Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin

MUTCH NJ et al: POLYPHOSPHATE MODIFIES FIBRIN AND LYSIS

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ABSTRACT
Activated platelets secrete a negatively charged polymer, polyphosphate (polyP). Here we explore the interactions of polyP with fibrin(ogen) and its impact on fibrin structure and fibrinolysis. Electrophoretic mobility and binding assays indicate that polyP interacts with fibrinogen and soluble fibrin. Clots formed in the presence of polyP exhibited reduced turbidity and permeability indicative of a tighter fibrin network but these changes were not related to cross-linking or fibrinopeptide release. Microscopy revealed a change in fibrin distribution in clots formed with polyP; with formation of tight aggregates of fibrin fibers interspaced with large pores in contrast to homogenous fiber distribution in control clots. Lysis by tPA and plasminogen or plasmin was delayed in clots formed with polyP and was dependent on both the activator and polyP concentration. Addition of polyP to the clot after fibrin formation or to repolymerising soluble fibrin did not affect lysis indicating that changes induced by polyP occur at the level of conversion of fibrinogen to fibrin. Surface plasmon resonance revealed that the presence of polyP reduced the binding of both plasminogen and tPA to partially-lysed fibrin surfaces. These data show that polyP directly influences fibrin architecture and attenuates fibrinolysis through reduced binding of fibrinolytic proteins.
INTRODUCTION

Coagulation culminates in the generation of thrombin which converts fibrinogen to fibrin. Fibrinogen is composed of two sets of three chains designated $A\alpha$, $B\beta$ and $\gamma$. The N-termini of all six chains are clustered in the centre of the molecule termed E region from which two sets coiled coils extend to the D regions. Thrombin cleaves short acidic N-terminal sequences on the $A\alpha$ and $B\beta$ chains, designated fibrinopeptides A and B respectively, thereby exposing polymerisation sites. Thrombin preferentially cleaves fibrinopeptide A spontaneously forming staggered, double-stranded protofibrils. Removal of fibrinopeptide B then liberates the C-termini of the $\alpha$ chain from the central E region resulting in lateral aggregation of the protofibrils through $\alpha\text{C}-\alpha\text{C}$ interactions. Fibers exhibit branching thus forming a three-dimensional scaffold.

The structure and mechanical properties of fibrin are important determinants for its breakdown and in vivo can translate into an increased risk of thrombosis or embolism. Fibrin clot structure is determined by fiber thickness and distribution of the fibres and branchpoints. Changes in these parameters influence clot stability and susceptibility to degradation by fibrinolytic proteases. Thrombin also activates factor (F)XIII, a process that is augmented by fibrin itself. Activated FXIII (FXIIIa) cross-links fibrin and incorporates inhibitors of fibrinolysis, such as $\alpha_2$antiplasmin, into the clot resulting in a network with increased resistance to lysis.
Fibrin not only acts as a cofactor in its stabilization by FXIII, but also in its dissolution, by binding tissue plasminogen activator (tPA) and plasminogen.\textsuperscript{11} Activation of plasminogen by tPA is inefficient in solution but the rate is increased dramatically on the fibrin surface.\textsuperscript{12} Thick fibers are lysed at a slower rate at the level of the individual fibers than thin fibers.\textsuperscript{13} Clots with thick fibers are however more susceptible to lysis on the whole, as there are a smaller number of fibers present per volume of clot and the fibers are less densely packed.\textsuperscript{14}

Fibrin structure is influenced by platelets.\textsuperscript{15-18} The platelet surface is a regulator of procoagulant activity and local thrombin concentration, parameters which have been shown to dictate clot structure.\textsuperscript{18} Platelets interact directly with fibrin via the $\alpha_{IIb}\beta_3$ integrin which alters fibrin structure and susceptibility to lysis.\textsuperscript{15-17} When stimulated, platelets release polyphosphate (polyP) from the dense granules.\textsuperscript{19} PolyP is a linear polymer of orthophosphate residues ($P_i$) linked via high energy phosphoanhydride bonds. Released polyP stimulates thrombin generation by enhancing factor V activation and activating the contact pathway.\textsuperscript{20} The changes in thrombin generation induced by polyP increase activation of thrombin activatable fibrinolysis inhibitor (TAFI) and down-regulate fibrinolysis.\textsuperscript{20} The proximity of polyP to the clot led us to question whether it has a direct impact on fibrin structure, independently of its effect on thrombin generation. Using a purified system we find that fibrin networks formed in the presence of polyP are heterogeneous in nature and are more resistant to degradation by fibrinolytic proteases.
MATERIALS AND METHODS

Materials

Human plasminogen-free fibrinogen, α-thrombin, plasminogen and plasmin were from Enzyme Research Laboratories (Swansea, UK). Polyphosphate (average chain lengths 5, 25, 45 & 65), atroxin, toluidine blue O were from Sigma. Tissue plasminogen activator (tPA) was from Technoclone (Vienna, Austria). Zirconia beads were from ZirChrom Separations, Inc. (Anoka, MN). Rabbit polyclonal antibody to fibrinogen was from DAKO (Stockport, UK). Handee mini-spin columns, polyvinylidene fluoride (PVDF) membrane and Gelcode were from Pierce. 4-12% NuPAGE gels, NuPAGE sample buffer and reducing agent, MOPS running buffer and 6% Tris-Glycine gels were from Invitrogen. Fibrogammin was from CSL Behring UK Limited. CM5 sensorchips and P-20 were from GE Healthcare (Buckinghamshire, UK). S2251 chromogenic substrate was from Diapharma (Axis-Shield, Dundee, UK). Unless otherwise stated Tris-buffered saline (TBS; 50 mM Tris-HCl pH 7.4, 100 mM NaCl) was used. PolyP concentration is expressed in monomer concentration throughout the manuscript (monomer formula NaPO₃).

