haematologica
Journal of the Ferrata Storti Foundation

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Received: September 26, 2024.
Accepted: November 8, 2024.

Citation: Robert A.S. Ariëns, Andrew S.P. Sharp, and Cédric Duval. Ultrasound-mediated catheter delivery of tissue plasminogen activator promotes thrombolysis by altering fibrin fibre thickness and clot permeability.
Haematologica. 2024 Nov 21. doi: 10.3324/haematol.2024.286684 [Epub ahead of print]

Ultrasound-mediated catheter delivery of tissue plasminogen activator promotes thrombolysis by altering fibrin fibre thickness and clot permeability

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Short title: Mechanisms of ultrasound-accelerated fibrinolysis

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Disclosures: This study was funded by the provider of the EkoSonic Ultrasound System®, but all the work was performed independently by the investigators. ASPS: Consultant to Medtronic, Boston Scientific, Philips, Angiodynamics, Penumbra and Recor Medical. Stock options in Althea Medical.

Contributions: CD and RASA designed the study, performed the research, interpreted the data, performed the statistical analysis, and wrote the manuscript. ASPS interpreted the data and wrote the manuscript. All authors critically reviewed and approved the manuscript.

Acknowledgements: This study was funded by an investigator-led research grant from Boston Scientific Ltd. Decisions on experimental set-up and interpretation of data were done independently by the investigators and was not subject to approval from Boston Scientific Ltd.

Data-sharing statement: Data that support the findings of this study are available from the corresponding author upon reasonable request.

ARTICLE SUMMARY

The evidence for the adjunctive use of ultrasound to catheter-based local delivery of fibrinolytic agent in pulmonary embolism patients is limited, and the mechanisms underpinning ultrasound-mediated modulation of fibrinolysis are largely unknown. Here, we show that ultrasound accelerates fibrin degradation by reversibly altering clot structure, and that ultrasound application facilitates lower doses of lytic agent to achieve similar efficiency as standard thrombolysis, indicating the potential for lowering bleeding risks associated with thrombolytic therapy.

ABSTRACT

It has been proposed that low power, high frequency ultrasound can augment the ability of thrombolytic agents to dissolve clot in patients with venous thromboembolism. We created a bench model to examine what role and mechanism ultrasound may have in this process.
Fibrin polymerisation was analysed through modified light-scattering experiments with the inclusion of catheter-mediated ultrasound application. We studied fibrin fibre diameters through scanning electron microscopy of ultrasound treated fibrin clots. Clot porosity was investigated using permeation tests, while fibrinolysis was analysed through light-scattering experiments, and by changes in porosity of lysing clots under flow.
Application of ultrasound did not change initial fibrin polymerisation but did induce a reversible change in maximal turbidity of already formed fibrin clots. This change in turbidity was caused by a reduction in fibrin fibre diameter and was associated with an increase in clot porosity. These reversible structural changes were associated with a linear increase in fibrinolysis rates under static conditions, while an exponential increase in rates was observed under flow.
The use of ultrasound augmentation of thrombolysis enhances clot dissolution through greater and more rapid fibrin degradation. This is due to conformational change created by the ultrasound in clot structure, a reversible phenomenon that may increase binding sites for lytic agent and could potentially allow the use of lower doses and shorter infusion times of ultrasound-assisted thrombolytic to treat venous thromboembolism *in-vivo*.

INTRODUCTION

Pulmonary Embolism (PE) is the third commonest cause of cardiovascular death and a major cause of morbidity worldwide[1]. Treatment of the majority of PE is based on simple anticoagulation. However, some patients with PE experience haemodynamic instability and, in such circumstances, thrombolysis is considered as an adjunct to care to accelerate clot dissolution and speed up clinical recovery. Thrombolysis can be delivered systemically at high doses by a peripheral vein and is the mainstay of care for PE that results in sustained low blood pressure. Whilst doing so can prevent or reverse cardiovascular collapse, it is associated with clinically important rates of bleeding complications, in particular the higher risk of intracranial haemorrhage[2], that can to some degree mitigate benefits.

Catheter-based delivery of lower doses of thrombolytic, given directly into the pulmonary arterial system, has been proposed as one mechanism of maintaining the therapeutic cardiovascular benefits of systemic reperfusion therapies, with the hope that local delivery could improve efficacy and/or reduce required doses, thereby potentially reducing the risk of bleeding complications[3]. One such mechanism of this is using the EKOS catheter, which utilises ultrasound-facilitated delivery of the drug to potentiate the dose of lytic used[4, 5]. In addition to PE, EKOS catheter-mediated thrombolysis is also utilised in cases of large deep vein thrombosis[6], which can present with life-threating symptoms. However, the evidence for the adjunctive use of ultrasound to catheter-based local delivery of fibrinolysis into the pulmonary vasculature is limited[7]. Furthermore, the mechanisms underpinning ultrasound-mediated modulation of fibrinolysis are also largely unknown.

In this study, we analysed the ability of the EKOS ultrasound catheter to affect speed and magnitude of lytic effect on a bench clot model, to quantify any benefit that might be gained. Moreover, we assessed the effects of ultrasound on fibrin clot structure, permeability and fibrin fibre architecture, to characterise underlying molecular and structural mechanisms that impact fibrinolysis rates.

METHODS

EKOS system for ultrasound and catheter mediated thrombolysis

The EKoSonic (EKOS) Endovascular ultrasound delivery system and catheters were obtained from Boston Scientific (Marlborough, MA, USA). The EKOS system uses a

rotating algorithm of pulses of differing wattages, and so the bench model was set-up to deliver the energy levels utilised in this algorithm - 0, 9, 15, 30 and 47W ultrasound pulses. These ultrasound wattages match those of the clinical algorithm consisting of 4 pulses of 9W, 15W, 30W and 47W. The EKOS system allowed for constant monitoring of the ultrasound power during delivery and temperature levels on the outside of the catheter. A constant temperature (average 31.7°C, range 31.0-32.9°C) was maintained within the catheter by circulating room temperature coolant at 0, 2, 2.5, 8.5, and 11mL/min for 0, 9, 15, 30, and 47W ultrasound, respectively.

Turbidity and lysis

Clot formation and Lysis was assessed by a modified turbidity assays protocol[8]. Plasma clots were formed in spectrophotometer cuvettes fitted with the EKOS catheter and ultrasonic core (Fig. S1). For turbidity assays, 4mL of clotting mixture were prepared with plasma diluted 1:6 in Tris-Buffered Saline (TBS; 50mM Tris, 100mM NaCl, pH 7.4) warmed to 32°C, 1U/mL Thrombin and 10mM $CaCl_2$. For lysis assays, tPA (Apollo Scientific; Stockport, UK) was added in the clotting mixture at 50ng/mL, unless specified. In all cases, the clotting mixture was transferred into the cuvette, which was then transferred into the thermostated cuvette compartment (32°C) of a Multiskan GO spectrophotometer (ThermoFisher Scientific; Altrincham, UK). Optical Density (OD) was measured at 340nm every 12sec for over 75min. Ultrasound was applied at i) 0-30min, ii) 30-45min, or iii) 30-75min from the activation of clotting for turbidity, and 25-60min from the activation of clotting for lysis (timepoints optimised with preliminary experiments), with coolant applied at the relevant speed. Experiments consisted of 6 repeats, each using a fresh catheter.

Permeation

Clot pore size was determined using a modified permeation protocol[9]. The bottom of spectrophotometer cuvettes were perforated with 4 holes of 2mm diameter, covered from the outside with parafilm and fitted with the EKOS catheter and ultrasonic core (Fig. S3). 1.5mL of clotting mixtures were prepared with plasma diluted 1:6 in TBS warmed to 32°c, 1U/mL Thrombin and 10mM $CaCl_2$. The clotting mixture was transferred into the cuvette, which was incubated at 32°C for 20min before a small layer of TBS (200µl) was added, with a further incubation of 100min. After 2hr, the parafilm was removed and TBS was added to the top of the clot up to 4cm height (distance from bottom of the clot to top of the liquid), which was kept constant throughout the experiment. The flow through was collected every 10min for 1hr and weighed. The Darcy's permeation constant, relating to

average pore size, was calculated as previously described[10]. Experiments consisted of 6 repeats, each using a fresh catheter.

Materials, Scanning electron microscopy, Lysis under flow, and Data Analysis

Details provided in supplemental methods.

Ethical statement

The study did not require ethical approval but respected the ethical rules of the country in which it has been performed.

RESULTS

Application of ultrasound on clot formation and structure

The effect of ultrasound delivery by EKOS on clot formation was assessed by a modified turbidity assay (Fig. S1). Initially, we analysed the effect of ultrasound on the initial step of clot formation (Fig. 1A). Ultrasound was continuously applied for the first 30min after addition of thrombin to plasma. None of the ultrasound powers tested affected the lag phase, or pre-gelation phase, of turbidity (Fig. 1B), and the resulting clots showed similar maximum absorbency, or fibre mass-to-length ratio (Fig. 1C), indicating ultrasound does not influence fibrin clot formation.

