Supplemental Materials, Methods & Data

Hemolytic Assays

Erythrocyte (RBC) lysis assays as previously described[1,2] were used to measure complement activity. For alternative pathway assays and for total hemolytic activity assays, unsensitized rabbit erythrocytes have been routinely used for over 30 years (reviewed in[1]). Although guinea pig erythrocytes could also be used, most investigators use rabbit erythrocytes, partly due to the latter generally being more readily accessible and more sensitive[3]. Furthermore, rabbit erythrocyte sensitivity varies little between batches and the cells remain stable for 2-4 weeks[4]. Chicken and guinea pig erythrocytes are ideal targets of the terminal pathway, whereas sheep, pig or rabbit erythrocytes are considered unsuitable due to high level of endogenous CD59[5].

Erythrocytes were washed three times in GVB with the reaction buffers as indicated, and counted using the Advia 120 Hematology System from Siemens (Siemens, Erlangen, Germany). Reactions in 300 µL were allowed to proceed for 30 min at 37°C, after which unlysed cells were pelleted by centrifugation at 600 x g. Erythrocyte lysis, reflected by the released hemoglobin in the supernatant, was quantified by measuring the absorbance at 405 nm using the Mithras LB 940 microplate reader from Berthold Technologies (Bad Wildbad, Germany). Percent lysis is relative to the control of 100% lysis with H_2O.

For total hemolytic activity assays, normal human serum (NHS) (4.5%), polyP, and rabbit erythrocytes (rRBC) ($6.0x10^7$ cells/mL) were added sequentially to initiate lysis. Reagents were diluted in GVB. Reactions were stopped by the addition of 30 mM EDTA in GVB. Lysis in the absence of NHS was subtracted as background.

For serum-based terminal pathway (TP) hemolytic assays, components were diluted in GVB containing 10 mM EDTA (GVB-E), the latter added to prevent upstream complement activation and generation of endogenous C5b,6. 2% NHS was used as the source of C7, C8, and C9, and hemolysis of chicken erythrocytes (cRBC) ($3.3x10^8$ cells/mL) was initiated by addition of exogenous purified C5b,6, the concentration of which was determined from pilot studies to yield ~70-80% lysis at 30 min. No C5b6 activity was detected in the NHS prior to initiating the reaction. For some experiments, instead of NHS, purified terminal pathway complement

components were used in the TP assays. In these assays, in a final volume of 300 ul GVB, lysis was achieved by the sequential addition of the following components: cRBC ($3.3x10^8$ cells/mL), C5b,6 (20 pM), C7 (15 nM), C8 (10 nM), and C9 (25 nM). The unlysed cell pellet was removed by centrifugation and lysis was measured immediately after the 30-minute reaction.

Calf Intestinal Alkaline Phosphatase (CIAP) Digestion of PolyP

600 µM polyP was incubated with 400 U/mL calf intestinal alkaline phosphatase (CIAP; Invitrogen Life Technologies Inc., Burlington, ON, Canada) for 18 hrs in TBS (20 mM Tris-HCl, 150 mM NaCl, pH 8.4) in the presence of 2 mM $MgCl_2$ and 0.2 mM $ZnCl_2$. Complete digestion of polyP into monomers was confirmed by TBE-urea polyacrylamide gel electrophoresis followed by staining with 0.05% toluidine blue and by the malachite green assay[6]. CIAP-treated polyP was added to the TP assay at a final concentration of 100 µM.

Visualization of PolyP in TBE-Urea Gels

PolyP can be visualized in both native and TBE-urea gels using the metachromatic stain toluidine blue which, upon binding to polyP, shifts the absorption peak from 630 nm to 530 nm. 10 nmol of phosphate were mixed with 5x sample buffer (15% Ficoll 400, 0.25% xylene cyanol FF, 0.25% bromophenol blue, 5x TBE) and loaded into 10% TBE-urea Precast Ready Gels (8.6 x 6.8) from Bio-Rad. Running buffer was 1x TBE containing 90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3. Samples were electrophoresed under constant voltage for 30 min at 150 V. Gels were stained with a fixative solution containing toluidine blue (0.05% toluidine blue, 25% methanol, 5% glycerol) for 10 min, destained with the same fixative without toluidine blue, and then imaged in white light on a flatbed scanner.