Fibrin(ogen) binding

PolyP was immobilized onto porous zirconia beads as described.²¹ Briefly, 250 mg zirconia beads were incubated with 10 mg/ml polyP₆₅ in water for 20 h at 37°C. The beads were washed with distilled water before blocking with 10% BSA
for 15 h at ambient temperature. After washing in water the zirconia beads were oven dried at 80°C for 2 h. Control beads were treated with water and BSA only.

PolyP-zirconia beads (10 mg dry weight) were equilibrated with 200 μl TBS containing 0.1% BSA before addition of fibrinogen (10 μg). After incubation at ambient temperature for 30 min the mixtures were centrifuged in mini-spin columns at 1677 g for 30 s. The flow-through was collected and the polyP-zirconia beads were washed with 200 μl TBS 0.1% BSA followed by elution with 200 μl TBS 1 M NaCl, 0.1% BSA. Recovery of fibrinogen from the beads was determined by separating samples on 4-12% NuPAGE gels with MOPS running buffer, transferring to PVDF and western blotting with an antibody to fibrinogen.

Gel Mobility Shift Assays were performed with fibrinogen and soluble fibrin, prepared as described. Proteins (10 μg/lane) were pre-incubated with polyP65 (100 μg) for 10 min at ambient temperature before mixing with 60 mM Tris-HCl pH 6.8, 10% glycerol, 0.01% bromophenol blue. Samples were resolved on 6% Tris-Glycine gels for 2.5 h at 125 V. Gels were stained for protein with Gelcode, or for polyP with 0.25% toluidine blue O in 25% methanol, 5% glycerol for 10 min and destained in the same solution without dye.

**Microscopy of fibrin clots**

Fibrinogen (3 μM) was incubated with 0.25 U/ml thrombin and 5 mM CaCl2 ± polyP65 (325 or 650 μM) in TBS. After 2 h at room temperature, clots were
washed in sodium cacodylate buffer and fixed overnight in 2% glutaraldehyde. Clots were dehydrated using an acetone gradient and sputter coated with platinum palladium. Samples were analyzed using a field-emission scanning electron microscope (LEO 1530 FEGSEM, Leo Electron Microscopy, Cambridge UK) in 10 different areas of the clot and recorded using Leo 32 version 03.0210 software.

For confocal microscopy, fibrinogen (3 μM) was added to chamber slides (Ibidi μ-slide VI-flow, Thistle Scientific, UK) and clotted ± polyP65 as described above. Samples were visualized using Leica TCS SP-2 laser scanning confocal equipment (Leica Microsystems, Wetzlar, Germany) on an inverted DM IRE 2 microscope with 63x water immersion objective (numeric aperture 1.2). Each sample was analyzed using reflected light mode in at least 10 different areas of 240 x 240 x 40 μm. Z-stacks were taken every 1-2 μm, and 2D reconstructed images were rendered of the fibrin clots using the Leica software.

**Fibrin permeation studies**

Fibrin clots were generated by incubating fibrinogen (3 μM) with 0.25 U/ml thrombin and 5 mM CaCl2 ± polyP65 (325 or 650 μM) in TBS for 2 h in a moist chamber at ambient temperature. Permeation of TBS through the clot was quantified as described23 with a pressure drop of 2 cm, from which the Darcy constant (Ks) was calculated.
Turbidity analysis

Purified human fibrinogen (2.4 μM) ± polyP₆₅ (325 -650 μM) in TBS was added in triplicate to 96-well polystyrene plates (Greiner). Clotting was initiated by thrombin (0.25 U/ml) and CaCl₂ (5 mM) and turbidity was monitored every 12 s at 350 nm for 60 min in a FLX-800 plate reader (Biotek Instruments, Winooski, VT).

Fibrin cross-linking

Clots were prepared as described for turbidity but with the addition of Fibrogammin (10 μg/ml) as a source of FXIII to fibrinogen prior to initiation of clotting. Commercial preparations of fibrinogen are contaminated with FXIII, so in some cases were subjected to further purification. Briefly, CaCl₂ (10 mM) was added to fibrinogen before addition of 20% ammonium sulfate. After incubation for 90 min the mixture was spun at 3000 g for 20 min at 4°C, the pellet discarded and the supernatant, containing FXIII- depleted fibrinogen, was precipitated by bringing the ammonium sulphate concentration to 33%. The pellet was dissolved in distilled water and dialyzed into TBS. Cross-linking by FXIIIa was investigated by SDS-PAGE as described²⁴. Briefly, fibrinogen (3 μM) ± polyP (325 μM) was clotted using thrombin (0.25 U/ml) and CaCl₂ (5 mM) with Fibrogammin (10 μg/ml). The reaction was stopped at 0, 5, 30, 60 min using reducing NuPAGE sample buffer. Samples were then separated on 4-12% gradient gels with MOPS running buffer and stained with GelCode.
**Fibrinopeptide release**

