ARTICLE - Coagulation & its Disorders

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

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SUPPLEMENTAL METHODS & FIGURES

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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

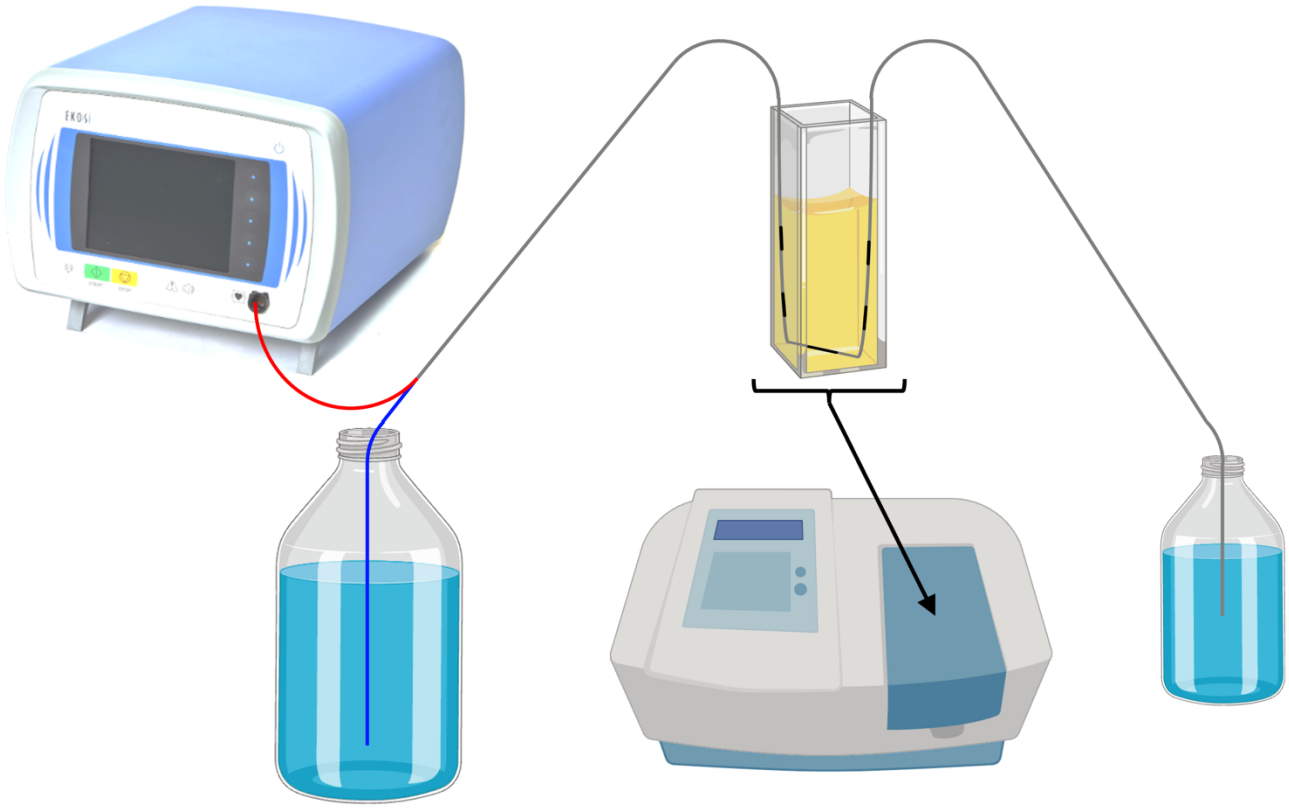

Figure S1: Turbidity and Turbidity&Lysis set-up. A plasma clot was formed in a spectrophotometer cuvette, with EKOS™ catheter and ultrasonic core positioned so that the light (λ340nm) was not obstructed. The experiments were performed at 32°c, with coolant applied at various rates depending on the ultrasound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