Next, we examined the effect of ultrasound on already formed clots (Fig. 1D), which is more relevant to the clinical setting of lysis of already established thrombi. Ultrasound was applied 30min after thrombin addition, for the whole duration of the experiment. After 30 minutes, the clot without, and with 9W or 15W, ultrasound showed a slow and steady increase in absorbency (Fig. 1E-F). However, for the first 5min of ultrasound application, 30W power significantly diminished this increase in absorbency (by 53%), whilst power of 47W induced a plateau in absorbency (Fig. 1E-F). After this period, absorbency increase rates were no longer affected by 30W and 47W ultrasound. These data suggest that higher ultrasound power alters clot structure, measurable by turbidity.

Finally, we tested whether this change in clot structure is reversible (Fig. 1G). Ultrasound was applied 30min after thrombin addition, for 15min only, and absorbency was measured for a further 30min. We found a similar effect at 30W and 47W immediately upon application of ultrasound, but upon stopping, absorbency rapidly and fully returned to pre-ultrasound levels (Fig. 1H). Upon quantification, both 30W and 47W ultrasound

significantly diminished absorbency between 30-45min, which was followed by a rapid increase in absorbency between 45-60min, and then matching again that of no ultrasound application between 60-75min (Fig. 1I). The effect of ultrasound on clot structure, and its reversibility, is extremely rapid, occurring within 5min of ultrasound application / switch-off.

Application of ultrasound reduces fibre thickness and increases clot pore size

With turbidity data indicating a significant effect on fibre mass-length ratio, especially at 47W, we next assessed clot and fibre ultrastructure by scanning electron microscopy (Fig. S2) and clot porosity by permeation assay (Fig. S3).

Clot fibre thickness was significantly decreased (34%) after application of 47W ultrasound compared to control (Fig. 2A-C).

With changes in fibre thickness affecting overall clot density, therefore pore size, we next investigated the effect of ultrasound on clot permeability. We found that application of 47W ultrasound significantly increased (24%) average pore size, compared to control (Fig. 3A). Turbidity experiments showed a transient effect on clot structure, so we investigated the effect of stopping ultrasound application on pore size. In agreement with the reversible effect observed by turbidity, the increase in pore size upon application of 47W ultrasound was fully reversible (Fig. 3B), with the pore size returning to its original value pre-ultrasound.

Application of ultrasound increases the rate of internal and external clot lysis

To confirm the effect of ultrasound on clot lysis, its main target for clinical application, we first used our modified turbidity set-up (Fig. S1), in the presence of tPA. Ultrasound was applied 30min after addition of thrombin and tPA, for a further 30min. We found that 30W and 47W increased the rate of lysis, whilst 9W and 15W had no effect on clot lysis (Fig. 4A). Times to 25%, 50%, 75% and 100% lysis were all decreased at 30W and especially 47W (Fig. 4B). 47W ultrasound significantly reduced all lysis time parameters, whilst significance was achieved at 15W and 30W for time-to-100%-lysis only (Fig. 4C). These data indicate that 47W ultrasound induced an immediate speeding-up of lysis, whilst lower powers (15W and 30W) appeared to only affect lysis after longer application time.

Next, we applied a modified permeation assay (Fig. S4) to assess the effect of ultrasound on clot lysis under flow, when tPA is added to a formed clot. 0.4mg/ml tPA was applied to the clot during ultrasound delivery, and permeation was measured whilst lysis was quantified by measuring D-dimers released into the flow-through. We found that permeation, increased exponentially and significantly after 30min of 47W ultrasound

application, demonstrated by increased flow-through over time, whilst remaining consistent in the absence of ultrasound (Fig. 5A). The start of the exponential increase in pore size matched that of the appearance of D-dimer in the flow-through at 30min (Fig. 5B). We also found that after 50min, D-dimer amount exponentially and significantly increased in the presence of 47W ultrasound, whilst remaining steady in the absence of ultrasound (Fig. 5B). Of note, all clots collapsed between 80-90min in the presence of 47W ultrasound, whilst remaining stable up to the 90min endpoint in the absence of ultrasound.

Application of ultrasound decreases the amount of tPA required to achieve similar lytic efficiency to higher dose

To assess whether ultrasound decreases the amount of tPA required to achieve the same lytic efficiency as higher concentrations, we performed lysis assays in our modified turbidity set-up (Fig. S1), in the presence of varying concentrations of tPA combined with the absence or presence of tPA. We found that a decrease of tPA concentration from 50ng/mL to 37.5ng/mL, in the absence of ultrasound, significantly prolonged the time to half-lysis from 28.3 to 75.3min (Fig. 6A&B). In the presence of ultrasound, the time to half-lysis was significantly reduced to 18.0 (50ng/mL tPA) and 32.37min (37.5ng/mL tPA), with the latter value similar to that of 50ng/mL tPA without ultrasound (28.3min). We therefore showed that compared to a standard dose of tPA (50ng/mL), we achieve a similar lytic efficiency with 25% less tPA (37.5ng/mL) when ultrasound is applied. These data indicate the potential for ultrasound to diminish lytic dose *in-vivo* whilst still achieving the same thrombolytic action.

DISCUSSION

Our study shows that *in-vitro* application of ultrasound through a catheter placed close to a plasma clot accelerates fibrinolysis rates, and that these effects are associated with changes in clot structure, based on thinning of fibrin fibres and increased porosity. These effects on clot structure provide a possible mechanism for the effects of ultrasound on fibrinolysis, since increased porosity and thinning of fibrin fibres increase the access of fibrinolytic enzymes to the core of the clot and its constituent fibrin fibres. The mechanistic effects were mainly present at ultrasound outputs of 30 and 47W in the context of this model system. Two key findings are of particular interest: firstly, the effects of ultrasound on clot structure were completely reversible, with onset of effects and return to baseline

occurring within minutes of application and discontinuation of ultrasound respectively. Secondly, the increase in fibrinolysis was measurable under static conditions but enhanced exponentially under flow conditions. These data suggest that ultrasounds provide a fully reversible effect on fibrin fibre architecture that increases lysis, particularly when blood flow has been (partially) restored.

Reversibility of ultrasound-mediated effects on fibrin structure provides evidence that there are no long-lasting changes in clot structure induced by the application of ultrasound. We further showed that ultrasound did not change the clot formation stage, but only had an effect after clots were fully formed. We observed a reversible thinning of fibrin fibres, which could be caused by disbanding or dispersing of internal fibrin structures. Ultrasound-induced fibre dispersion is then reflected by smaller fibre diameters after dehydration and fixation in SEM. A previous study showed that fibrin fibres are remodelled by reversibility of binding interactions that underpin fibre formation, as shown by re-arrangements of fluorescently labelled fibrin molecules in fully assembled fibres[11]. However, this type of fibrin remodelling was abrogated after fibrin crosslinking by activated FXIII[11]. Since our experiments are performed with blood plasma, the clots of which are fully crosslinked by FXIII, the reversibility observed here is likely due to another process. A possible mechanism for the reversible fibre dispersing by ultrasound is based on increased thermodynamic structural kinesis. We have shown that clot structure is reversibly altered by ultrasound shortly after formation, however PE occlusion results from a breakdown of preformed ‘older’ deep vein thrombi. Our current experimental set-up did not allow for these effects to be assessed on ‘older’ thrombi, which will need further investigation.

In agreement with the hypothesis of thermodynamic structural kinesis, our data indicate that catheter-mediated ultrasound application increases access of fibrinolytic factors to the interior of fibrin fibres. Moreover, the rates of fibrinolysis accelerated exponentially when we applied flow. A key mechanism underpinning this accelerated lysis under flow is that there is a constant supply of fresh tPA applied to the clot, whereas a fixed concentration of tPA was used in the static system. Furthermore, as the fibrin clot was degraded by tPA, flow increased, resulting in delivery of growing quantities of tPA to the lysing clot, and an exponential increase in clot breakdown.

The reversible changes in fibrin structure underpinning increased fibrinolysis prompted us to study whether similar fibrinolysis rates could be achieved with lower doses of tPA in the presence of ultrasound. Indeed, when ultrasound was applied, we could lower the dose of tPA by ~25% to achieve the same rate of clot breakdown under static conditions. It is likely that an even greater reduction in dose may be achieved when flow has been partially

restored. Note that the concentrations of tPA in these *in-vitro* experiments (ng/mL) are different from those applied clinically (mg/kg). Our findings have several important clinical implications. Catheter-directed thrombolysis is selectively used in two principal scenarios for the treatment of pulmonary embolism. The first is in patients in whom there are signs of right ventricular dysfunction, and catheter lytic is considered as a preferential strategy to the use of anticoagulation alone, with the aim of accelerating recovery in PE patients with right ventricular dysfunction and/or hypoxia. The second is in patients with haemodynamic instability caused by PE, in whom concerns exist over the bleeding risks associated with high dose systemic thrombolysis[12]. Our findings suggest a potential role for ultrasound facilitation of thrombolysis with respect to both of these groups, albeit with potentially important caveats.