Cleavage of fibrinopeptides A and B from fibrinogen by thrombin was monitored by high-performance liquid chromatography (HPLC) as described\textsuperscript{23, 25} with minor modifications. Fibrinogen (3 \(\mu\)M), thrombin (0.25 U/ml), \(\text{CaCl}_2\) (5 mM) ± polyP\textsubscript{65} (325 & 650 \(\mu\)M) were incubated at 37°C for 0, 0.5, 1, 2.5, 5, 10 and 20 min before stopping with 1:10 (vol/vol) of 3 M \(\text{HClO}_4\). Samples were spun for 15 min at 1677 \(g\) and supernatants applied to a 0.46 X 25 cm silica C18 (bead size 5 mm; pore size 30 nm [300 Å]) column (Pepmap C18) on a BioCad Sprint chromatography system (both Perseptive Biosystems). Fibrinopeptides were eluted with a linear gradient from 10% acetonitrile and 90% 0.083 M \(\text{NaH}_2\text{PO}_4\), pH 3.1 to 40% acetonitrile and 60% 0.083 M \(\text{NaH}_2\text{PO}_4\), pH 3.1, and UV absorbancy determined at 205 nm. The area under the curve was determined and converted to molar concentration against the maximal fibrinopeptide release at 45 min. Samples were performed in triplicate and expressed as mean ± SD of the ratio of fibrinopeptide concentration over maximal release ([Fp]/[Fp]\textsubscript{max}).

**Fibrinolysis**

Clots were generated as described for turbidity with the addition of plasminogen (0.24 \(\mu\)M) and tPA (20 pM) or plasmin (11 nM), before initiation of clotting with thrombin (0.25 U/ml) and \(\text{CaCl}_2\) (5 mM). In some cases thrombin was replaced with atroxin (0.5 \(\mu\)g/ml) or clots were formed by repolymerizing soluble fibrin. The effect of polyP chain length was analyzed by including \(\text{NaH}_2\text{PO}_4\) or polyP of different average chain lengths (5-, 25-, 45- and 65-mer) at a constant phosphate
monomer concentration (325 μM). The concentrations of thrombin 0.0125-5 U/ml, CaCl₂ 0-20 mM, tPA 10-500 pM and polyP₆₅ 0.065-3.25 mM were varied. Lysis was monitored at 340 nm. Turbidity was normalized and the time to 50% lysis was calculated as the time between maximal and half-maximal turbidity. In some experiments clots were generated in the absence of lytic proteins, and were incubated for 30 min before overlaying with tPA (10 nM) and plasminogen (0.55 μM). In these experiments polyP₆₅ (325 μM) was included with fibrinogen prior to clotting, in the overlay or in both.

**Surface plasmon resonance**

Binding of plasminogen and tPA to fibrin was analysed using a Biacore 3000 (Biacore, Stevenage, UK) as previously described²⁶ with the following modifications. All samples were analyzed in 10 mM Hepes, 140 mM NaCl and 2.5 mM CaCl₂ with 0.05% P-20, pH 7.4 (HBSC) and the experiments were performed at 25°C. Fibrinogen was covalently attached to a carboxymethyl dextran coated biosensor chip (CM5) by amine coupling, to yield approximately 1000 response units (RU). Immobilized fibrinogen was converted to fibrin by running 1 U/ml thrombin, ± 325 μM polyP₆₅, at 2 μl/min for 20 min. Thrombin was removed by injecting 40 μl of 1M NaCl, 10 mM Hepes, pH 7.4 followed by 35 μl of HBSC (30 μl/min). Plasminogen (0-1 μM) or t-PA (0-2.2 μM) was injected for 6 min at 30 μl/min and the dissociation monitored for 3 min. The surface was regenerated using 1M NaCl, 10 mM Hepes, pH 7.4 at 30 μl/min, followed by HBSC (35 μl) and re-equilibrated with running buffer for 5 min before the next
run. Benzamidine (2.5 mM) was included in the buffer during plasminogen binding to prevent activation. Plasmin (30 nM) was applied to the fibrin surface at 2 μl per min for 5 min and binding studies with plasminogen and tPA were repeated on partially-lysed fibrin.

**Plasmin activity**

Fibrinogen (2.4 μM) and plasminogen (0.55 μM) were added to microtitre plates (Greiner) ± 325 μM polyP₆₅ in 10 mM Tris (pH 7.4) with 140 mM NaCl and 0.01% Tween-20. Clotting was initiated with thrombin (1 U/ml) and CaCl₂ (5 mM) and the plate was incubated for 30 min at 37°C before lysing with tPA (5 nM) in the presence of S2251 (0.12 mM). Absorbance at 405 nm was recorded every 30 s and the amount of plasmin within the clot quantified. The direct effect of polyP on plasmin (0-200 nM) was analysed ± polyP₆₅ (325 μM) by monitoring hydrolysis of S2251 (0.12 mM) at 405 nm for 10 min.