Native Polyacrylamide Gel Electrophoresis (PAGE)

Native PAGE was used to assess polyP-protein interactions (known as electromobility shift assay). Mini gels were handcast according to Laemmli's gel system in the absence of detergent and reducing agent. 4x resolving and stacking gel buffer consisted of 1.5 M Tris-HCl (pH 8.8) and 1.0 M Tris-HCl (pH 6.8), respectively. Running buffer contained 25 mM Tris and 192 mM glycine (pH 8.3).

1-2 µg of protein was incubated with 3 µg polyP (or buffer as control) at ambient temperature for 10 min before adding sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 0.02% bromophenol blue). Proteins were electrophoresed at 100V constant voltage for 2 hours, stained with EZBlue Coomassie Brilliant Blue G-250 from Sigma-Aldrich and destained with several changes of water, and then imaged in white light on a flatbed scanner.

Gel Filtration

Proteins were stored in their stock solution buffers. The C5b,6 buffer consisted of 10 mM HEPES, 120 mM NaCl, pH 7.2. The C7 buffer consisted of 10 mM Na_3PO_4, 145 mM NaCl, at pH 7.3 (PBS). The buffers were filtered through a 0.22 µm pore diameter Stericup and Steritop Express™PLUS filter from Millipore (Billerica, Massachusetts, USA).

Proteins were thawed at 37°C for 10 min. An excess amount of protein was removed from the stock vial and transferred to a clean 600 µL microtube. C7 was diluted to 50 µg/µl. The proteins were centrifuged at 20,817 g for 15 min and under a Class 2A Biosafety Cabinet, 100 µL fractions were transferred to clean microtubes. The following samples were analyzed by gel filtration:

a. C5b,6 alone
b. C5b,6 with $polyP_{1000}$
c. C5b,6 with monophosphate (P_1)
d. C7 alone
e. C7 with $polyP_{1000}$

For all samples, 20 µg of the protein in 100 µL of their respective buffers was prepared. For samples containing polyP, 8 µL of 400 mM $polyP_{>1000}$ was mixed with 2 µL of 5x concentrated HBS (for C5b,6) or PBS (for C7), yielding a solution of 320 mM $polyP_{1000}$ in HBS or PBS. 3.13 µL of the polyP solution was added to 100 µL of either C5b,6 or C7, for a final $polyP_{>1000}$ concentration of 9.71 mM. The same protocol was used for the sample containing monophosphate (P_1), adding 4.17 µL of the 240 mM P_1 solution into 100 µL of C5b,6, for a final P_1 concentration of 9.61 mM. Both the $polyP_{>1000}$ and P_1 were centrifuged at 20,817 xg for 15 min prior to adding to the proteins.

The gel filtration apparatus, ÄKTAmicro System from G.E. Healthcare (Buckinghamshire, UK), was equilibrated with either HBS (0.22 μm filtered) for C5b,6 or PBS (0.22 μm filtered) for C7 for at least 1 hour, at 5 μL per minute. The column was a Superose 6 PC 3.2/30 from G.E. Healthcare (Buckinghamshire, UK). Protein was monitored with UV absorbance at 280 nm. The entire volume of each sample was injected onto the column after equilibration with buffer. Data for each curve were normalized to the point where the peak starts.

