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uPA-mediated plasminogen activation is enhanced by polyphosphate

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ABSTRACT

Tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA) differ in their modes of action. Efficient tPA-mediated plasminogen activation requires binding to fibrin. In contrast, uPA is fibrin independent and activates plasminogen in solution or when associated with its cellular receptor urokinase protease activated receptor (uPAR). We have previously shown that polyphosphate (polyP), alters fibrin structure and attenuates tPA and plasminogen binding to fibrin, thereby down-regulating fibrinolysis. Here we investigate the impact of polyP on uPA-mediated fibrinolysis. As previously reported polyP of an average chain length of 65 ($polyP_{65}$) delays tPA-mediated fibrinolysis. The rate of plasmin generation was also delayed and reduced 1.6-fold in $polyP_{65}$-containing clots (0.74±0.06 *vs.* 1.17±0.14 pM/s in $P<0.05$). Analysis of tPA-mediated fibrinolysis in real-time by confocal microscopy was significantly slower in $polyP_{65}$-containing clots. In marked contrast, $polyP_{65}$ augmented the rate of uPA-mediated plasmin generation 4.7-fold (3.96±0.34 *vs.* 0.84±0.08 pM/s; $P<0.001$) and accelerated fibrinolysis (t1/2 64.5±1.7 min *vs.* 108.2±3.8 min; $P<0.001$). Analysis of lysis in real-time confirmed that $polyP_{65}$ enhanced uPA-mediated fibrinolysis. Varying the plasminogen concentration (0.125-1 μM) in clots dose-dependently enhanced uPA-mediated fibrinolysis, while negligible changes were observed on tPA-mediated fibrinolysis. The accelerating effect of $polyP_{65}$ on uPA-mediated fibrinolysis was overcome by additional plasminogen, while the down-regulation of tPA-mediated lysis and plasmin generation was largely unaffected. $polyP_{65}$ exerts opposing effects on tPA- and uPA-mediated fibrinolysis, attenuating the fibrin cofactor function in tPA-media-ted plasminogen activation. In contrast, polyP may facilitate the interaction between fibrin-independent uPA and plasminogen thereby accelerating plasmin generation and downstream fibrinolysis.

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Introduction

Polyphosphate (polyP) is a biomolecule composed of orthophosphate residues (Pi) linked by phosphoanhydride bonds.[1] polyP of average chain length of 60-100-mers is a constituent of platelet dense granules and is released following stimulation of platelets with different agonists.[1,2] polyP acts at numerous points in the coagulation cascade to augment clot formation, including stimulating factor XII (FXII) activation, thrombin-mediated factor XI (FXI) activation and interfering with the function of TFPI.[3] Our work has shown that polyP interferes with fibrin polymerization by stunting protofibril growth, producing a heterogeneous network of dense 'knotted' regions interspersed by pores with altered mechanical properties.[4]

Plasmin is the serine protease responsible for degradation of fibrin. Two forms of the zymogen precursor plasminogen circulate in plasma, the native more abundant form, Glu-plasminogen, and the intermediate form Lys-plasminogen, formed by cleavage of the N-terminal peptide from Glu-plasminogen.[5] Lys-plasminogen exists in flexible open conformation, with an approximately 10-fold higher binding affinity for plasminogen activators thereby facilitating its activation.[6-9] Two-chain plasmin is formed by enzymatic cleavage of plasminogen at Arg561-Val562.[5,10] The two

main physiological plasminogen activators are the serine proteases tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Efficient plasminogen activation by tPA requires fibrin as a cofactor, acting as a template for its own dissolution by binding tPA and plasminogen.[11] uPA is fibrin-independent and efficiently activates plasminogen in solution, but is often found in association with its cellular receptor, urokinase protease activated receptor (uPAR).[12] uPAR does not have a catalytic role but acts to localize plasminogen and uPA to the cell surface increasing local reactant concentration.

We have previously shown that binding of tPA and plasminogen to fibrin is downregulated by polyP, in turn, delaying tPA-mediated fibrinolysis.[4] Here, we characterize the impact of polyP on uPA-mediated plasminogen activation. Our data reveal that polyP has a strong accelerating effect on uPA-mediated plasminogen activation that is highly dependent on concentration of polyP and plasminogen. Enhanced uPA-mediated plasminogen activation in the presence of polyP translates as a robust profibrinolytic effect in clot lysis assays. These data exemplify the complexity of polyP's action on hemostasis and illustrate that the presence of different activators and substrates in the local milieu can direct functional activity.

Methods

Additional experimental details can be found in the *Online Supplementary Appendix*.

Ethical Consent

Ethical approval was obtained from the University of Aberdeen College Ethics Review Board.

Materials

$PolyP_{70}$ was a kind gift from Dr Thomas Staffel BK Giulini GmbH (Ludwigshafen, Germany). PolyP 14, 60, 130 were a kind gift from Dr Toshikazu Shiba Regenetiss Inc. Medium chain (p100) polyP with and without biotin-labeling was from Kerafast Inc (Boston, MA, USA). PolyP concentrations are expressed as monomer concentrations throughout (monomer formula $NaPO_3$). All reactions were carried out in TBST (50 mM Tris, 100 mM NaCl, 0.01% Tween-20, pH 7.4).

Plasmin generation and uPA activity

Purified human plasminogen-free fibrinogen (2.4 μM), Glu- or Lys-plasminogen (0–1 μM), tPA (20 pM) or uPA (180 pM) ± $polyP_{65}$ (328 μM) in TBST was added in triplicate to 96-well polystyrene plates. Clotting was initiated by thrombin (0.25 U/mL) and $CaCl_2$ (5 mM), and activity quantified using the fluorogenic substrate D-Val-Leu-Lys 7-amido-4-methylcoumarin (D–VLK-AMC [0.35 mM]) by measuring fluorescence release (excitation 360/40 nm, emission 460/40 nm) every minute (min) for 5 hours (h) in a Biotek Flx800 fluorescence microplate reader at 37 °C. The rate of plasmin generation was calculated using; Longstaff C, 2016, Shiny App for calculating zymogen activation rates, version 0.6 (*https://drclongstaff.shinyapps.io/zymogenactnCL/*).