**Statistical analysis**

Data are expressed as mean and standard deviation (SD) or standard error of the mean (SEM) of at least three separate experiments. Statistical analyses were performed with Graph Pad Prism 5 using t-test (Graph Pad Software Inc.) and P-values less than 0.05 were considered statistically significant.
RESULTS

Polyphosphate binds to fibrinogen and fibrin

Binding of polyP to fibrinogen was studied using a column-based technique where polyP$_{65}$ was pre-immobilized onto zirconia dioxide beads to form a stable solid phase. PolyP-zirconia or control-zirconia (treated with BSA only) were incubated with fibrinogen (10 μg) for 30 min at ambient temperature. The flow-through and eluates at low and high salt conditions were analyzed by Western blot using an antibody to fibrinogen. Fibrinogen could be detected in the flow-through of both polyP-zirconia and control beads (Figure 1A) but beads coated with polyP$_{65}$ retained fibrinogen which could only be eluted with 1 M NaCl. Binding between polyP and fibrin(ogen) was also investigated using a gel shift assay. Fibrinogen or soluble fibrin ± polyP$_{65}$ was subjected to native gel electrophoresis and the gel stained with toluidine blue O, a metachromatic dye which binds polyP. Free polyP$_{65}$ migrated with the dye front but pre-incubation with either fibrinogen or soluble fibrin retarded movement of the polymer through the gel; showing an interaction of polyP with these proteins (Figure 1B). In the absence of polyP$_{65}$ negligible staining was observed for the proteins.

Polyphosphate influences fibrin structure

PolyP$_{65}$ did not influence the rate of fibrin polymerization during clot formation (Figure 2A) even when the thrombin concentration was varied (not shown). However, inclusion of polyP$_{65}$ during clot formation reduced maximum turbidity of the fibrin clot compared to control (0.45 ± 0.014 vs 0.51 ± 0.015; p<0.005).
next measured the permeation constant $K_s$ which is a direct measure of the average pore size in the fibrin network. Fibrin clots formed in the presence of polyP$_{65}$ showed an approximately 1.5 fold reduction in $K_s$ ($3.35 \pm 0.15 \times 10^{-9}$ cm$^2$) compared to control ($5.02 \pm 0.26 \times 10^{-9}$ cm$^2$), $p<0.0005$. The lower turbidity and reduction in $K_s$ of clots formed in the presence of polyP$_{65}$ show a direct effect of polyP on the structure of the fibrin network.

We investigated whether changes in fibrin structure are related to an effect of polyP on fibrin cross-linking by FXIIIa. Purified fibrinogen preparations are contaminated with FXIII and therefore the assays performed are assumed to contain small amounts of the transglutaminase. Inclusion of purified FXIII did not result in any additional changes in the turbidity of the fibrin clots formed ± polyP. When fibrinogen was purified, to remove contaminating FXIIIa, no differences in the response to polyP$_{65}$ were observed (not shown). SDS-PAGE of the fibrin crosslinks generated by FXIIIa did not reveal differences in $\gamma$-$\gamma$ dimers or $\alpha$-polymers ± polyP$_{65}$ (not shown). These observations support previous work that polyP does not interfere with fibrin cross-linking$^{27}$ and that FXIIIa activity is not directly affected by polyP$^{28}$.

Fibrin structure is influenced by factors that interfere with the kinetics of fibrinopeptide cleavage. Using HPLC we measured release of fibrinopeptide A and fibrinopeptide B by thrombin ± polyP$_{65}$ (325 or 650 $\mu$M) and found equivalent (Figure 2B; only 325 $\mu$M polyP is shown for clarity). The similar kinetics of
fibrinopeptide A cleavage in the presence and absence of polyP$_{65}$ is consistent with the lack of effect on polymerization in turbidity measurements. The fact that fibrinopeptide B release was also unaffected indicates that the altered fibrin structure observed in the presence of polyP does not result from fibrinopeptide B-dependent changes in lateral association of fibrin fibers. These data are consistent with a thrombin-independent effect of polyP on fibrin formation.

Fibrin clots prepared in the absence of polyP showed a homogenous structure by laser scanning confocal and scanning electron microscopy (Figure 3A, C, E). In contrast, clots formed in the presence of polyP showed an abnormal and distinct pattern of fibrin distribution with tightly knotted regions that were interspersed by large pores (Figure 3B, D, F). Despite the irregular appearance of the clots, no major effect of polyP was observed on the average fiber diameter.

**Polyphosphate delays fibrinolysis**

Changes in the structural and mechanical properties of fibrin can have profound effects on its degradation by fibrinolytic enzymes. We found that clots formed with polyP were significantly more resistant to lysis by tPA and plasminogen or plasmin (Figure 4A & B). There was a strong dose-dependent effect of polyP in delaying tPA-mediated fibrinolysis with high concentrations of the polymer conferring considerable resistance to degradation (Figure 4C). Down-regulation of lysis was dependent on polymer size with significant lengthening of the 50% lysis time on inclusion of 25-, 45- and 65-mer polymers (polyP$_{65}$ being most
effective) while very short polymers (5-mer) or inorganic phosphate did not delay lysis (Figure 4D). This experiment also illustrates that the effect of polyP does not result from changes in ionic strength, as the same phosphate monomer concentration (325 μM) was present in each reaction mixture.

PolyP-induced changes in fibrinolysis were independent of thrombin concentration (0.0125 – 5 U/ml) but dependent on calcium concentration (0-20 mM) (Figure 5). Down-regulation of lysis by polyP was most pronounced at calcium concentrations of 2.5 and 5 mM, with little effect at higher concentrations or in the absence of calcium. PolyP is known to chelate metal ions but the molar concentration of calcium used in these experiments is significantly higher than the polyP concentration (325-650 μM). The ability of polyP to down-regulate fibrinolysis could also be overcome by including high concentrations of tPA (Figure 5C).

