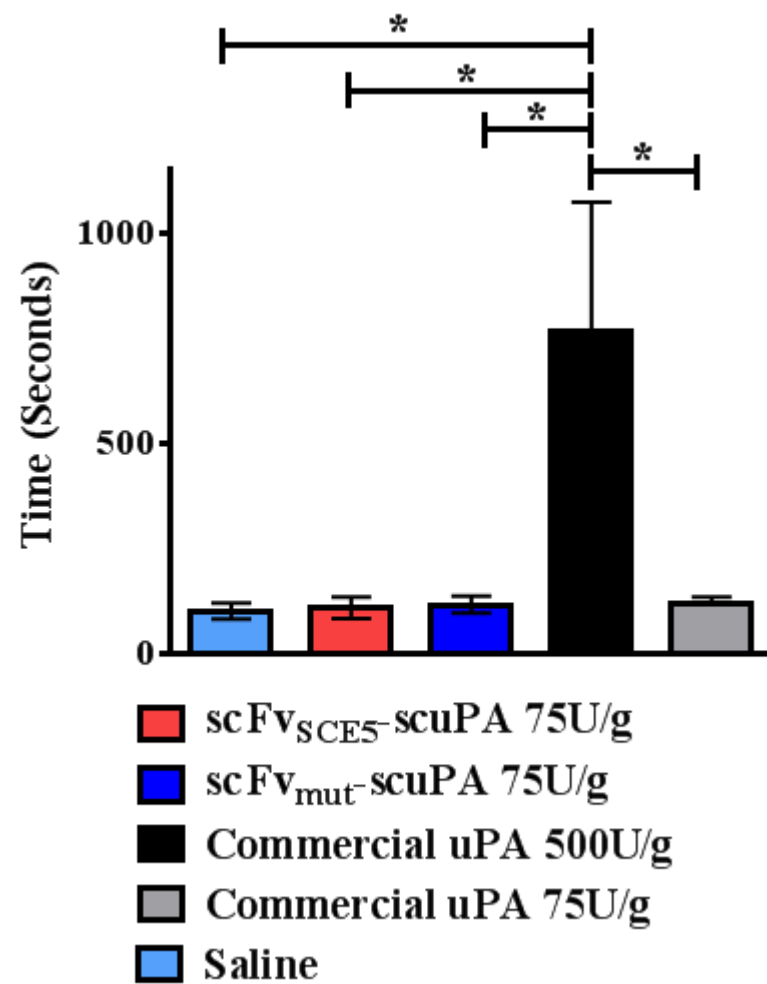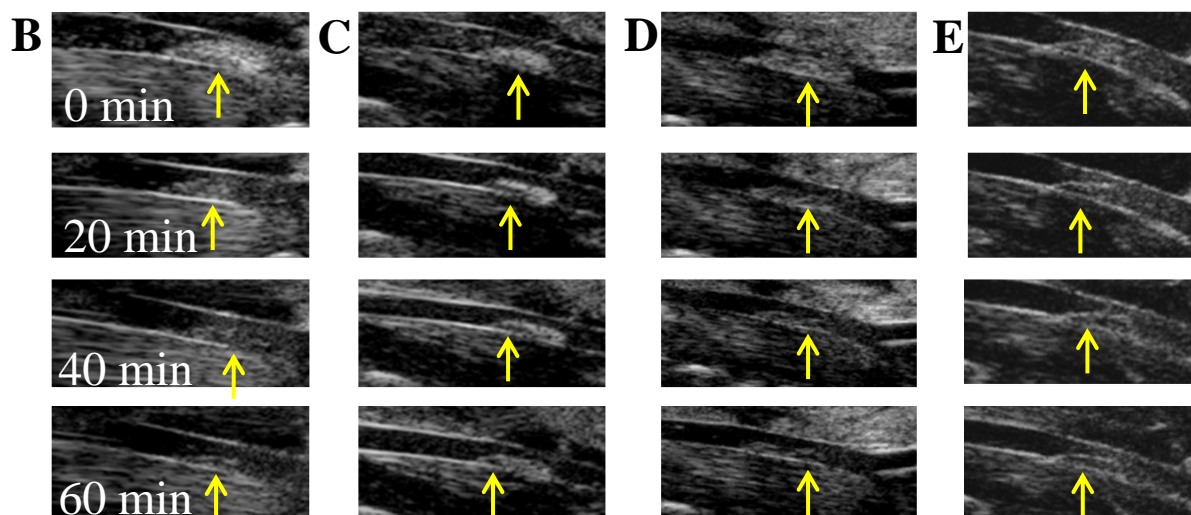Figure 7

**Figure 8**



**A**

% thrombus size vs Time from scFv-scuPA injection (minutes)

*** *** *** *** *** ***

Plasminogen injection 100μg/ml

*** p < 0.001

scFv$_{SCE5}$-scuPA (n=3)    scFv$_{mut}$-scuPA (n=3)

0 min        20 min        40 min        60 min

**B**

**C**