UPA activity was determined by incubating the enzyme (180 pM) ±328 μM $polyP_{65}$ with the chromogenic substrate CS-61 44 (1.25 mM). Change in absorbance was measured every 30 seconds (s) at 405 nm for 200 min.

Protein binding assays to biotin-labelled polyP

Binding of tPA, uPA, plasmin, FXII and activated FXII (FXIIa) to biotin-labelled polyP (71 μM) were performed using an adaptation of the protocol described by Choi *et al.*[13] Bound tPA, uPA or plasmin was detected with chromogenic substrates (1.2 mM S2288, CS-61 44, or 0.6 mM S2251, respectively). FXII(a) was detected using a peroxidase conjugated goat anti-human FXII antibody.

Turbidimetric fibrinolysis assays

Purified human plasminogen-free fibrinogen (2.4 μM), Glu- or Lys-plasminogen (0–1 μM), tPA (20 pM) or uPA (180 pM) with or without polyP (0–1.3 mM) in TBST was added in triplicate to 96-well polystyrene plates. Clotting was initiated by thrombin (0.25 U/mL) and $CaCl_2$ (5 mM), and turbidity monitored every min at 340 nm for 5 h at 37°C in a FLX-800 plate reader (Biotek Instruments).

Confocal Microscopy

Clots were prepared with human plasminogen-free fibrinogen (2.65 μM) of which 9% was DyLight 488-labeled, Glu-plasminogen (1.25 μM) of which 20% was DyLight 633-labeled, ±328 μM $polyP_{65}$ in TBST in Ibidi -slides VI$^{0.4}$. Thrombin (0.25 U/mL) and $CaCl_2$ (5 mM) were added and fibrinolysis initiated by addition of tPA or uPA (75 nM).

Cascade blue ethylenediamine (CB)-labeled $polyP_{70}$ was prepared as described.[13] Clots were formed ±328 μM CB-polyP by polymerizing fibrinogen (2.65 μM, with 9% labeled with DL550-fibrinogen) as above.

Statistical analysis

Statistical analysis was performed in GraphPad Prism® 5.04 using one-way analysis of variance or two-way analysis of variance with Bonferroni *post hoc* test or an unpaired Student's *t*-test (2-tailed). $P<0.05$ was considered to be significant.

Results

PolyP promotes uPA-mediated plasmin generation

Our work has previously established that polyP interferes with the plasminogen activator function of tPA[4] while augmenting plasminogen activation by FXIIa.[14] Here we address the role of polyP on uPA-mediated plasminogen activation using the fluorogenic substrate D–VLK-AMC. PolyP dramatically enhanced the rate of plasmin generation (3.96±0.34 *vs.* 0.84±0.08 pM/s; $P<0.001$) during uPA-mediated fibrinolysis (Figure 1A). In contrast, and consistent with our previous results4, a decrease in the rate (0.74±0.06 *vs.* 1.17±0.14 pM/s in $P<0.05$) and amount of plasmin generation was observed during tPA-mediated fibrinolysis (Figure 1B). The ability of polyP to enhance the rate of uPA-mediated lysis was dose-dependent up to 32.8 μM after which it decreased before increasing again at 164 μM (Figure 1C). Above this concentration the rate of plasmin generation was greatly enhanced with a 6-fold increase at 328 μM polyP. In marked contrast, downregulation of tPA-mediated plasmin generation required concentrations of greater than 70 μM and was not strongly does-dependent (Figure 1D).

Plasminogen concentration attenuates the cofactor function of polyP in uPA-mediated plasmin generation

We next analysed the impact of plasminogen concentration on the cofactor function of polyP. Plasmin generation was monitored during clot lysis at various concentrations of plasminogen. The rate of plasmin generation by uPA and tPA in clots was directly proportional to the Glu-plas-

minogen concentration (Figure 2A-B). The presence of polyP significantly increased the rate of uPA-mediated plasmin generation by around 75–82% at all Glu-plasminogen concentrations (Figure 2A). In marked contrast, polyP attenuated tPA-mediated plasmin generation at all Glu-plasminogen concentrations, with a maximal reduction at 1 μM Glu-plasminogen (Figure 2B). Cleavage of the activation peptide of Glu-plasminogen by plasmin forms an intermediate form, Lys-plasminogen, which displays increased affinity for fibrin[15] and is more readily cleaved by plasminogen activators.[7,16,17] Overall plasmin generation by uPA occurs at a significantly faster rate in clots formed with Lys-plasminogen compared to Glu-plasminogen and is directly proportional to the concentration (Figure 2C-D). Interestingly, Lys-plasminogen overcame the cofactor function of polyP on uPA-mediated plasmin generation, significantly decreasing the magnitude of the effect from 79% at 0.125 μM Lys-plasminogen to 9% at 1 μM Lys-plasminogen (Figure 2C). With tPA there was a similar attenuation of plasmin generation (approximately 37%) at all Lys-plasminogen concentrations analysed (Figure 2B, D).

polyP binds with a higher affinity to uPA than tPA

We have previously demonstrated that polyP binds to Glu-plasminogen.[14] Here we use biotin-labelled polyP in a plate-based assay to determine the binding affinity to (A) uPA and (B) tPA. polyP bound with approximately 25-fold higher affinity to uPA (K_d=9.8 nM) than tPA (K_d=245.3 nM; Figure 3). Interestingly, binding of polyP did not alter the activity of uPA and no specific binding of polyP to plasmin was detected (*data not shown*). We have shown that polyP stimulates autoactivation of FXII to an active single chain FXII (scFXIIa)[18] and enhances the plasminogen activator capacity of FXIIa.[14] We found that the high affinity interaction of uPA and polyP was similar to the interaction of polyP with FXII (K_d=7.9 nM) and FXIIa (Kd=4.0 nM) (*Online Supplementary Figure S1*).

polyP enhances uPA-mediated lysis in a concentration and polymer length dependent manner

Incorporation of polyP during clot formation shortened the uPA-mediated 50% clot lysis time by 43.7±1.7 min (P<0.0001; Figure 4A). In contrast, tPA-mediated clot lysis was delayed on inclusion of polyP (Figure 4B), as previously reported.[4] The cofactor function of polyP on uPA-mediated lysis was concentration dependent and required 66 μM or higher to significantly stimulate the rate of lysis (Figure 4C). The use of polyP of more refined chain length revealed that polymers of >14-mer were required to promote uPA-mediated lysis (Figure 4D).