The effect of polyP on fibrin structure was independent of fibrinopeptide release by thrombin. Consistent with these observations, fibrin generated using atroxin, which only cleaves fibrinopeptide A, was more resistant to tPA-mediated fibrinolysis when formed in the presence of polyP₆₅ (Figure 6A). Clots generated from soluble fibrin, which was allowed to re-polymerise ± polyP₆₅, lysed at the same rates regardless of the presence of polyP (Figure 6B). We next analyzed fibrin clots formed ± polyP₆₅ that were lysed externally using an overlay of plasminogen and tPA. PolyP induced down-regulation of fibrinolysis occurred
only when clots were formed in the presence of the polymer and not when it was added to the overlay with tPA and plasminogen (Figure 6C). Inclusion of polyP in both the clot and overlay did not generate any additional effects (not shown). These data demonstrate that polyP down-regulates fibrinolysis by altering the arrangement of the fibrin network during clot formation.

**Plasmin generation and binding of both tPA and plasminogen is attenuated by polyphosphate**

The effect of polyP on the activation of plasminogen by tPA was assessed by incorporating the chromogenic substrate S2251 during clot formation. Plasmin generation was slower on fibrin formed in the presence of polyP$_{65}$ (325 μM) compared to control fibrin (Figure 7A; P<0.0001), consistent with a slower rate of lysis (Figure 6C). Reduced plasmin generation by polyP$_{65}$ was not observed in the absence of fibrin and there was no direct effect of polyP$_{65}$ on the activity of preformed plasmin (not shown).

We next used surface plasmon resonance to investigate the effects of polyP on the binding of plasminogen and tPA to fibrin. Fibrinogen, captured on a CM5 sensor chip, was converted to fibrin ± polyP$_{65}$ (325 μM) by injecting thrombin (1 U/ml) in the presence of calcium (2.5 mM). The surface was treated with 1 M NaCl to remove fibrin-bound thrombin. Binding of plasminogen (0-1 μM; Figure 7B, dashed lines) and tPA (0-250 nM; Figure 7C, dashed lines) to fibrin formed in the presence of polyP$_{65}$ was only slightly reduced compared to control surfaces.
Exposure of surfaces to plasmin (30 nM) resulted in a drop in the baseline, consistent with fibrin degradation. This effect was less pronounced on fibrin surfaces formed in the presence of polyP$_{65}$ (37.5% assuming 100% for control). Plasmin degradation of fibrin significantly enhanced binding of both plasminogen (Figure 7B, solid lines) and tPA (Figure 7C, solid lines). The presence of polyP$_{65}$ during fibrin formation dramatically impairs the ability of plasminogen (Figure 7B) and tPA (Figure 7C) to bind to partially-lysed fibrin; a similar fold-decrease was noted for both proteins relative to control values (Table 1). Collectively these results suggest that polyP down-regulates fibrinolysis by a two tier process (Figure 7D), whereby changes in fibrin structure delay plasmin degradation of the clot. This, in turn, reduces the availability of lysine binding sites on partially-lysed fibrin thereby attenuating binding of tPA and plasminogen and further decreasing plasmin generation and fibrinolysis.
DISCUSSION

The fibrin matrix is a dynamic environment that dictates the stability and resistance of a clot to degradation by fibrinolytic enzymes. This study shows that polyP, a negatively charged polymer that is released from platelet dense granules upon activation, binds to fibrin(ogen) generating heterogeneous clots characterised by knotted regions interspersed with large pores. We found that lysis of these clots by tPA and plasminogen or plasmin is impaired and that plasmin generation is attenuated. Binding studies revealed a reduction in the association of plasminogen and tPA to partially-lysed fibrin surfaces formed in the presence of polyP. These experiments show that polyP induces changes in fibrin structure that interfere with its ability to be degraded by plasmin and bind fibrinolytic proteases thereby interfering with its role as a cofactor in tPA-mediated activation of plasminogen. Both these processes result in a more stable fibrin clot.

The lagphase of fibrin polymerization was not affected by polyP suggesting that fibrinopeptide A release, oligomer formation and conversion to protofibrils occurs in a normal manner. Release of fibrinopeptide B stimulates lateral aggregation which arises once protofibrils reach a sufficient length. Experimentally it is reflected by a rapid increase in turbidity that is directly related to the average fiber cross-sectional area. The maximal turbidity was lower in the presence of polyP indicating a possible shift in average fiber diameter. However, measurements of fiber diameter on SEM and confocal microscopy demonstrated
that fiber thickness remained unaltered in the presence of polyP. Instead, clusters of tightly knit fibers were found interspersed with looser fibrin structures. The ratio of these clusters over the areas of loose structures may explain the differences in maximal absorbancy observed in turbidity experiments. Consistent with the decrease in maximal turbidity, the permeability constant $K_s$ of clots formed with polyP was lower.