Clot turbidity assay

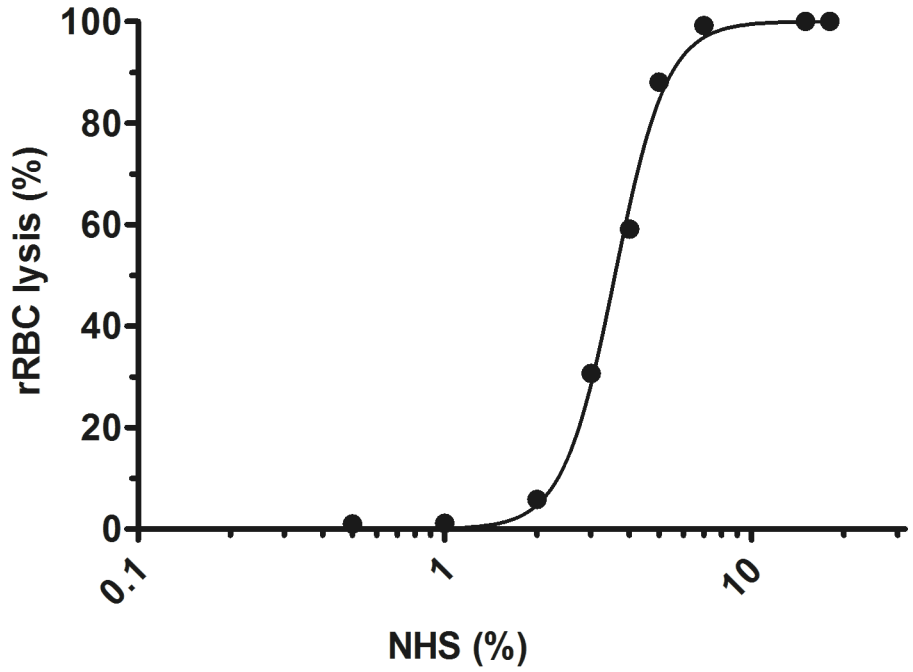
Clot turbidity assays were performed in untreated 96-well microplates (Corning, Tewksbury, MA, USA) that were blocked with 1% BSA overnight at 4^{o}C and washed before using. Poly$P_{>1000}$ was diluted in a lipid mixture containing 125 mM imidazole (pH 7.0) and 75 μM unilamellar phospholipid vesicles consisting of 20% 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS) and 80% 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC; both from Avanti Polar Lipids, Alabaster, AL, USA), that was freshly made using the Avestin Liposofast apparatus (Ottawa, ON, Canada) according to the manufacturer's instructions. 50 μL poly$P_{>1000}$ was incubated with 50 μL normal pooled plasma (Affinity Biologicals, Ancaster, ON, Canada) at 37^{o}C for 3 minutes, and clotting was initiated by adding 50 μL of 25 mM CaCl2. Turbidity was measured at A405 using a SpectraMax 190 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) every 20 seconds for 60 minutes until absorbance from all wells plateaued. Clot time was defined as the time at which the horizontal line across baseline absorbance intersects with a fitted line to the steepest linear region of the curve during clot formation.

Measuring effect of polyP on C5b-8 and C5b-7 binding to erythrocyte membranes

Chicken erythrocytes (cRBC) (Colorado Serum Company, Denver, CO) were washed four times with gelatin veronal buffer (GVB), and resuspended in GVB at a final of concentration 3.00 x 10^{9} cells/mL. Varying concentrations of polyP from 0 μM to 10 mM were added to the cells and incubated for 5 minutes at room temperature. C5b,6 was then added to the reaction mixtures (final concentration 2.5 nM) and incubated for 5 minutes. Finally, C7 and C8 (Supplemental Figure S4a) or C7 (Supplemental Figure S4b) were added at final concentrations of 2.5 nM each and incubated for a further 5 minutes. The cells were then pelleted at 300 g for 3 minutes and

40 µL of the supernatant was transferred to another microfuge tube. This was again centrifuged at 300 g for 3 minutes to remove any contaminating cRBC. 32 µL of this supernatant containing any unbound complement components was mixed with 8 µL of Laemmli buffer with beta-mercaptoethanol for separation by SDS-PAGE on a 10% acrylamide gel. The transferred gel was Western immunoblotted with goat-anti-human C5 primary antibody (Complement Technology, Inc. Tyler, TX) with detection accomplished with 680RD donkey-anti-goat secondary antibody from LI-COR Biosciences (Lincoln, NE). The α'-chain of C5b was quantified by densitometry using the Odyssey Software from LI-COR Biosciences (Lincoln, NE). Values were normalized to the experimental conditions in which cRBC were incubated with maximal concentrations of polyP, but without C7 or C8. The amount of C5b under these conditions was considered to be 100%. The results as shown in Supplemental Figure S4a and S4b reflect the averages of 3 independent experiments, with standard deviations indicated.