Plasminogen modulates polyP-mediated effects on fibrinolysis

The mode of action of tPA and uPA differ significantly, with tPA relying on the interaction with fibrin for efficient plasminogen activation, while uPA is fibrin independent. We investigated the relationship between plasminogen and polyP-mediated effects on fibrinolysis. A clear concentration dependence was observed with Glu-plasminogen on uPA-mediated lysis, with maximal lysis at 0.75 μM plasminogen (Figure 5A). The impact of Glu-plasminogen on tPA-mediated lysis was less pronounced, but a small effect was observed with maximal lysis at 0.5 μM plasminogen (P<0.01; Figure 5B). polyP significantly accelerated uPA-mediated lysis at all Glu-plasminogen concentra-

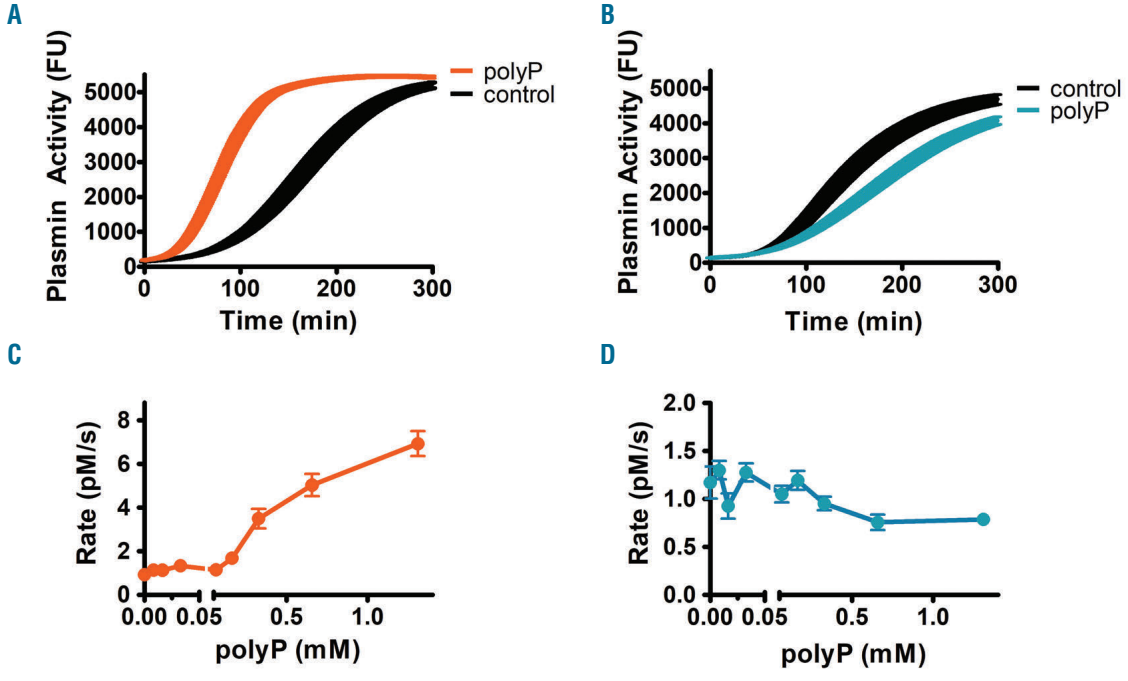

Figure 1. PolyP stimulates uPA-mediated plasmin generation in a concentration dependent manner. Fibrin clots were prepared containing 2.4 μM fibrinogen, 0.24 μM Glu-plasminogen, 20 pM tPA or 180 pM uPA ± 328 μM (polyP$_{ea}$). Clotting was initiated with thrombin (0.25 U/mL) and CaCl$_2$ (5 mM) and plasmin generation was quantified by incorporating the fluorogenic substrate D–VLK-AMC and monitoring fluorescence release (FU; Ex 360 nm Em 460 nm). (A) uPA and (B) tPA mediated plasmin generation curves. Rate of plasmin generation in clots formed with varying polyP concentration (0-1.3 mM polyP$_{ea}$) for (C) uPA and (D) tPA. Data are expressed as mean ± standard error of the mean, n≥ 3. PolyP: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator; FU: fluorescence units, Ex: excitation; EM: emission.

tions tested, however, the cofactor function of polyP was tempered at high concentrations. Inclusion of polyP delayed tPA-mediated lysis at all Glu-plasminogen concentrations tested by an average of 40%; therefore, increasing the Glu-plasminogen concentration did not alter the efficacy of polyP on tPA-driven lysis.

Clots containing Lys-plasminogen lyse significantly faster than those containing Glu-plasminogen (Figure 5C-D). A dose-dependent relationship exists between Lys-plasminogen concentration and uPA-mediated lysis, with 50 % lysis times decreasing from 51.1±1.36 min at 0.125 μM to 17.2±0.32 min at 1 μM (Figure 5C). In contrast, there is no relationship between Lys-plasminogen concentration and tPA-mediated lysis, exemplifying the different modes of action of these plasminogen activators (Figure 5D). Similar to the results with plasmin generation,