Firstly, in patients with PE and haemodynamic disturbance, the risk of death or destabilisation is most pronounced within the first 48-72 hours of presentation[13]. This is due to a 'spiral of shock' whereby the longer the right ventricle is under strain, the worse the haemodynamic disturbance becomes[3]. A pharmacomechanical strategy of reperfusion – using both ultrasound and thrombolytic – could therefore accelerate clot dissolution and, by consequence, increase recovery of the failing right ventricle when compared to a strategy of catheter or peripheral thrombolysis alone – both in terms of speed and volume of clot dissolution, as evidenced by D-dimer and time to dissolution[14, 15]. The second advantage of a combined strategy with ultrasound is the facilitation of lower doses of thrombolytic to dissolve a given amount of clot. In the case of patients with high bleeding risk, the advantages are clear – the circulating volume of lytic would be lower and therefore in patients such as the elderly, or those with cancer, the bleeding risk might also be lower.

This concept is supported by several human clinical trials of EKOS. Initial published studies of catheter lysis with the EKOS system had utilised 20mg of alteplase over 15 hours[4], or 24mg over 12 hours[16]. However, the OPTALYSE study randomised patients to differing doses of alteplase (from 8mg to 24mg for bilateral PE) with shorter durations of delivery (from 2 to 6 hours). Interestingly, just 8mg (over 2 hours or 4 hours) and 12 mg (over 6 hours) of alteplase with EKOS were all able to successfully offload the right ventricle on CT at 48 hours, with durable clinical benefit at one year[17], although the absence of an anticoagulation control arm prevented comparison to lytic-free treatment in that trial. In the ULTIMA study, however, there were no changes at 24 hours in the degree of echocardiographic RV ventricular dilatation in patients treated with anticoagulation

alone, suggesting that resolution of RV strain is a relatively slow process when treated with anticoagulation alone, when compared to EKOS with alteplase[13].

In KNOCOUT PE, 489 patients were enrolled in a real-world prospective observational study, reporting data on outcomes and bleeding from real-world use of EKOS[18]. The majority of patients were enrolled after publication of OPTALYSE, and so lower doses of lytic given over shorter time periods were increasingly used in clinical practice, with a median dosage across the study of 18mg of alteplase. Major bleeding rates across the full cohort at 72 hours were 1.6% - markedly lower than those observed in the earlier SEATTLE II and OPTALYSE studies, which the authors speculate may have been due to lower dose protocols in higher bleeding risk patients. The 8mg and 12mg protocols tested within OPTALYSE were utilised in 31% of patients in the KNOCOUT study[18].

Combining bench data from the current manuscript with the available prior *in-vivo* clinical data on the use of ultrasound-assisted catheter-based thrombolysis therefore raises the possibility that ultrasound could accelerate clot dissolution and/or reduce the dose of lytic. A single head-to-head comparison of catheter lysis versus ultrasound-assisted catheter lysis showed no difference in clot burden reduction on CT scanning performed at 48 hours, but the study was underpowered (n=81) and infusion times were not standardised[7]. Larger studies are needed to test either clinical outcome and/or benefit on RV strain resolution or bleeding risk reduction. The largest trial to date of ultrasound-assisted catheter lysis - a randomised outcome trial of EKOS plus anticoagulation versus anticoagulation alone - is currently ongoing, aiming to recruit 544 patients[19]. However, this trial is not modulating lytic dosing, and will not compare ultrasound-facilitated lysis against either passive catheter lysis or systemic lysis. Nevertheless, it will test the role of ultrasound facilitation of lytic against current standard of care in intermediate-high risk PE, and we await the results with interest.

Finally, the question of ultrasound power algorithm remains open. In the current study, 30W and 47W demonstrated highly reversible changes in clot structure. Lower wattages were less effective. The only available commercial system to utilise this pharmacomechanical strategy, the EKOS system, deploys a rotating algorithm of wattage, cycling through low wattages, below the threshold of 30W, up to a maximum of 47W[5]. Further studies of whether a rotating algorithm offers improved safety or efficacy over and above a fixed algorithm at 30W to 47W would be of interest. The manufacturers of the EKOS system are currently conducting clinical trials with a new algorithm deploying higher Wattages, designed to further shorten time and dose of ultrasound-assisted lytic delivery in

PE (ClinicalTrials.gov ID NCT06310018). Additional studies would be required to assess the impact of this new protocol.

In conclusion, in our bench model, low-power ultrasounds reversibly altered clot configuration and increased the rate of clot dissolution in the presence of thrombolytic agent. These findings support the concept of ultrasound-facilitated pharmacomechanical treatment of venous thromboembolism, although the optimal algorithm of ultrasound wattage for safety or efficacy, and the optimal dosage of adjunctive lytic to be used in patients to gain the greatest net clinical benefit requires further investigation.

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FIGURE LEGENDS

Figure 1: EKOS does not affect clot formation, but does alter clot structure. Plasma clot formation was measured by turbidity experiments, in the absence or presence of ultrasound (grey-shaded areas) at 9, 15, 30 and 47W. Application of ultrasound during the first 30min of clotting (**A**) did not affect the lag phase (**B**) or MaxOD (**C**). Application of ultrasound after 30min of clotting (**D**) resulted in a decrease in MaxOD that was significant at 47W (**E**). When comparing the change in OD between various timepoints (**F**), the reduction in δOD was significant for 30W and 47W after the first 15min, whilst remaining unchanged across all ultrasound powers between 45-75min. Transient application of ultrasound between 30-45min (**G**,**H**) resulted in a significantly decreased δOD at 30W and 40W (**I**), that was reversible once the ultrasound was turned off (**H**, **I**). Interestingly, the transient application of ultrasound resulted in a significantly increased MaxOD at 47W (**G**-**I**). Group comparisons were analysed using two-way ANOVA with Dunnett's multiple comparison test. Data are represented as mean ± standard deviation (n=6). * $p<0.05$, $^{\$}$ $p<0.001$, $^{\#}$ $p<0.0001$.

Figure 2: EKOS reduces fibrin fibre thickness. Plasma clot structure was assessed by scanning electron microscopy, in the absence (**A**) or presence (**B**) of ultrasound. 47W ultrasound significantly reduced fibrin fibre thickness (**C**). Group comparisons were analysed using unpaired Student's t-test. Data are represented as mean ± standard deviation (n=6). $^{\#}$ $p<0.0001$.

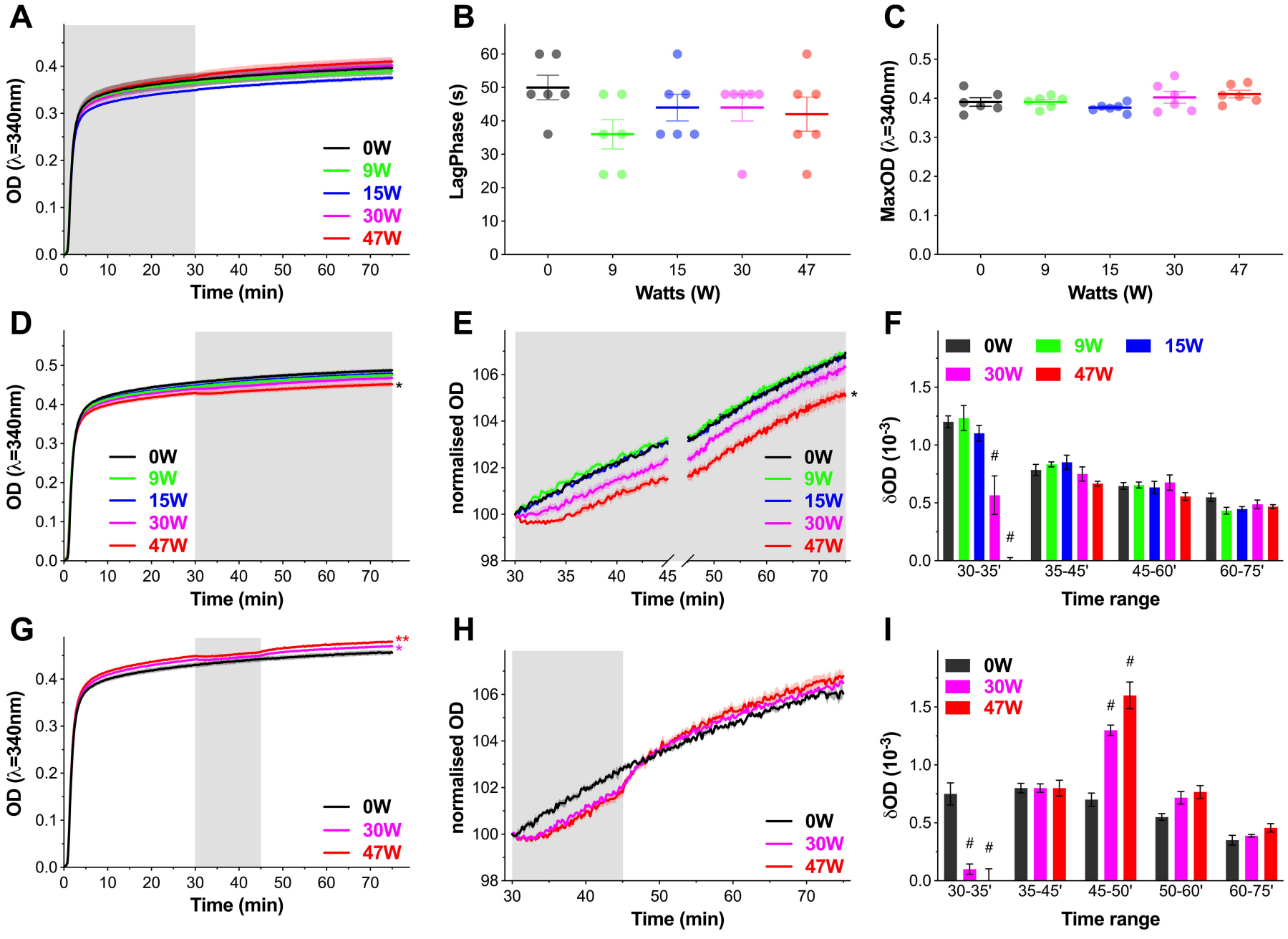
Figure 3: EKOS increases clot permeability. Plasma clot pore size was assessed by permeation experiments, in the absence or presence of ultrasound. 47W ultrasound significantly increased the pore size within the clots (**A**), an effect that was reversible once ultrasound (grey-shaded area) was stopped (**B**). Group comparisons were analysed using one-way ANOVA with Turkey's multiple comparison test. Data are represented as mean ± standard deviation (n=6). $^{\#}$ $p<0.0001$.