Microscopy studies$^{30}$ following fibrin formation in real time show that initial scaffold formation corresponds to the lag phase in turbidity curves with branching, lateral and longitudinal growth of fibers continuing until network formation is complete. The effects of polyP on fibrin structure were clearly evident at the microscopic level with formation of distinct bundles of fibers rather than the homogenous distribution observed in control samples. These effects on fibrin structure were apparent using both scanning electron microscopy on fixed, processed clots and laser scanning confocal microscopy on fully hydrated clots. High magnification images did not reveal clear differences in the diameter of individual fibers but showed structures of highly knotted fibers. Remarkably, the changes in fibrin fiber arrangement that we observe with polyP appear similar to the structure of fibrin surrounding platelets, as found by Collet et al.$^{16}$ This suggests that polyP released by platelets may contribute to the tightly knitted fibrin structures surrounding these cells, an observation that requires further investigation. Our data on fibrin clot structure in part contrasts a previous report,$^{27}$ which revealed a significant increase in the diameter of the fibrin fibers
in the presence of polyP. These discrepancies perhaps result from the higher concentrations of polyP in this study, generally 1 mM compared to 0.325 mM in our study, although lower concentrations were found to increase fiber diameter albeit at a more modest level. Another explanation could be the 10-15 min pre-incubation of fibrinogen, calcium and polyP, necessary for the increase in fiber thickness to be observed. In our study the changes in fibrin structure were independent of the order of addition of components, as long as polyP was added prior to clot formation. It is noteworthy that purified fibrinogen, like that used here and in Smith et al, is preloaded with calcium as no steps were taken to chelate ions from fibrinogen or the buffer before clotting was initiated.

Identical patterns of fibrinopeptide release from fibrinogen were observed by HPLC in the presence and absence of polyP, confirming the turbidimetric analysis and indicating that this is not the point at which polyP influences fibrin formation. Similarly, the effect of polyP is not mediated through cross-linking as depletion of contaminating FXIII or addition of purified FXIII did not alter the ability of polyP to modify fibrin structure. These data confirm previous reports that polyP does not alter fibrin cross-linking or FXIIIa activity. The fibrin(ogen) αC-regions, which are liberated from the central E region after removal of fibrinopeptide B, have been reported to influence lateral aggregation of fibrils. This region has a positive charge, in contrast to the net negative charge of fibrin(ogen), and has been shown to be involved in lateral association. It is therefore feasible that the αC regions provide an appropriate surface for a highly
negatively charged molecule, such as polyP, to bind. Interestingly, a study including homo poly(L-amino acids) during fibrin formation illustrated altered fibrin networks on inclusion of positive but not negative polymers.\textsuperscript{33} The negatively charged glycosaminoglycan heparin, which binds to the N-terminus of the $\beta$ chain of fibrin\textsuperscript{34, 35}, generates clots with thicker fibers and larger pores which results in increased susceptibility to fibrinolytic degradation.\textsuperscript{36} These divergent effects of anionic polymers on fibrin structure indicate that while charge may be important, there are more subtleties to be considered. It is plausible that polyP bridges fibers during assembly or acts as a point of nucleation but further studies will be required to define the underlying mechanism.

The composition of the fibrin network affects its susceptibility to fibrinolysis, with the number of fibers per volume having more effect than individual fiber diameter.\textsuperscript{37} PolyP induced a dose-dependent delay in time to 50\% lysis when included in clots at the time of formation, but not if added to pre-formed fibrin clots alongside the lytic agents. When soluble fibrin was allowed to repolymerize in the presence of polyP lysis was unchanged. These experiments are consistent with polyP exerting its effect during conversion of fibrinogen to fibrin. For the most part, this study investigated intrinsic lysis, when the components of the fibrinolytic system are incorporated during clot formation. This mode of lysis is akin to situations \textit{in vivo}, when clotting and fibrinolysis are triggered simultaneously, and act side-by-side balancing fibrin deposition and breakdown. Increasing the tPA concentration annihilated the differences in lysis observed
with polyP, most likely due to the rapid fibrin-independent conversion of plasminogen to plasmin. Nevertheless, delayed lysis of clots was still observed with preformed plasmin, suggesting the effect was related to the structure of the fibrin network and/or the ability of plasmin to interact with the fibrin surface. Plasmin activity toward a chromogenic substrate was not affected by polyP, but fibrin-enhanced tPA-stimulated plasminogen activation was down-regulated. Binding of plasminogen and tPA to fibrin formed in the presence of polyP was only slightly attenuated but following plasmin treatment of the fibrin surface this effect was dramatically magnified. These data indicate that polyP affects fibrinolysis at two levels, as represented in the schematic in Figure 7D. Plasmin is less efficient at cleaving fibrin formed in the presence of polyP which reduces the exposure of lysine binding sites. This ultimately decreases the capacity of fibrin to act as a cofactor in tPA-mediated plasminogen activation thereby reducing plasmin generation and further delaying fibrinolysis.

Delayed fibrinolysis of clots formed in the presence of polyP was evident over a range of thrombin concentrations, further illustrating that the ability of this polymer to influence fibrin structure and lysis was independent of thrombin or fibrinopeptide cleavage. These observations were substantiated using snake venom atroxin, which cleaves only fibrinopeptide A from fibrinogen, and showed that polyP can induce a similar down-regulation of fibrinolysis. Calcium binds to several sites in fibrinogen. Calcium binding has been shown, using a variant fibrinogen lacking the β2 binding site, to regulate lateral aggregation. PolyP is
known to chelate divalent metal ions such as calcium. The effects of polyP on fibrin structure cannot, however, be explained by simple sequestration, as the experiments were performed using a large molar excess of calcium (5 mM) over the concentration of phosphate groups (typically 650 μM, which could bind 0.33 mM Ca²⁺). Furthermore, effects on lysis were not observed by addition of a similar concentration of the monomer which would sequester equal amounts of calcium. Dose responses to calcium revealed a bell-shaped response with a minimal effect of polyP on lysis at either end of the scale. A possible explanation for these observations is that polyP is binding at or near a calcium binding site and is interfering with fibrin formation leading to altered network that is cleaved less effectively by plasmin. The similar fiber diameters in the presence of polyP suggest that lateral aggregation is unaffected, in agreement with an effect that is independent of calcium modulation. The observation that fibrin structure in the presence of polyP shows areas of tightly knit fibers interspersed by more loosely packed structures suggests that polyP interferes with fiber branching rather than lateral aggregation.