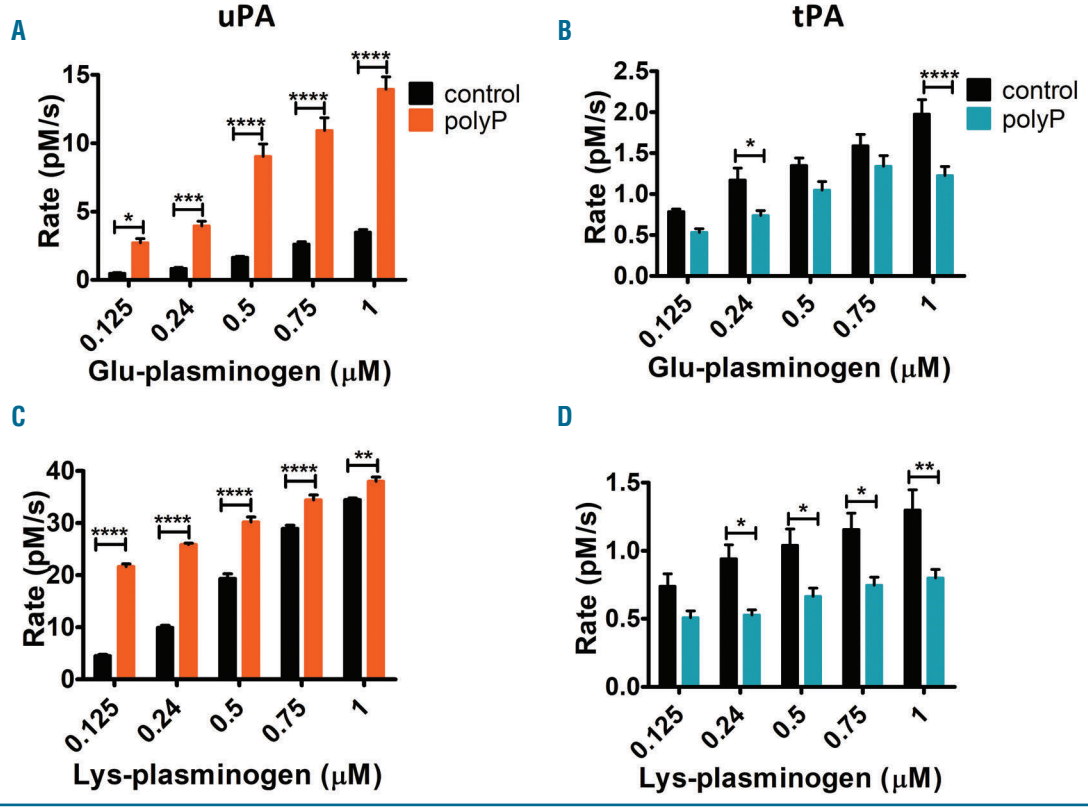

Figure 2. High concentrations of plasminogen attenuate the cofactor function of polyP in uPA-mediated plasminogen activation. Fibrin clots were prepared with 2.4 μM fibrinogen, 0 -1 μM (A-B) Glu-plasminogen or (C-D) Lys-plasminogen, (A, C) 180 pM uPA or (B, D) 20 pM tPA ± 328 μM polyP$_{45}$. Clotting was initiated with thrombin (0.25 U/mL) and $CaCl_2$ (5 mM). Plasmin generation in clots was quantified by incorporating the fluorogenic substrate D–VLK-AMC and monitoring fluorescence release (FU; Ex 360 nm Em 460 nm). *P<0.05; **P<0.01, ***P<0.001 and ****P<0.0001 compared with control clots. Data are expressed as mean ± standard error of the mean, n≥3. polyP: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator; FU: fluorescence units; Ex: excitation; EM: emission.

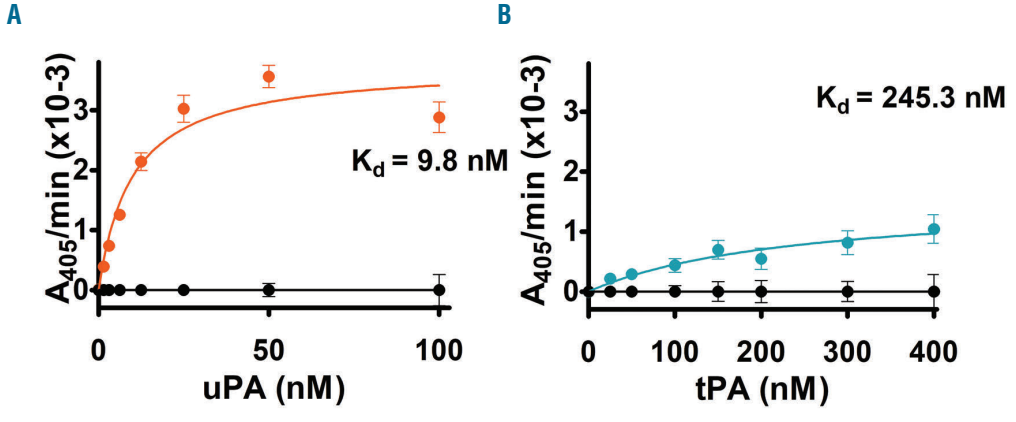

Figure 3. polyP binds to uPA with a significantly higher affinity than tPA. Binding of uPA or tPA (0–400 nM) to biotin-labelled polyP (71 μM) bound to streptavidin coated stripwells. Bound uPA (orange) or tPA (blue) was detected with chromogenic substrates (S2288 or CS-61 44 respectively) by reading the change in absorbance at 405 nm every 30 seconds (s) for 200 minutes (min). No unspecific binding was detected in the absence of biotin-labelled polyP (black lines). Data are expressed as baseline corrected nonlinear fit as mean ± standard error of the mean (SEM), n=4. PolyP: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator.

the cofactor function of polyP on uPA-mediated lysis is lost at high Lys-plasminogen concentrations (Figure 5C). Whereas, polyP delayed tPA-mediated lysis by approximately 20 % at all Lys-plasminogen concentrations (Figure 5B, D).

polyP localization during fibrinolysis

polyP is found to co-localize with fibrinogen, as previously reported,[19] and plasminogen in fibrin clots. During fibrinolysis polyP concentrates within the fibrin dense knots of the clot alongside plasminogen and fibrinogen (Figure 6A). Colocalization was observed with both uPA and tPA but only the data for uPA is shown. Lysis was initiated at the edge of the clot, by addition of plasminogen activator, and as it progressed through the fibrin network polyP and plasminogen were found to remain colocalized within the lysis front (Figure 6B).

Visualization of the cofactor function of polyP during uPA-mediated lysis in real-time

Fluorescent confocal microscopy was used to visualize lysis in real-time following application of either uPA or tPA to the edge of fibrin clots. The rate of fibrinolysis in the control clot was similar in the presence of uPA or tPA (Figure 7A-B). As uPA-mediated lysis progresses plasmin(ogen) is dispersed throughout the clot, with a small concentrated zone at the leading edge (Figure 7A), in contrast plasminogen is restricted to a distinct zone at the leading edge in tPA-mediated lysis (Figure 7B). The incorporation of polyP significantly accelerates uPA-mediated lysis (Figure 7A) with full lysis observed on average at 6 min (*Online Supplementary Video S1B*) compared to greater than 9 min in the control (*Online Supplementary Video S1A*).