Supplemental Figure S1. **Standard curve for the total complement-mediated hemolytic assay**. This was generated by incubating increasing concentrations of normal human serum with rabbit erythrocytes for 30 min at 37^{o}C, quenching the reaction with excess EDTA, and then measuring the A_{405} of the supernatant. Increasing absorbance corresponds to increasing hemoglobin release from lysed red blood cells. The linear region of the curve was determined to be between 20-80% lysis, corresponding to 2.5-4.5% serum. Since the effects of polyP were determined in pilot studies to be inhibitory, baseline lysis was fixed at ~80% (using 4.5% serum). The effects of polyP over a range of concentrations under these experimental conditions was subsequently evaluated (see manuscript – Figure 1).



Supplemental Figure S1
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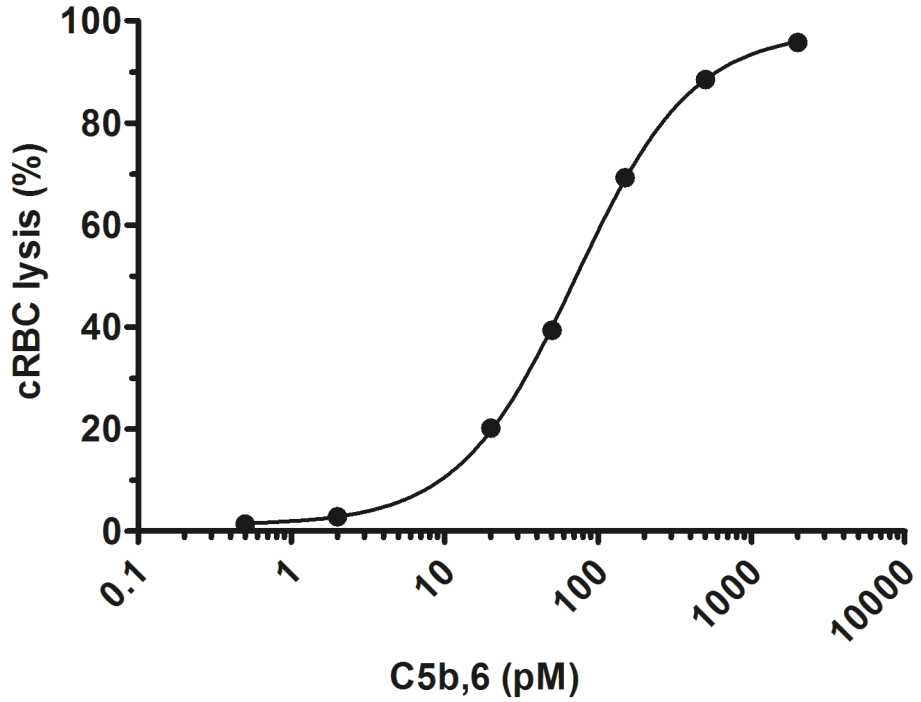
Supplemental Figure S2. **Standard curve for the terminal pathway assay using serum as the source of complement.** Serum concentration was fixed at 2%, and increasing concentrations of purified C5b,6 dose-dependently lysed chicken erythrocytes after 30 min at 37^{o}C. The linear region of the curve was achieved with 20-250 pM purified C5b,6. 250 pM of purified C5b,6 was therefore used to fix baseline lysis at ~80% to allow assessment of the inhibitory effects of polyP in serum.