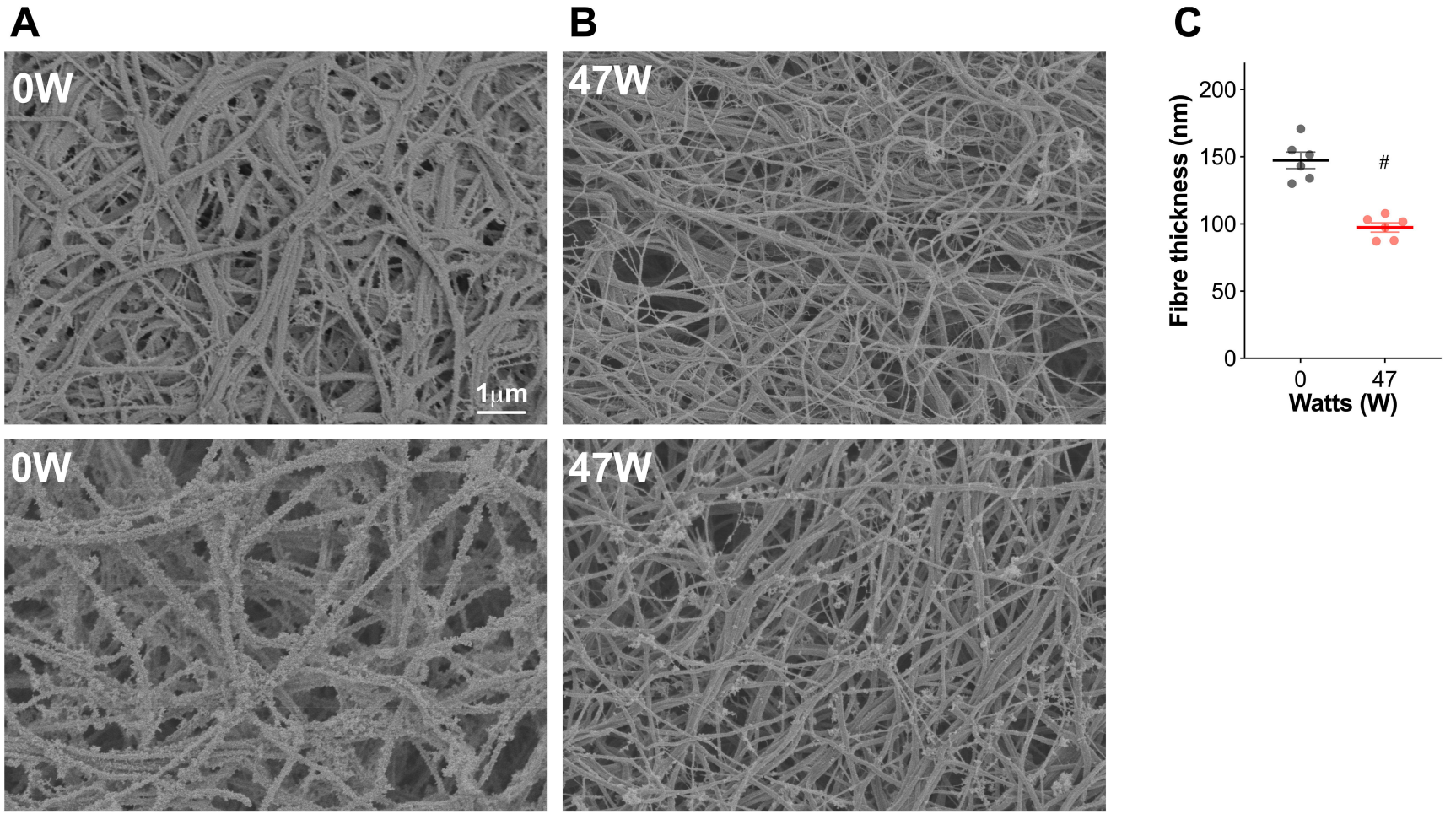
Figure 4: EKOS increases clot lysis rates. Plasma clot lysis was measured by turbidity experiments, in the absence or presence of ultrasound. Ultrasound (grey-shaded area) at 9, 15, 30 and 47W was applied after 30min of clotting, for 30min. Turbidity curves normalised to 100% OD at 30min (**A**) show that 30W and 47W ultrasound sped up clot lysis. 30W and 47W ultrasound reduced the time to reach 25%, 50%, 75% and 100% lysis

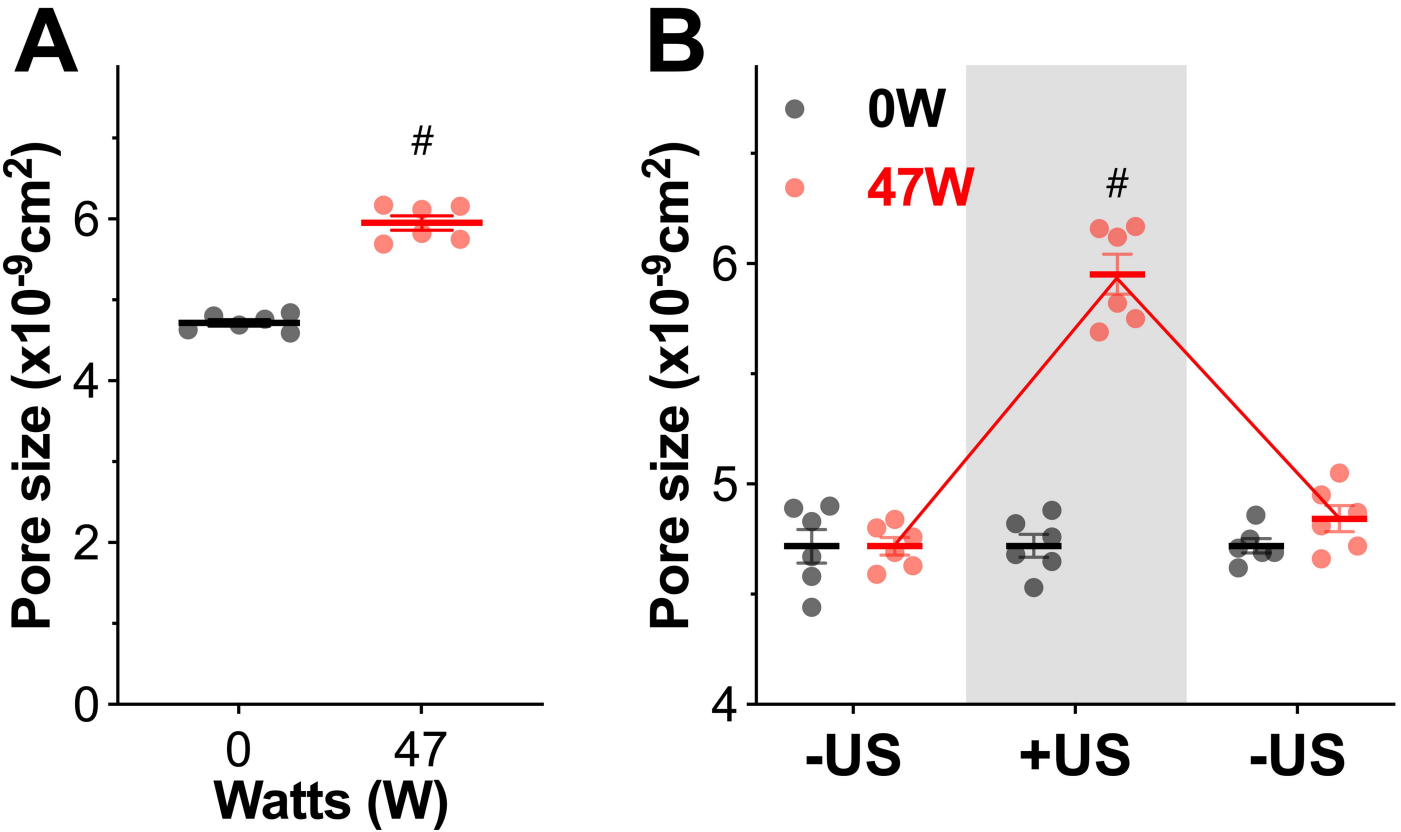
(**B**,**C**). Group comparisons were analysed using two-way ANOVA with Dunnett's multiple comparison test. Data are represented as mean ± standard deviation (n=6). * $p<0.05$, ** $p<0.01$, $^{\$}$ $p<0.001$, $^{\#}$ $p<0.0001$.