Discussion

Over the last decade a role for the biomolecule polyP in the regulation of hemostasis has been exposed. The ability of polyP to modulate fibrinolysis manifests on several levels. We and others[4,20] have demonstrated altered fibrin structure in the presence of polyP, a consequence of which is tempered binding of tPA and plasminogen to fibrin and subsequent down-regulation of fibrinolysis.[4] In contrast, in this study we show that that polyP significantly accelerates uPA-mediated plasminogen activation thereby augmenting fibrinolysis. The rate of plasmin formation was approximately 5-fold faster in the presence of polyP and was dependent on concentration and polymer length, with polymers of 60-mer or greater required to accelerate lysis. We determined that polyP binds with a 25-fold higher affinity to uPA than tPA and potentially accelerates conversion of Glu-plasminogen to the intermediate form Lys-plasminogen *via* a template mediated effect. Importantly, for the first time we visualize polyP during real-time lysis of fibrin clots and find it to be localized within fibrin dense areas alongside plasminogen, consistent with its known binding to these proteins.[4,14,19]

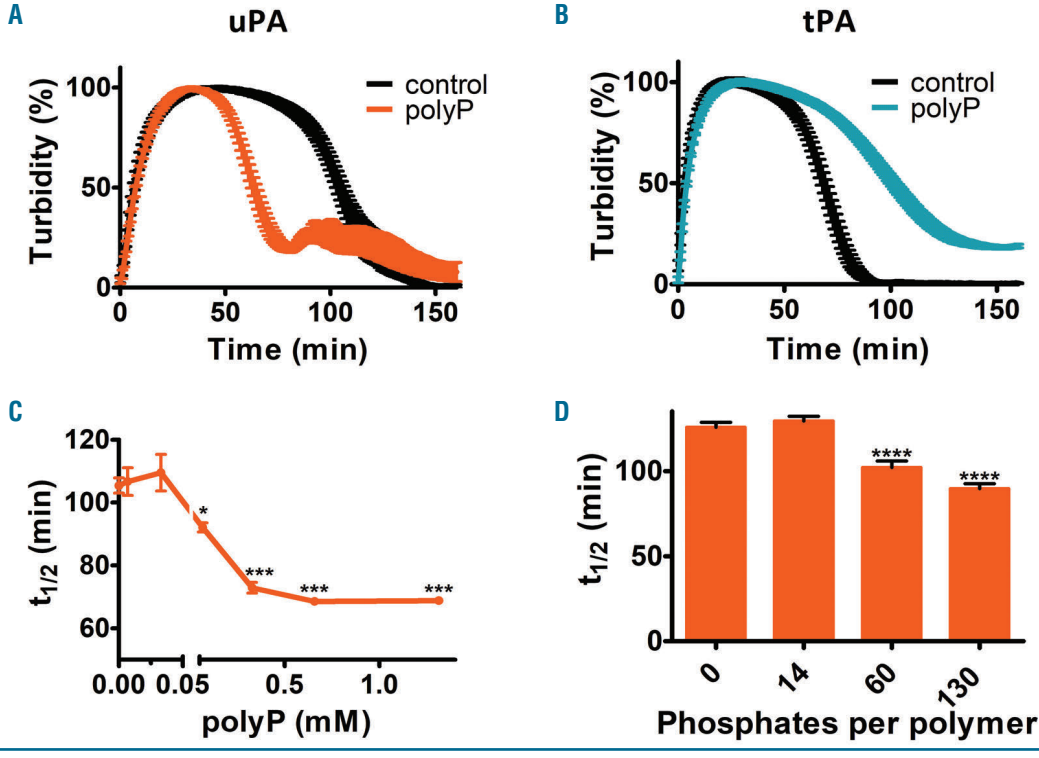

Figure 4. polyP acts as a cofactor to accelerate uPA-mediated fibrinolysis in a concentration and polymer size dependent manner. Fibrin clots were prepared containing 2.4 μM fibrinogen, 0.24 μM Glu-plasminogen, 20 pM tPA or 180 pM uPA ±328 μM polyP$_{ea}$. Clotting was initiated with thrombin (0.25 U/mL) and CaCl$_2$ (5 mM) and fibrinolysis monitored at 340 nm shown as percentage turbidity over time with (A) uPA or (B) tPA. (C) uPA-mediated fibrinolysis with 0-1.3 mM polyP$_{ea}$ or (D) polyP of various chain lengths at equivalent concentration of monomer (328 μm). *P<0.05, ***P<0.001 and ****P<0.0001 compared with control clots. Data are expressed as mean 50 % lysis time ($t_{1/2}$) ± standard error of the mean, n≥3. polyP: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator.