The concentration of polyP in serum is reported to reach approximately 50 μM and therefore it is plausible that local concentrations of 5-10 fold higher could be readily achieved during clot formation. Our data indicate that polyP produces prothrombotic fibrin structures with increased resistance to fibrinolysis. These effects result from architectural differences in the clot that occur during conversion of fibrinogen to fibrin. These changes impact the ability of plasmin to
cleave the network and reduce exposure of lysine binding sites thereby modulating the cofactor capacity of fibrin in tPA-mediated plasminogen activation. Secretion of polyP by platelets during clot formation will therefore lead to a more stable fibrin clot and this may have important implications for thrombotic disease.

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Author Contributions

NJM performed research, analyzed data and wrote the manuscript. RE and SUdW performed research. HP analyzed data and RASA analyzed data and wrote the manuscript.

Conflict of Interests

The authors have no conflicts of interest to disclose.
REFERENCES


Table 1. The increase in binding of plasminogen and tPA to fibrin upon limited proteolysis by plasmin is impaired in the presence of polyP. Binding of plasminogen and tPA to fibrin surfaces formed ± polyP$_{65}$ was analyzed pre- and post-partial lysis with plasmin. The mean fold-increase in binding ± SEM is calculated after degradation of the fibrin surface with plasmin from four concentrations of plasminogen (125, 250, 500, 1000 nM), and tPA (31.25, 62.5, 125, 250 nM).

<table>
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<tr>
<th></th>
<th>control</th>
<th>polyP$_{65}$ (325 μM)</th>
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<tr>
<td>plasminogen</td>
<td>3.9 ± 0.13</td>
<td>2.6 ± 0.11**</td>
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<tr>
<td>tPA</td>
<td>3.1 ± 0.09</td>
<td>2.2 ± 0.13*</td>
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* $P<0.005$; **$P<0.0005$ when comparing control to polyP$_{65}$ values
FIGURE LEGENDS

Figure 1. PolyP binds to fibrinogen and soluble fibrin. (A) Fibrinogen (10 μg) was incubated with zirconia beads coated with either polyP65 or BSA. The starting material (SM), flow-through (FT), low salt (LS) and high salt (HS) washes were subjected to SDS-PAGE on 4-12% NuPAGE gels with MOPS running buffer and blotted with an antibody to fibrinogen. (B) PolyP65 was incubated with 10 μg of soluble fibrin (SF), fibrinogen (FG) or buffer alone for 10 min before separating on 6% Tris-glycine gels for 2.5 h under native conditions. The gels were stained with toluidine blue O which binds polyP. Free polyP65 migrates with the dye front and cannot be visualised.

Figure 2. PolyP-induced changes in fibrin formation and structure. (A) Fibrinogen (2.4 μM) was clotted in the absence (grey line) and presence (black line) of polyP65 (325 μM) by an activation mix of thrombin (0.25 U/ml) and CaCl2 (5 mM). The turbidity was monitored at 340 nm every 12 s for 30 min and the results are expressed as mean ± SEM (n=4). (B) Fibrinopeptide release from fibrinogen during polymerization with thrombin (0.25 U/ml) and CaCl2 (5 mM) was monitored over time by reverse-phase HPLC in the absence (open symbols) and presence (closed symbols) of polyP65 (325 μM). The concentration of fibrinopeptide A (circles) and fibrinopeptide B (triangles) at each time point is
expressed as a fraction of maximal release ([FP]/[FP]_{max}, so that complete release is equal to 1). The inset shows the linear part of the curve.

**Figure 3. PolyP-induced changes in fibrin ultrastructure.** Fibrin clots were prepared by incubating fibrinogen (3 μM) with thrombin (0.25 U/ml) and CaCl_2 (5 mM) in the absence, (A, C, E) and the presence (B, D, F) of polyP_{65} (325 μM). Panels (A) and (B) represent fibrin clots analyzed by laser scanning confocal microscopy and panels (C - F) were analyzed by scanning electron micrographs. A total of 10 different areas were visualized in three replicate clots and representative pictures are shown. The high magnification images (E & F) show details of a tightly knotted fibrin region in the presence of polyP (F).

**Figure 4. PolyP delays fibrinolysis.** Fibrin clots were formed with purified fibrinogen (2.4 μM) in the absence (grey line) and presence (black line) of polyP_{65} (325 μM). Lysis was induced by including 20 pM tPA and 0.24 μM plasminogen (A) or 11 nM plasmin (B) with fibrinogen prior to activation with thrombin (0.25 U/ml) and CaCl_2 (5 mM). Lysis was monitored by changes in absorbance at 340 nm for 4 h with time points every 1 min. Results are normalized to account for polyP induced changes in turbidity and expressed as mean ± SEM of the percentage lysis (n=6). (C) Lysis was followed as described above for tPA and plasminogen but with the addition of various polyP_{65} concentrations (0-3.25 mM). The time to mean time to 50% lysis ± SEM is shown (n=3). (D) Clots were formed in the presence of monophosphate or
polyphosphate of varying chain lengths (5-65 phosphate residues) at equivalent monomer concentration (325 μM). Lysis by tPA and plasminogen was monitored and the mean 50% lysis time ± SEM shown (n=4). 50% lysis times of statistical significance are denoted with ** (P<0.001) and *** (P<0.0001).