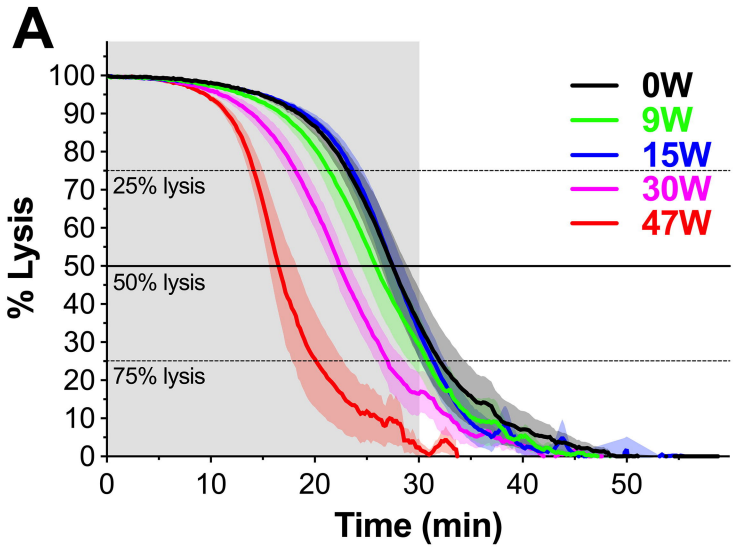
Figure 5: EKOS increases clot lysis under flow. Permeation experiments were performed on plasma clot, with application of tPA once the clot has formed, in the absence or presence of ultrasound. 47W ultrasound significantly and exponentially increased the rate of permeation as tPA flowed through the clot, compared to the absence of ultrasound (**A**). The amount of D-Dimer in the flow-through also significantly and exponentially increased with 47W ultrasound, compared to the absence of ultrasound (**B**). Of note, all clots exposed to 47W ultrasound collapsed between the 70-80min timepoints, whilst those without ultrasound remain structurally intact during the 90min measurement period. Group comparisons were analysed using one-way ANOVA with Turkey's multiple comparison test. Data are represented as mean ± standard deviation (n=6). * $p<0.05$, ** $0<0.01$, $^{\#}$ $p<0.0001$.

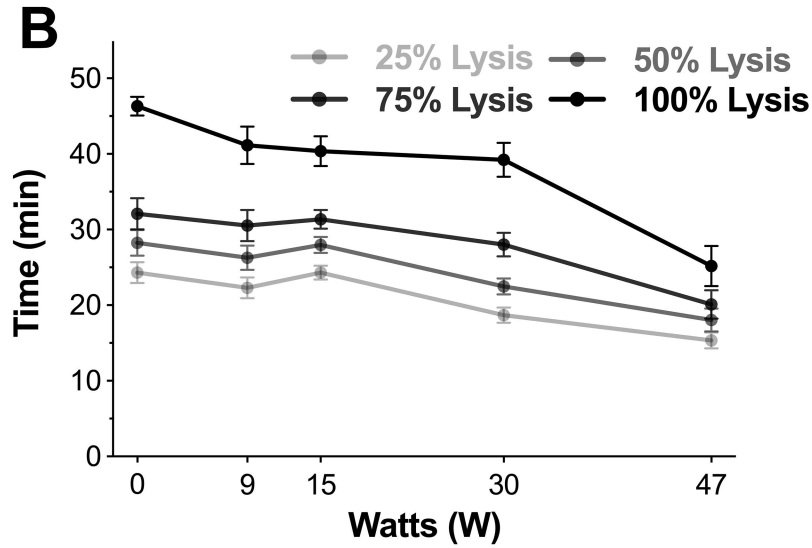
Figure 6: Lower tPA doses are required for similar efficacy when ultrasound is applied. Plasma clot lysis was measured by turbidity experiments, in the absence or presence of ultrasound with various doses of tPA. Ultrasound was applied after 30min of clotting, for the duration of the lysis, at 47W. Turbidity curves normalised to 100% OD at 30min (**A**). At 50ng/mL tPA, 47W ultrasound significantly reduced lysis time, whilst lysis with 37.5ng/mL tPA alone showed a significant reduction in lysis time (**A**,**B**). However, application of ultrasound in the presence of 37.5ng/mL tPA increased lysis efficiency to similar level to 50ng/mL tPA without ultrasound (**A**,**B**). Group comparisons were analysed using one-way ANOVA with Turkey's multiple comparison test. Data are represented as mean ± standard deviation (n=6). $^{\$}$ $p<0.001$, $^{\#}$ $p<0.0001$.

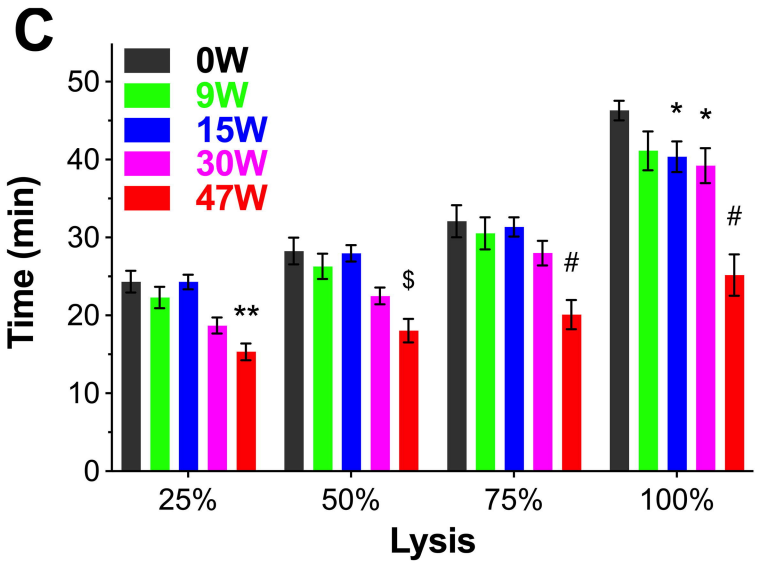

A
OD (λ=340nm)
Time (min)
0W
9W
15W
30W
47W
B
LagPhase (s)
Watts (W)
C
MaxOD (λ=340nm)
Watts (W)
D
OD (λ=340nm)
Time (min)
*
E
normalised OD
Time (min)
*
F
δOD (10-3)
#
#
30-35'
35-45'
45-60'
60-75'
Time range
G
OD (λ=340nm)
Time (min)
**
*
H
normalised OD
Time (min)
I
δOD (10-3)
#
#
#
30-35'
35-45'
45-50'
50-60'
60-75'
Time range