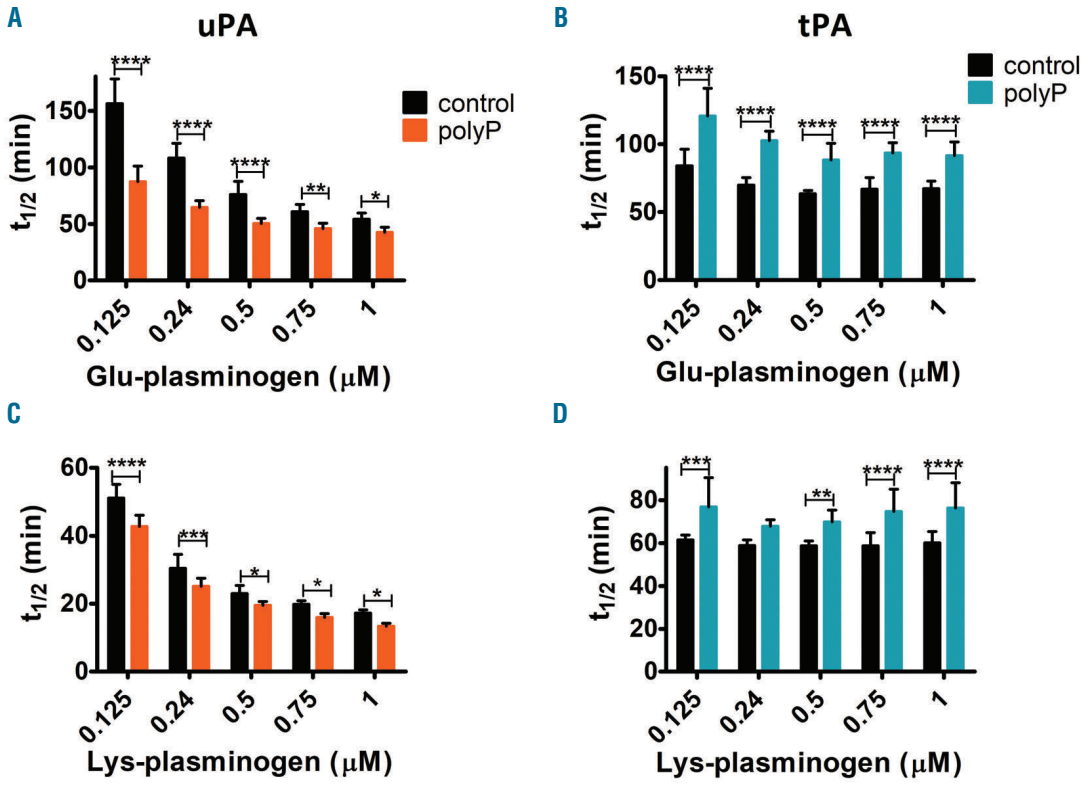

Figure 5. The cofactor function of polyP in uPA-mediated fibrinolysis is modulated by plasminogen concentration and form. Fibrin clots were prepared containing 2.4 μM fibrinogen, 0-1 μM (A-B) Glu-plasminogen or (C-D) Lys-plasminogen, (A, C) 180 pM uPA or (B, D) 20 pM tPA ±328 μM polyP$_{\infty}$. Clotting was initiated with thrombin (0.25 U/mL) and $CaCl_2$ (5 mM) and fibrinolysis monitored at 340 nm. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 compared with control clots. Data are expressed as mean ± standard error of the mean, n≥3. polyP: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator.

Despite their homology in structure, the mechanism of tPA and uPA-mediated plasminogen activation are very different. Efficient plasminogen activation by tPA requires its association with the fibrin surface, that is fibrin orchestrates its own destruction by plasmin. Binding of tPA to fibrin is largely attributed to its finger domain,[21,22] while plasminogen associates with fibrin via kringle domains.[23] The colocalization of enzyme and substrate on fibrin, as a surface, is crucial, as tPA is a poor plasminogen activator in solution. Circulating fibrinogen cannot accelerate plasminogen activation by tPA, as the sites are only exposed in fibrin[24] thereby localizing plasmin formation to the fibrin clot. In marked contrast uPA does not bind fibrin and is reasonably efficient at activating plasminogen in solution. However, its cellular receptor, uPAR, localizes uPA via its amino terminal fragment to the cell surface, thereby augmenting plasminogen activation due to an increase in local reactant concentration.

The high affinity interaction of uPA and plasminogen[14] with polyP and dependence on substrate and template concentration are indicative of a template mechanism of activation. In line with this polyP polymers of around 60-mer were required to stimulate plasminogen activation by uPA, suggesting this length is critical to accommodate binding of both enzyme and substrate to the same template. Indeed, a template effect of polyP in thrombin-mediated activation of FV to FVa has previously been reported.[25] Polymers of around 80-100-mer are secreted following platelet stimulation[2] with insoluble divalent cation bound polyP nanoparticles remaining associated with the activated platelet membrane.[26] Platelet-derived polyP could actively participate in these template-mediated reactions within the milieu of a thrombus where local concentrations of the polymer will be high.

Previously an inhibitory effect of polyP on uPA-mediated fibrinolysis was described in a plasma based system.[25] The data presented here delineates the role of polyP specifically on plasminogen activation by uPA using a purified system. The roles of polyP in hemostasis are multifaceted and coagulation and fibrinolysis are inextricably linked. It is therefore difficult, at this current time, to exclude the fact that these differences cannot be attributed to different experimental setups, reagents and indeed an impact of polyP on a different part of the pathway.

Intriguingly, the cofactor function of polyP described here in uPA-mediated plasminogen activation is markedly more pronounced than in uPA-mediated fibrinolysis. This can potentially be explained by an increase in the number of binding sites, that is plasminogen has the capacity to bind both polyP and lysine residues exposed on partially degraded fibrin generated following onset of lysis.[23] Thus, the increase in surface binding sites on fibrin for plasminogen tempers the cofactor function of polyP. Therefore, there is a more evident effect at low plasminogen concentrations, where fewer lysine residues will be generated. Given the anionic nature of polyP it is feasible that it associates with positively charged lysine residues on fibrin, thereby obscuring the kringle-dependent binding of plasminogen to fibrin, however, high plasminogen concentrations eliminate the need for fibrin binding.