Supplemental Figure S2
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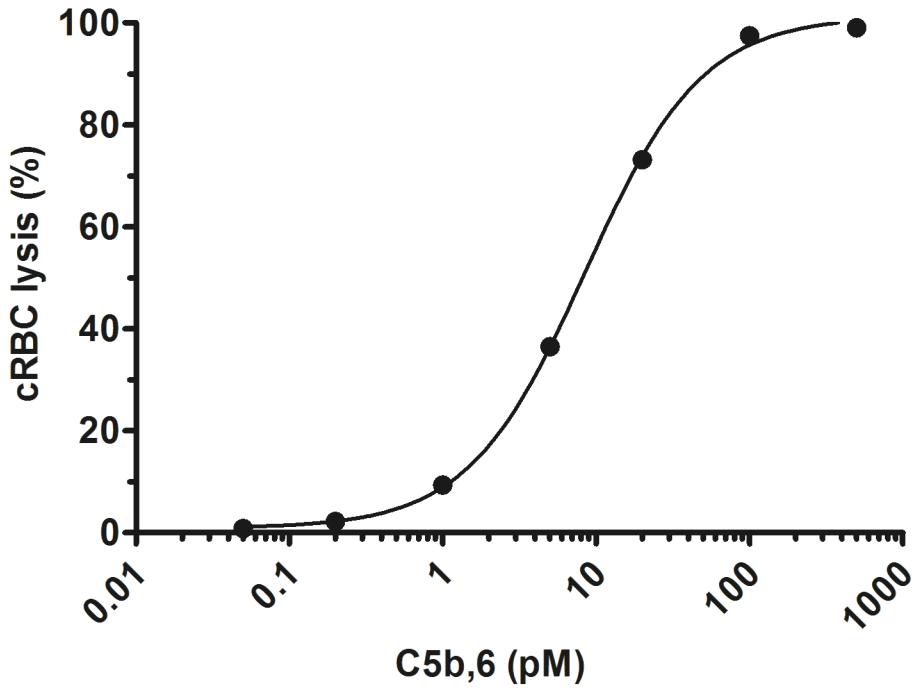
Supplemental Figure S3. Standard curve for the terminal pathway assay using purified complement components. C7 (15 nM), C8 (10 nM), and C9 (25 nM) were in excess, and hemolysis of chicken erythrocytes after 30 min at 37 ^{o}C was determined as dependent on the concentration of purified C5b,6. The linear region of the curve was achieved with 2-20 pM purified C5b,6. 20 pM of purified C5b,6 was therefore used to fix baseline lysis at ~80% and this was used to assess the inhibitory effects of polyP.



Supplemental Figure S3
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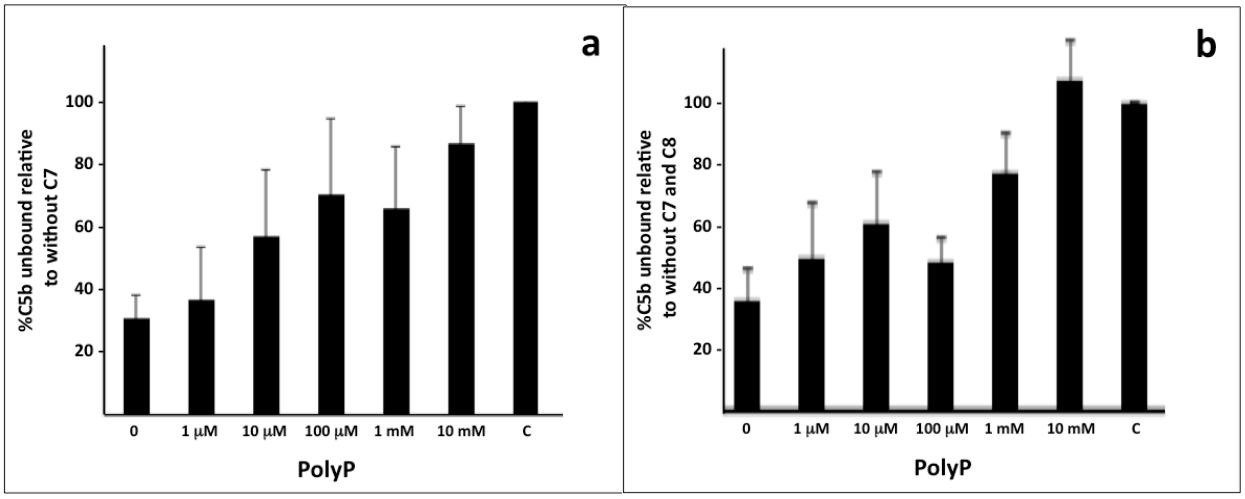
Supplemental Figure S4. PolyP interferes with binding of C5b-8 and C5b-7 complexes to erythrocyte membranes. Varying concentrations of polyP were incubated for 5 minutes with cRBC, followed by a 5 minute incubation with C5b,6, and 5 minutes later by the addition of equimolar concentrations C7 and C8 (**a**) or C7 alone (**b**). C (control) represents the condition to which results were normalized, i.e., where polyP 10 mM was incubated with cRBC and C5b,6, but no C7 or C8 was added. Unbound C5b was quantified by Western blot and densitometry as described in the methods above within the Supplemental Data section. Results reflect 3 independent experiments. Error bars indicate standard deviation.