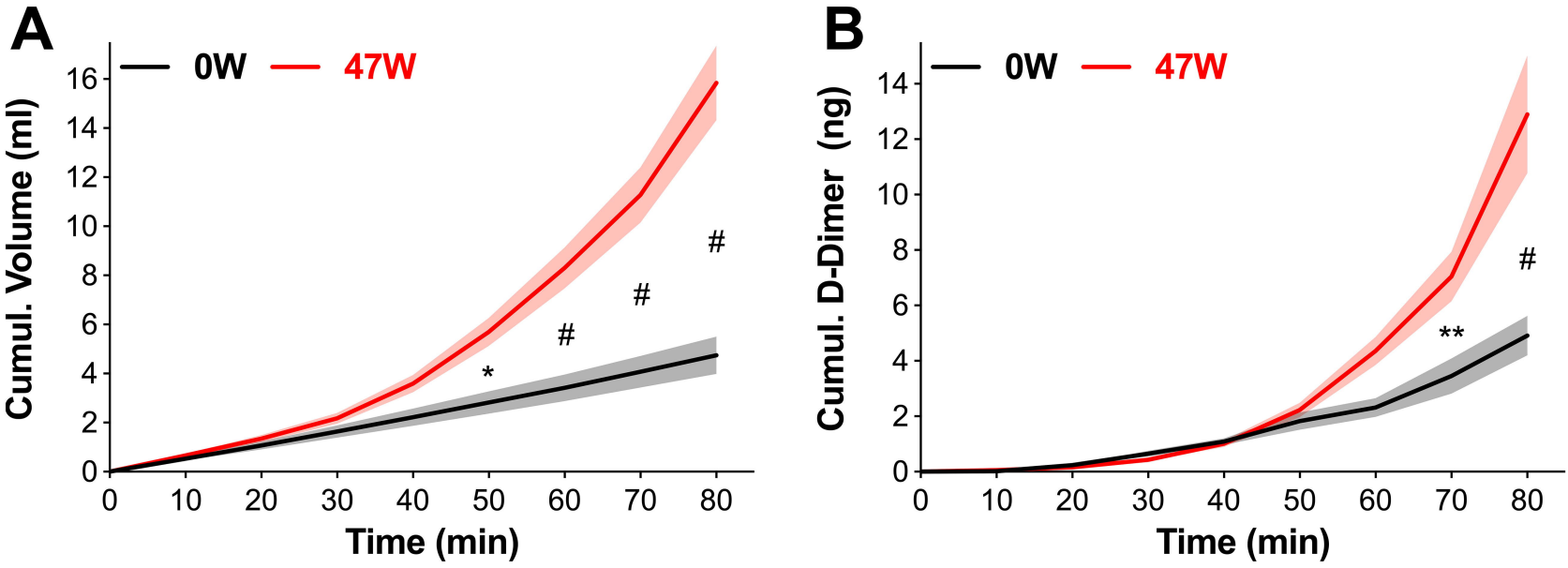

A
B
C
0W
47W
1μm
0W
47W
Fibre thickness (nm)
200
150
100
50
0
#
0
47
Watts (W)

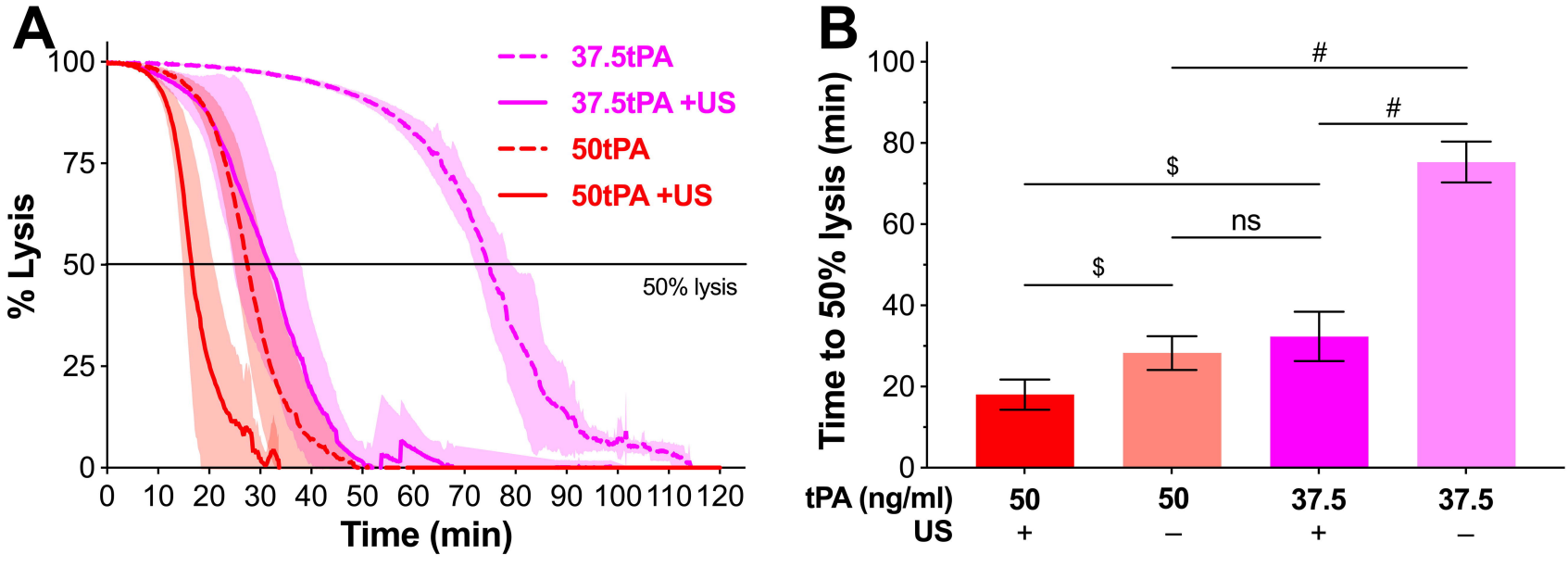

A
Pore size (x10-9cm2)
0
2
4
6
#
0
47
Watts (W)
B
Pore size (x10-9cm2)
4
5
6
0W
47W
#
-US
+US
-US


A
% Lysis
Time (min)
0W
9W
15W
30W
47W
25% lysis
50% lysis
75% lysis


B
Time (min)
Watts (W)
25% Lysis
50% Lysis
75% Lysis
100% Lysis


C
Time (min)
Lysis
0W
9W
15W
30W
47W
25%
50%
75%
100%


A
0W
47W
Cumul. Volume (ml)
0
2
4
6
8
10
12
14
16
0
10
20
30
40
50
60
70
80
Time (min)
*
#
#
#
B
0W
47W
Cumul. D-Dimer (ng)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60
70
80
Time (min)
**
#


A
100
75
50
25
0
% Lysis
0 10 20 30 40 50 60 70 80 90 100 110 120
Time (min)
37.5tPA
37.5tPA +US
50tPA
50tPA +US
50% lysis
B
Time to 50% lysis (min)
100
80
60
40
20
0
#
#
$
ns
$
tPA (ng/ml)
50
50
37.5
37.5
US
+
–
+
–

Ultrasound-mediated catheter delivery of tissue plasminogen activator promotes thrombolysis by altering fibrin fibre thickness and clot permeability

Ariëns RAS, Sharp ASP, Duval C*

SUPPLEMENTAL METHODS & FIGURES

SUPPLEMENTAL METHODS

Materials

Frozen mixed-pool plasma was obtained from First-Link (UK) Ltd (Wolverhampton, UK) and all other reagents were from Merck (Gillingham, UK) unless otherwise stated.

Scanning electron microscopy

Fibrin fibre thickness was assessed by a modified scanning electron microscopy protocol[1] (Fig. S2). 1mL of clotting mixtures were prepared with plasma diluted 1:6 in TBS warmed to 32°c, 1U/mL Thrombin and 10mM $CaCl_2$. The clotting mixture was transferred into the large end of a cut 1mL stripette fitted with the EKOS catheter and ultrasonic core positioned in the middle, and the clot was left to form for 2hrs in a thermostated humidity chamber. The clots were transferred into thermostated TBS (32°C), and ultrasound applied for 15min, with coolant applied at the relevant speed. The clots were then bathed in thermostated 2% glutaraldehyde (diluted in TBS), with the ultrasound and coolant applied for a further 5min, before the clots were left in fixative overnight. Next day, the catheters were removed from the clots, which were then removed from the stripettes. The ends of the clots were cut off 4mm deep and placed in the perforated lid of a 0.5mL Eppendorf, oriented so that the free end of the clots was exposed up during the remaining step of the protocol. Clots were washed in 50mM sodium cacodylate (in TBS) 3x 20min, and dehydrated by 15min incubations in 30%, 40%, 50%, 70%, 80%, 90%, 95%, 3x 100% acetone. The clots then underwent critical point drying using a Polaron E3000 CPD Unit (Quorum Technologies; Newhaven, UK), performed by Martin Fuller (Astbury Centre for Structural and Molecular Biology, University of Leeds, UK), before being mounted onto 13mm diameter aluminium stubs (Agar Scientific; Stansted, UK) and coated with 5nm layer of iridium using a High-Resolution Sputter Coater 208HRD coupled to a Thickness Controller MTM-20 (Cressington Scientific Instruments; Watford, UK). Each clot was imaged at x5k, x10k, x20k magnification using a SU8230 Ultra-High-Resolution Scanning Electron Microscope (Hitachi; Maidenhead, UK). Image processing was performed using the ImageJ software v2.9.0 (National Institutes of Health; Bethesda, MD, USA). A 4x6 lines grid was applied for each image, and the thickness of the fibres crossing each of the 24 intersections was measured using the scale bar as reference. Experiments consisted of 6 repeats, each using a fresh catheter.