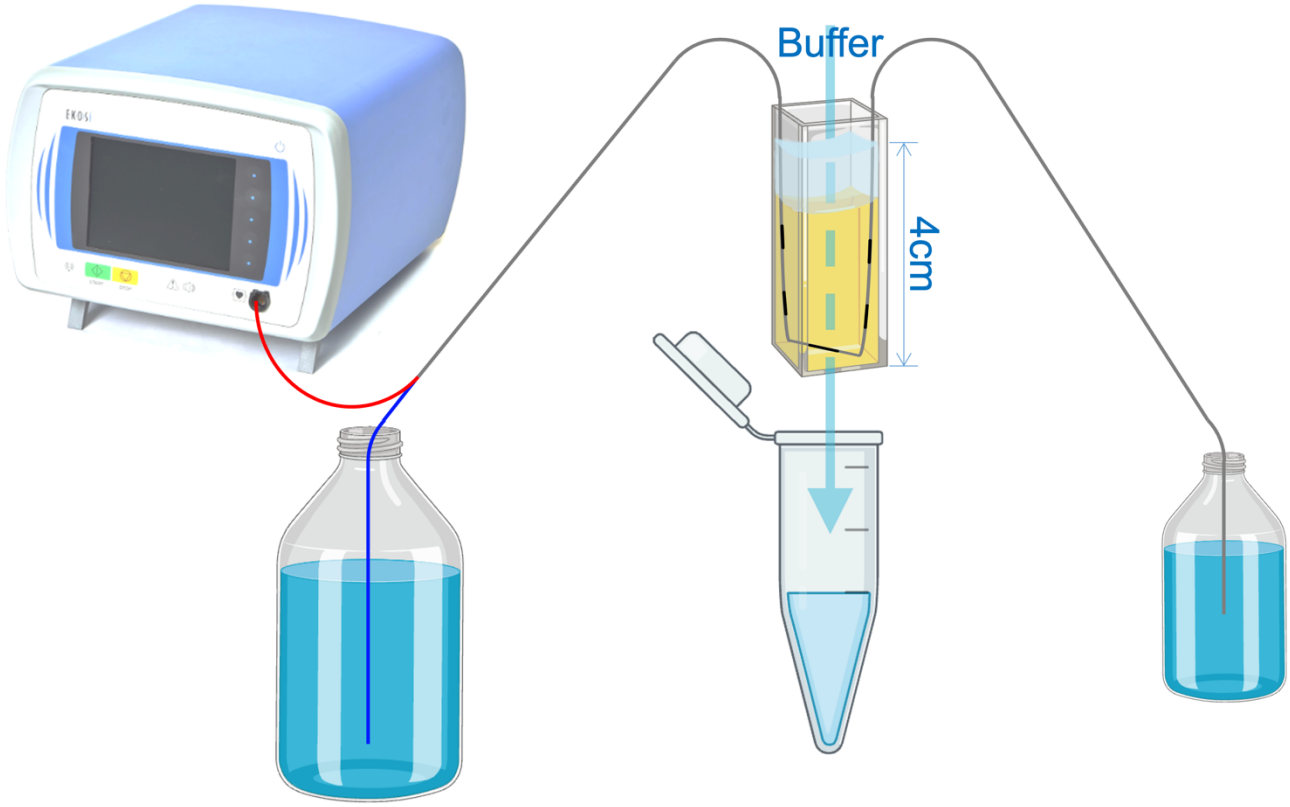

Figure S2: Permeation set-up. A spectrophotometer cuvette was modified with 4x1mm holes drilled at the bottom. The plasma clot was formed in the cuvette containing the EKOS™ catheter and ultrasonic core. After clotting for 60min in a humidity chamber, TBS was applied at the top of the clot, to a constant 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).