Figure 5. Different parameters affect the ability of polyphosphate to modulate lysis. Fibrin clots were formed from fibrinogen (2.4 μM), plasminogen (0.24 μM) and tPA in the absence (open circles) and presence (closed circles) of polyP₆₅ (325 μM) by addition of thrombin and CaCl₂. (A) The concentration of thrombin used to induce clotting was varied from 0.016 U/ml to 5 U/ml with constant CaCl₂ (5 mM) and tPA (20 pM) concentration. (B) The concentration of CaCl₂ was varied from 0-20 mM in the presence of constant thrombin (0.25 U/ml) and tPA (20 pM) concentrations. (C) The tPA concentration was varied from 10-500 pM in the presence of constant thrombin (0.25 U/ml) and CaCl₂ (5 mM) concentrations. All the results are expressed as the mean ± SEM time to 50% lysis (n=4).

Figure 6. Polyphosphate only affects lysis if present during polymerization of fibrinogen. (A) Fibrin clots were formed from fibrinogen (2.4 μM), plasminogen (0.24 μM) and tPA (20 pM) in the absence (grey line) and presence (black line) of polyP₆₅ (325 μM) by addition of atroxin (10 μg/ml) and CaCl₂ (5 mM) (n=4). (B) Soluble fibrin was repolymerized in the presence of plasminogen (0.24 μM) and tPA (20 pM) in the absence (grey line) and presence (black line) of
polyP<sub>65</sub> (325 μM) (n=5). (C) Clots were generated with purified fibrinogen clotted with (black line) and without (pale gray line) polyP<sub>65</sub> (325 μM). Clots were incubated for 30 min at 37°C before overlaying with a mixture of plasminogen (0.55 μM) and tPA (10 nM) with (dark grey line) and without polyP<sub>65</sub> (325 μM) (n=8). Results are normalized and expressed as the mean ± SEM.

Figure 7. Polyphosphate attenuates the binding of fibrinolytic enzymes and the cofactor activity of fibrin. (A) Fibrin clots containing plasminogen (0.55 μM) were formed with purified fibrinogen (2.4 μM) in the absence (grey line) and presence (black line) of polyP<sub>65</sub> (325 μM) by addition of thrombin (1 U/ml) and CaCl<sub>2</sub> (5 mM). Clots were lysed by overlaying with tPA (5 nM) in the presence of S2251 (0.12 mM) and readings were recorded at 405 nm. Plasmin generation at 405 nm over time is shown and results are expressed as mean ± SEM, n=3. (B) Fibrinogen was captured on the surface of a CM5 chip and converted to fibrin by thrombin (1 U/ml) in the absence and presence of polyP<sub>65</sub> (325 μM). Binding of 125 nM (black), 250 nM (orange), 500 nM (blue) and 1000 nM (green) plasminogen was analyzed (dashed lines). The surface was then treated with plasmin (30 nM) and binding of plasminogen, at the concentrations described above, repeated (solid lines). (C) As described for panel (B) with 31.25 nM (black), 62.5 nM (orange), 125 nM (blue) and 250 nM (green) tPA instead of plasminogen. Experiments in (B) and (C) were performed in triplicate and are expressed as mean ± SEM. (D) Schematic representation of polyP-induced down-regulation of fibrinolysis. 1. Fibrin formed in the presence of polyP has a
heterogeneous structure composed of tightly knotted regions interspersed with large pores. 2. Plasmin (yellow) is less efficient at cleaving this heterogeneous fibrin structure thereby reducing the exposure of C-terminal lysines (K) on the fibrin surface. 3. The capacity of fibrin to sequester tPA and plasminogen on its surface is subsequently reduced and the co-factor role of fibrin in tPA-mediated plasminogen activation diminished. 4. This leads to less plasmin formation. 5. As a result fibrinolysis is down-regulated in clots formed in the presence of polyP.
Figure 1

A

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<tr>
<td></td>
<td>SM FT LS HS</td>
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B

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Figure 2

A

B

A

\[ A_{340} \]

\[ \frac{[FP]}{[FP]_{\text{max}}} \]

Time (min)

Time (min)
Figure 3

A

B

C

D

E

F

10 μm

10 μm

10 μm

10 μm

200 nm

200 nm

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Figure 4

A

B
Figure 5

A

$\frac{t}{2}$ (min)

0.016 0.063 0.25 1 4

Thrombin (U/ml)

B

$\frac{t}{2}$ (min)

0 1 2 4 8 16

CaCl (mM)

C

$\frac{t}{2}$ (min)

10 100 1000

tPA (pM)
Figure 6

A

Turbidity (%)

Time (min)

B

Turbidity (%)

Time (min)

C

Turbidity (%)

Time (min)
Polyphosphate modifies the fibrin network and down-regulates fibrinolysis by attenuating binding of tPA and plasminogen to fibrin

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