Lysis under flow

Experiments to study lysis under flow consisted of the same set-up as the permeation, with a few modifications (Fig. S4). Once the clot was formed in the cuvette, TBS was added to the top of the clot up to a constant 4cm height (distance from bottom of the clot to top of the liquid), and the flow through was collected every 10min for 30min. After that time, the TBS on top of the clot was replaced with 0.4g/ml tPA (in TBS), up to 4cm height, which was kept constant throughout the experiment. The flow through was collected every 10min for 90min, weighed and stored at -20°C. The content of D-Dimer in the flow through aliquots was measured using a D-Dimer ELISA kit (RayBiotech; Peachtree Corners, GA, USA). Experiments consisted of 6 repeats, each using a fresh catheter.

Data analysis

Data are represented as mean ± standard deviation. Graphs and statistical analyses were performed using GraphPad Prism v10.0.3 (GraphPad Software; San Diego, CA, USA). Data normality was assessed using the Shapiro-Wilk test, and found to show normal distribution. Therefore, the means were compared by Student's t-test (2 groups) or one-way / two-way ANOVAs (>2 groups), as appropriate. * $p<0.05$, ** $p<0.01$, $^{\$}$ $p<0.001$, $^{\#}$ $p<0.0001$.

SUPPLEMENTAL REFERENCES

1. Duval C, Allan P, Connell SD, Ridger VC, Philippou H, Ariens RA. Roles of fibrin alpha- and gamma-chain specific cross-linking by FXIIIa in fibrin structure and function. Thromb Haemost. 2014;111(5):842-50.

SUPPLEMENTAL FIGURES

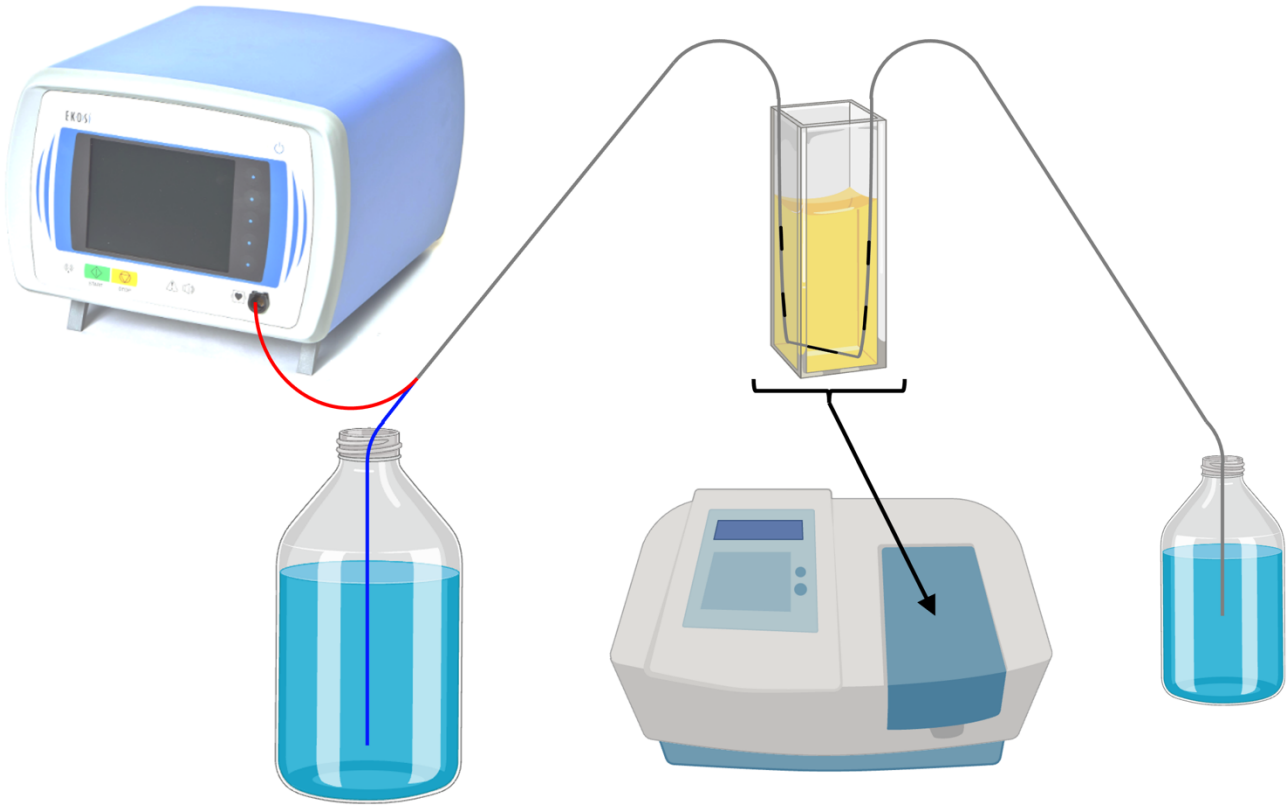


Figure S1: Turbidity and Turbidity&Lysis set-up. A plasma clot was formed in a spectrophotometer cuvette, with EKOS™ catheter and ultrasonic core positioned so that the light (λ340nm) was not obstructed. The experiments were performed at 32°c, with coolant applied at various rates depending on the ultrasound power. OD (λ340nm) was measured over 75min. Red lines: EKOS™ catheter and ultrasonic core. Blue line: coolant (water).

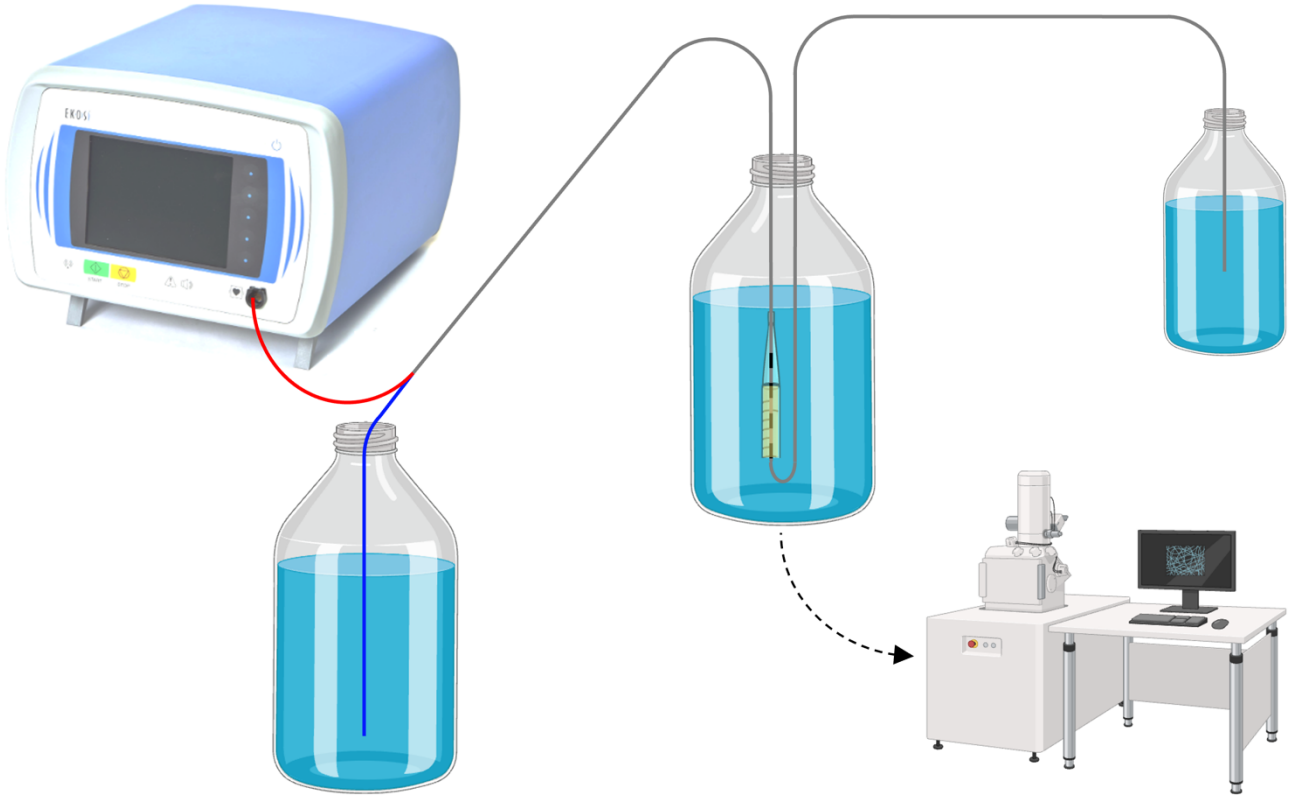


Figure S2: Electron Microscopy set-up. A plasma clot was formed in a 1ml stripette, containing the EKOS™ catheter and ultrasonic core so that the ultrasonic core was positioned at the edge of the clot. After clotting for 60min in a humidity chamber, the set-up was immersed into TBS and ultrasound was applied. The experiments were performed at 32°C, with coolant applied at various rates depending on the ultrasound power. Immediately after the end of ultrasound period (15min), the set-up was transferred into 2% glutaraldehyde, with the ultrasound applied for a further 5min. The clots were then removed from the set-up and freed of the catheter, before being incubated overnight in 2% glutaraldehyde. Next day, the clots were sliced so that the end part exposed to ultrasound could be imaged by scanning electron microscopy, after processing. Red lines: EKOS™ catheter and ultrasonic core. Blue line: coolant (water).