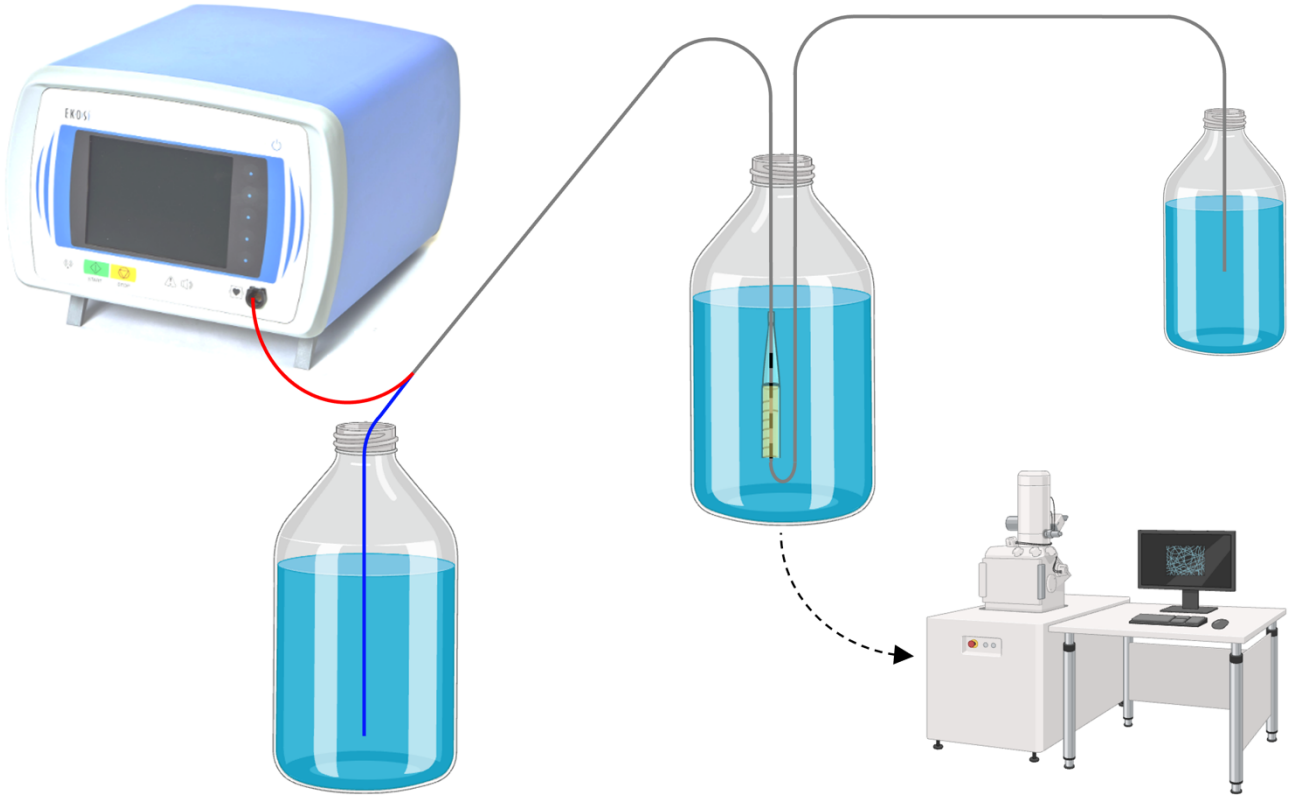

Figure S3: Electron Microscopy set-up. A plasma clot was formed in a 1ml stripette, containing the EKOS™ catheter and ultrasonic core so that the ultrasonic core was positioned at the edge of the clot. After clotting for 60min in a humidity chamber, the set-up was immersed into TBS and ultrasound was 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).

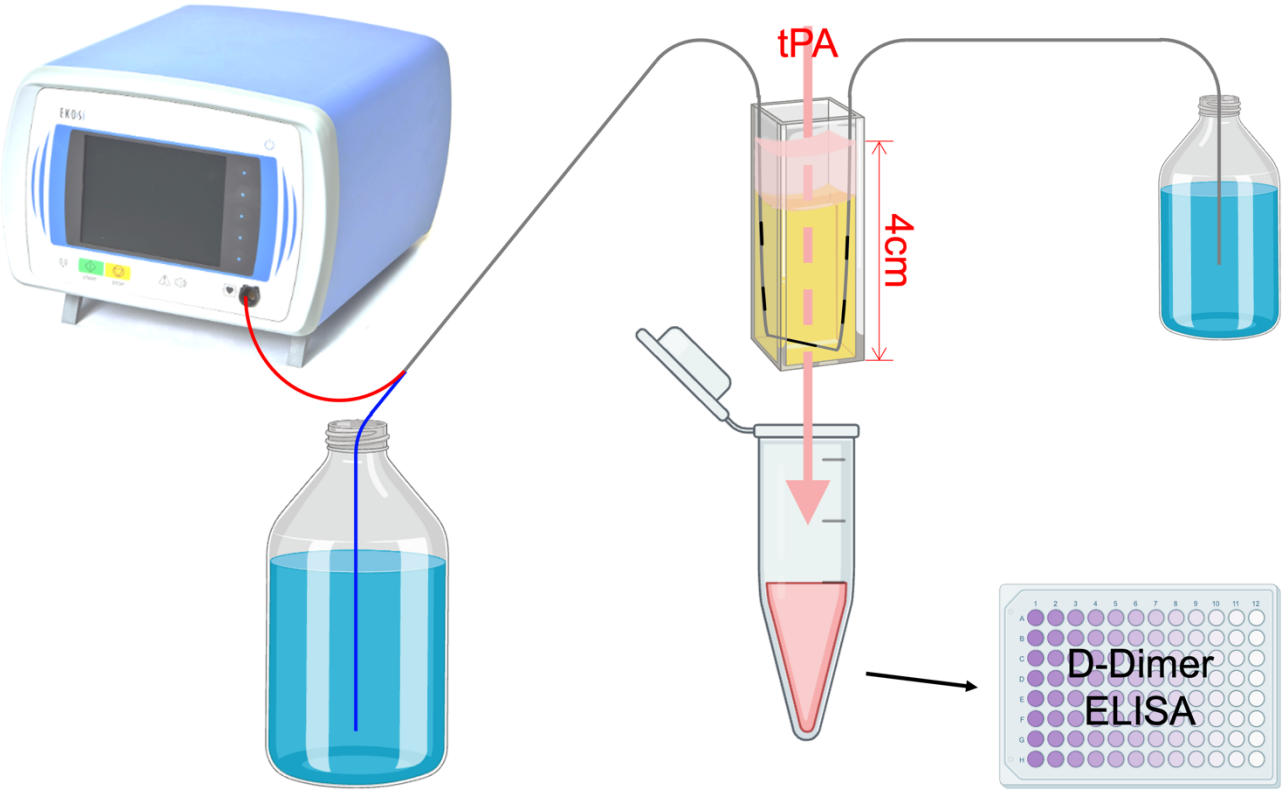

Figure S4: Lysis under flow set-up. A spectrophotometer cuvette was modified with 4x1mm holes drilled at the bottom. The plasma clot was formed in the cuvette containing the EKOS™ catheter and ultrasonic core. After clotting for 60min in a humidity chamber, TBS was applied at the top of the clot, to 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™ catheter and ultrasonic core. Blue line: coolant (water).