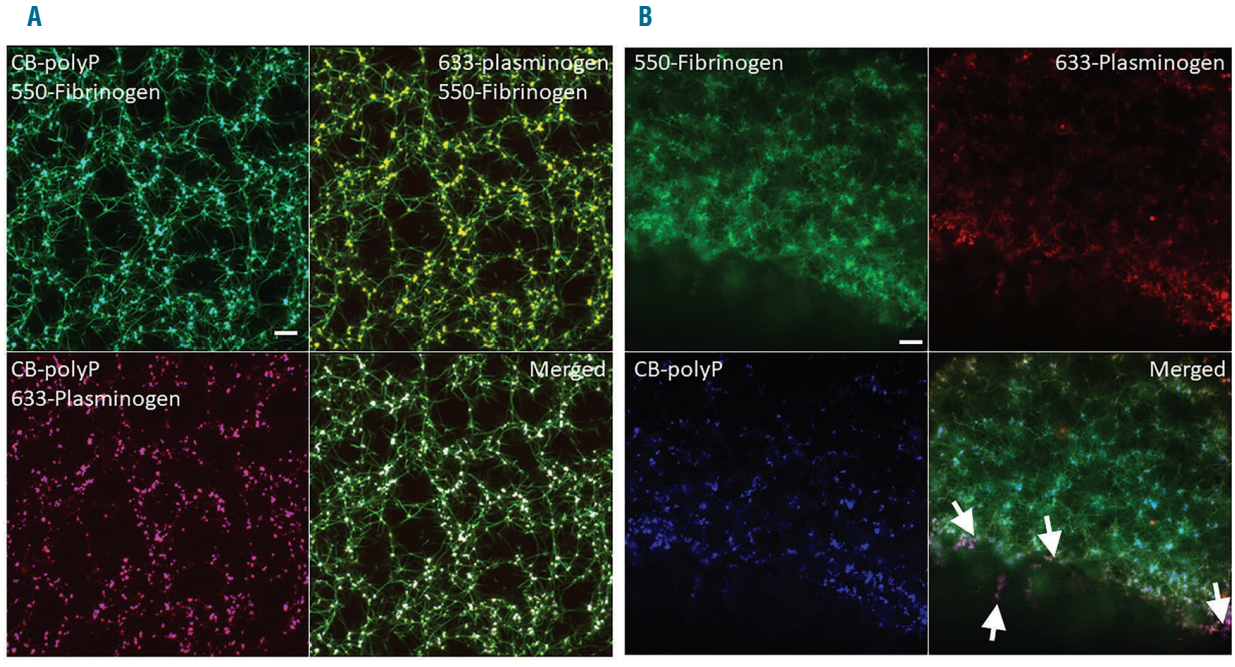

Figure 6. polyP colocalizes with fibrinogen and plasminogen during fibrinolysis. Fibrin clots were formed containing fibrinogen (2.65 μM, 9% DyLight 550-labeled), Glu-plasminogen (1.25 μM, 20% DyLight 633-labeled) 328 μM Cascade-blue labeled polyP70 (CB-polyP), thrombin (0.25 U/mL), CaC_2 (5 mM). (A) polyP and plasminogen largely accumulate in the knotted regions of fibrin. (B) Fibrinolysis was initiated by exogenous uPA (75 nM). Plasminogen and polyP strongly colocalize at the lysis front during clot lysis, as indicated by the arrows. Scale bars =10 μm. polyP: polyphosphate; uPA; urokinase plasminogen activator.

Our recent study[19] investigating the molecular mechanisms underpinning the changes in fibrin structure in the presence of platelet-derived polyP revealed a significant change in fibrin polymerisation which stunts protofibril growth; thus providing an explanation for the characteristic 'knotted' appearance.[4] Downstream this impacts on the mechanical properties of a clot, reducing overall stiffness and increasing its ability to deform plastically.[19] Here, we find that polyP is localized within these 'knotted' regions of fibrin alongside plasminogen. Analysis of lysis in real-time reveals significant acceleration of uPA-mediated fibrinolysis presumably due to the direct colocalization of cofactor, enzyme and substrate. Previous work has shown that co-assembly of uPA and plasminogen on the same surface is not a prerequisite to stimulate plasmin formation.[27] This crosstalk mechanism permits localization of plasminogen and uPA on different cellular surfaces or binding of plasminogen to fibrin while uPA is associated with cellular uPAR. A similar mechanism could explain our current observations; that is uPA is localized on polyP while plasminogen is bound to either polyP or partially degraded fibrin. Future work is necessary to ascertain the effect polyP may have on plasminogen activation on the surface of monocytes and neutrophils, which express both uPA and its cellular receptor uPAR.

Plasminogen circulates in the native or Glu-plasminogen form but can be cleaved by plasmin at Lys^{77}-Lys^{78} to generate Lys-plasminogen. This cleavage prompts changes in the properties of the zymogen thereby providing a positive feedback mechanism.[28] Lys-plasminogen is a considerably better substrate for both tPA[29] and uPA[30] and displays enhanced affinity for fibrin.[31,32] Surface-bound Glu-plasminogen is more readily cleaved to Lys- plasminogen than in solution.[33] We have previously shown,[14] and confirmed in this study, that plasminogen, but not plasmin, bindsdirectly to polyP, as does the activator uPA. Co-assembly of Glu-plasminogen and uPA on the polyP surface will augment local concentrations of the reactants. An initial spark of plasmin formation will drive cleavage of Glu-plasminogen to Lys-plasminogen. Once formed Lys-plasminogen is more readily activated to plasmin than native Glu-plasminogen. Stimulation of uPA-mediated plasminogen activation by polyP is diminished when Lys-plasminogen is used, indicating that its cofactor function may lie in initial surface-mediated conversion of Glu- to Lys-plasminogen, ultimately accelerating plasmin formation. We have shown that plasmin does not bind to polyP[4] or impact on its enzymatic activity indicating that the enhanced lysis arises at the level of plasminogen activation.

The lysine analogue, tranexamic acid (TXA), downregulates lysis by blocking tPA-mediated plasmin generation. The effect of TXA on uPA-mediated plasmin generation is more complex, with high concentrations of TXA augmenting plasmin generation, despite this fibrinolysis is still inhibited. Enhanced activation of plasminogen by uPA in the presence of TXA is indicative of a conformational change in plasminogen, from a closed structure to an open and more readily activated form.[34] Our observations with polyP are similar, in that binding of Glu-plasminogen appears to augment its susceptibility to uPA-mediated cleavage indicative of a conformational change in the protein.

The fibrin specificity of tPA has led to the view that it is the dominant plasminogen activator in hemostasis, whereas, uPA has been implicated in plasmin-mediated cell migration, tissue remodelling and activation of latent growth factors and cytokines.[35] TPA has been implicated in the degradation of deep vein thrombi in humans[36] but genetic deficiency in mice has no impact. In marked contrast, uPA deficiency in mice markedly impairs venous thrombus resolution[37] and conversely delivery of uPA to