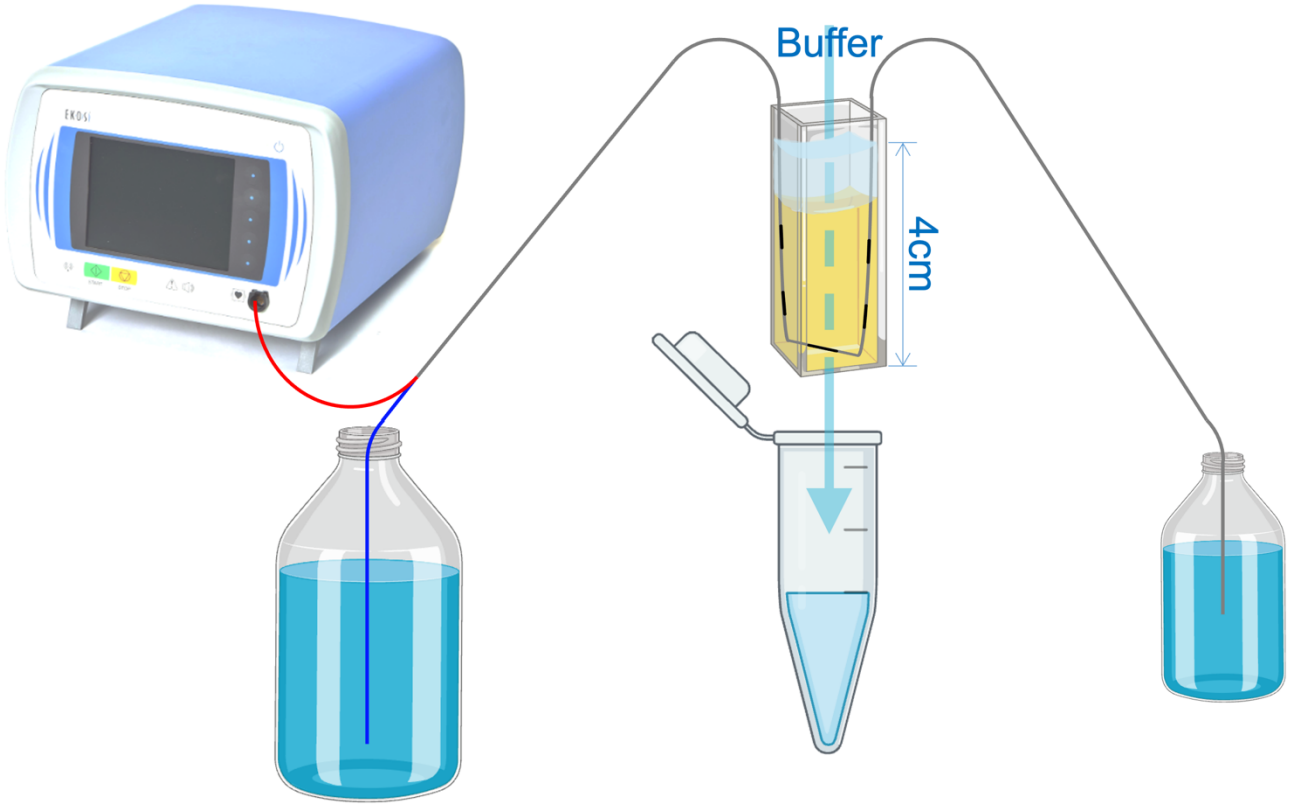


Figure S3: Permeation set-up. A spectrophotometer cuvette was modified with 4x1mm holes drilled at the bottom. The plasma clot was formed in the cuvette containing the EKOS™ catheter and ultrasonic core. After clotting for 60min in a humidity chamber, TBS was applied at the top of the clot, to a constant height of 4cm, and the volume of flow-through was measured every 10min for 90min. The experiments were performed at 32°C, with coolant applied at various rates depending on the ultrasound power. Red lines: EKOS™ catheter and ultrasonic core. Blue line: coolant (water).

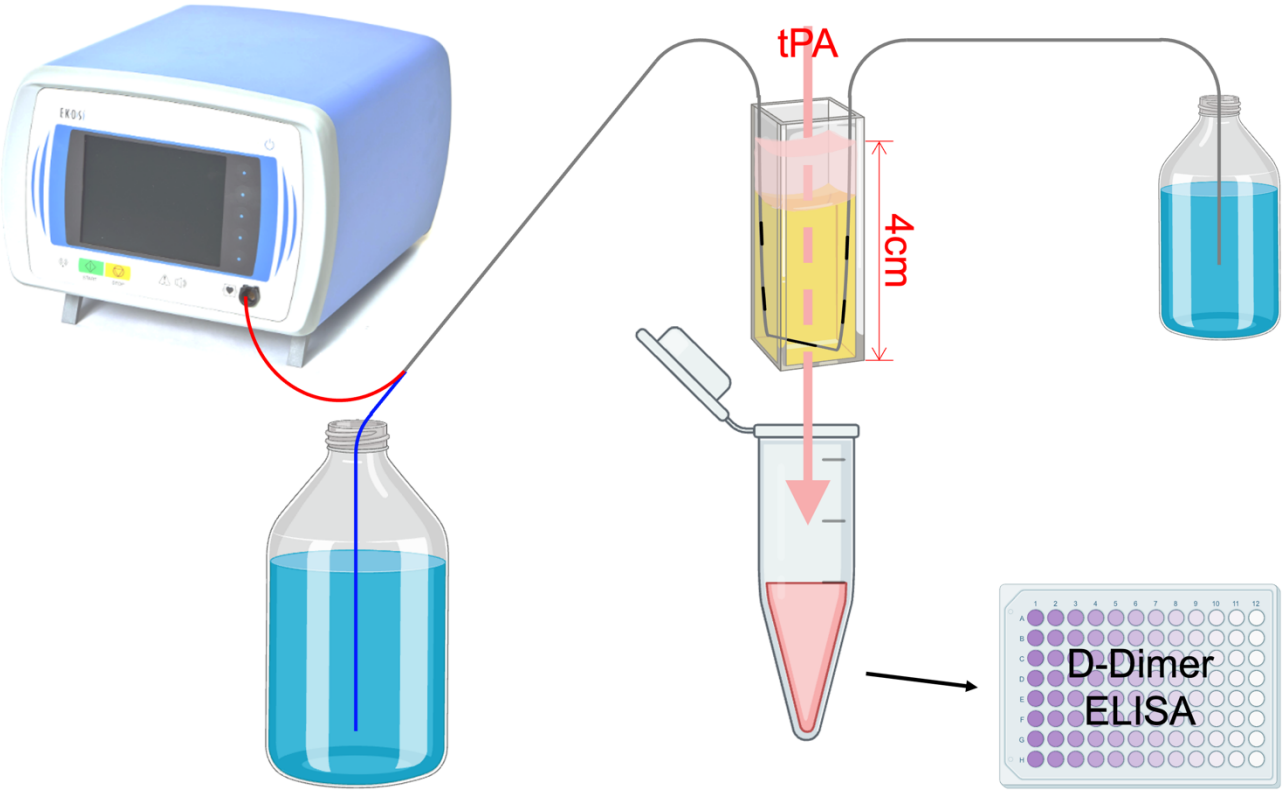


Figure S4: Lysis under flow set-up. A spectrophotometer cuvette was modified with 4x1mm holes drilled at the bottom. The plasma clot was formed in the cuvette containing the EKOS^{TM} catheter and ultrasonic core. After clotting for 60min in a humidity chamber, TBS was applied at the top of the clot, to a constant height of 4cm, and the clot was washed for 60min. The TBS was then replaced by tPA (in TBS) to a constant height of 4cm, and the volume of flow-through was measured every 10min for 90min. The amount of D-Dimer was quantified in each flow-through tube to assess the rate of lysis. The experiments were performed at 32°C, with coolant applied at various rates depending on the ultrasound power. Red lines: EKOS^{TM} catheter and ultrasonic core. Blue line: coolant (water).