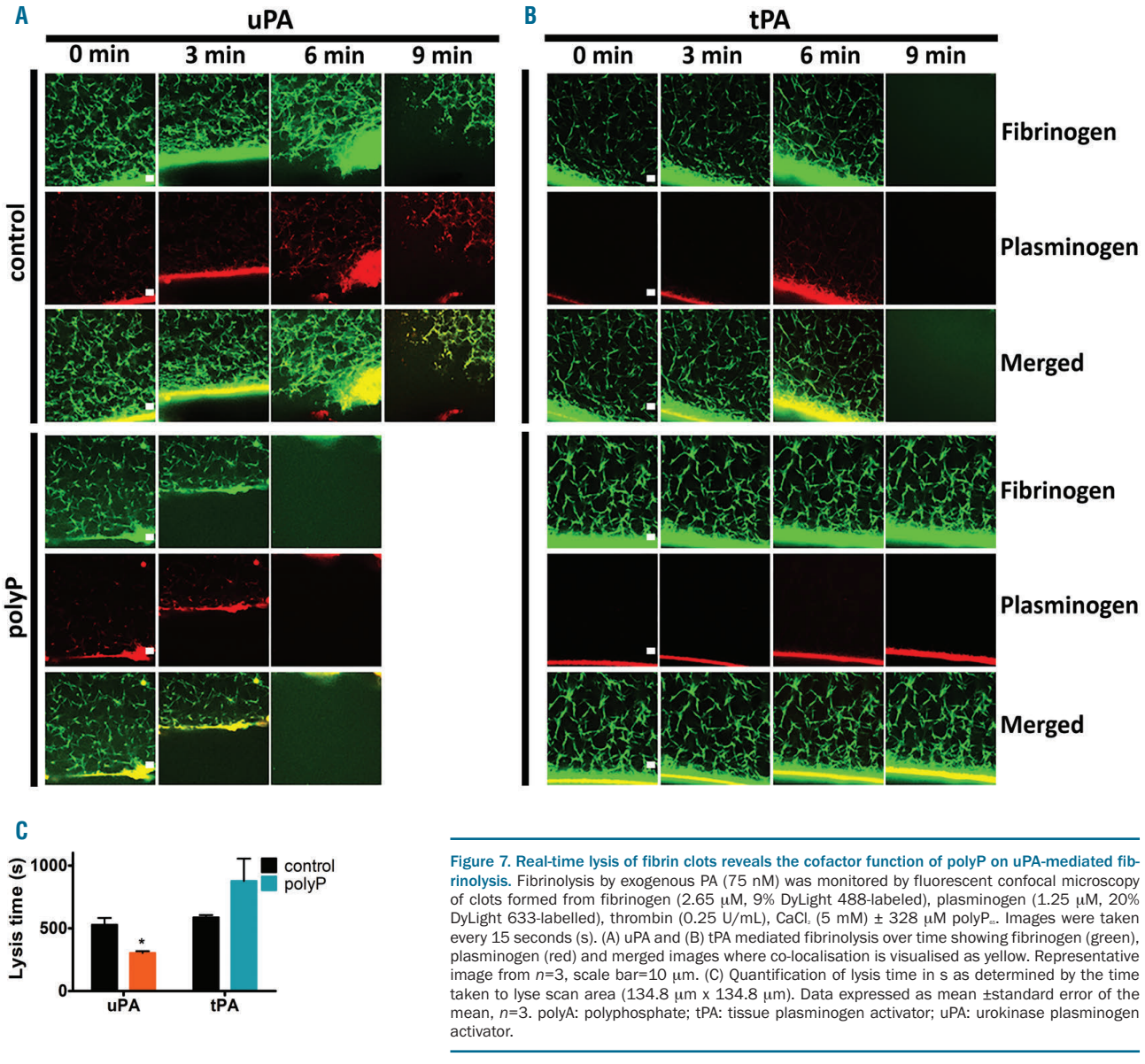

Figure 7. Real-time lysis of fibrin clots reveals the cofactor function of polyP on uPA-mediated fibrinolysis. Fibrinolysis by exogenous PA (75 nM) was monitored by fluorescent confocal microscopy of clots formed from fibrinogen (2.65 μM, 9% DyLight 488-labeled), plasminogen (1.25 μM, 20% DyLight 633-labelled), thrombin (0.25 U/mL), $CaCl_2$ (5 mM) ± 328 μM $polyP_{45}$. Images were taken every 15 seconds (s). (A) uPA and (B) tPA mediated fibrinolysis over time showing fibrinogen (green), plasminogen (red) and merged images where co-localisation is visualised as yellow. Representative image from *n*=3, scale bar=10 μm. (C) Quantification of lysis time in s as determined by the time taken to lyse scan area (134.8 μm x 134.8 μm). Data expressed as mean ±standard error of the mean, *n*=3. polyA: polyphosphate; tPA: tissue plasminogen activator; uPA: urokinase plasminogen activator.

thrombi, either directly[38] or *via* uPA expressing monocytes,[39] improves thrombus dissolution. Cross-talk between neutrophils, monocytes and platelets contributes to deep vein thrombosis[40] and it is feasible that platelet-derived polyP could augment monocyte-derived uPA-mediated plasmin formation and facilitate thrombus resolution.

Platelets contain approximately 0.74 nmol/10^8 platelets[1] therefore concentrations in whole blood are estimated to reach around ≈3 μM following platelet activation.[25] However, in platelet dense regions of thrombi concentrations could far exceed this; particularly as we and others have also shown that polyP remains bound to the activated platelet membrane.[26,41] Elegant studies have revealed that solute transport within the core regions of thrombi is restricted[42-44] and as such the diffusion rate of platelet-derived polyP within this milieu will be restricted. PolyP nanoparticles formed on the activated platelet membrane trigger contact activation[26] and our laboratory has described a role for polyP in augmenting FXIIa-mediated plasminogen activation, with the platelet surface acting as a focal point for colocalization.[14] By acting as a template for plasminogen activation by FXIIa or uPA, platelet-associated polyP nanoparticles could, under certain conditions, function to limit thrombus size.

Here we show that polyP binds to uPA with a high affinity and enhances uPA-mediated plasmin generation thereby augmenting the rate of fibrinolysis. This is in sharp contrast to the inhibitory effect of this polyanion on tPA-mediated plasminogen activation.[4] The strong binding of uPA and plasminogen is suggestive of a template mechanism that results in enhanced conversion of Glu-plasminogen to Lys-plasminogen and is supported by the colocalization of the reactants on fibrin. The interaction of Glu-plasminogen with polyP may additionally result in a conformational change thereby facilitating its activation by uPA. Our data define a novel cofactor function of polyP in regulation of plasminogen activation by uPA that drives fibrinolysis in real time and may have important implications for physiological processes such as thrombus resolution, cell migration and tissue remodeling.

Disclosures

No conflicts of interests to disclose.

Contributions

CSW performed the research, analysed the data and wrote the manuscript; NJM designed the research, analysed the data and wrote the manuscript.

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