Supplemental Figure S4
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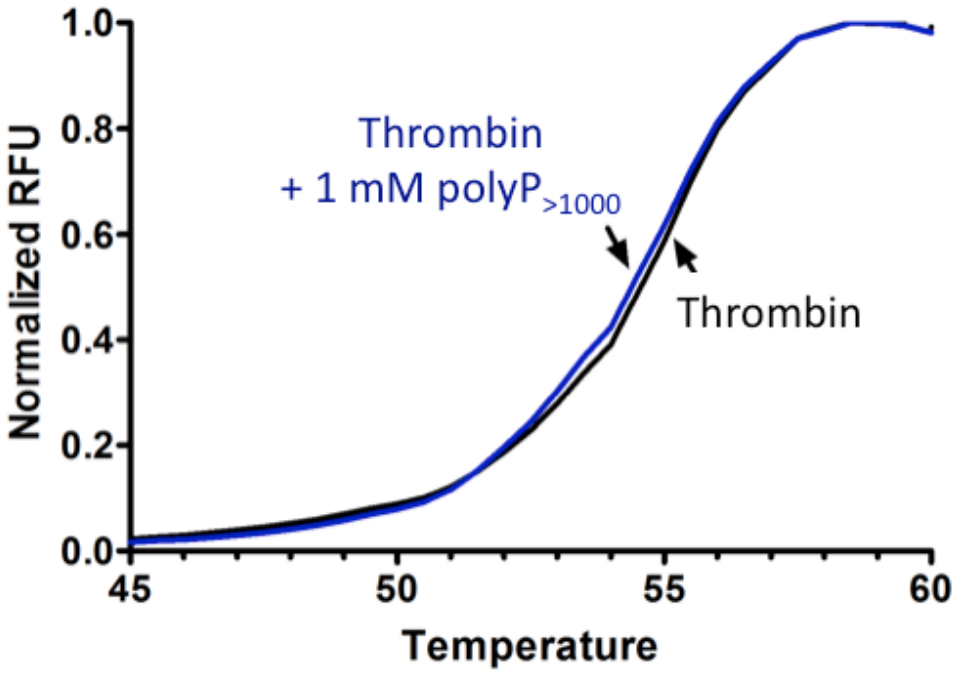
Supplemental Figure S5. PolyP does not alter the stability of thrombin. Thrombin was incubated with a range of concentrations of $polyP_{>1000}$ and relative fluorescence (RFU) was measured as the proteins were thermally denatured. In spite of the finding that thrombin binds to polyP, and in contrast to the effect of polyP on C5b,6 (Figure 7 of manuscript), polyP has no effect on the stability of thrombin (1 μM), i.e., there is no shift in the curve even with a high concentration of polyP (1 mM).



Supplemental Figure S5
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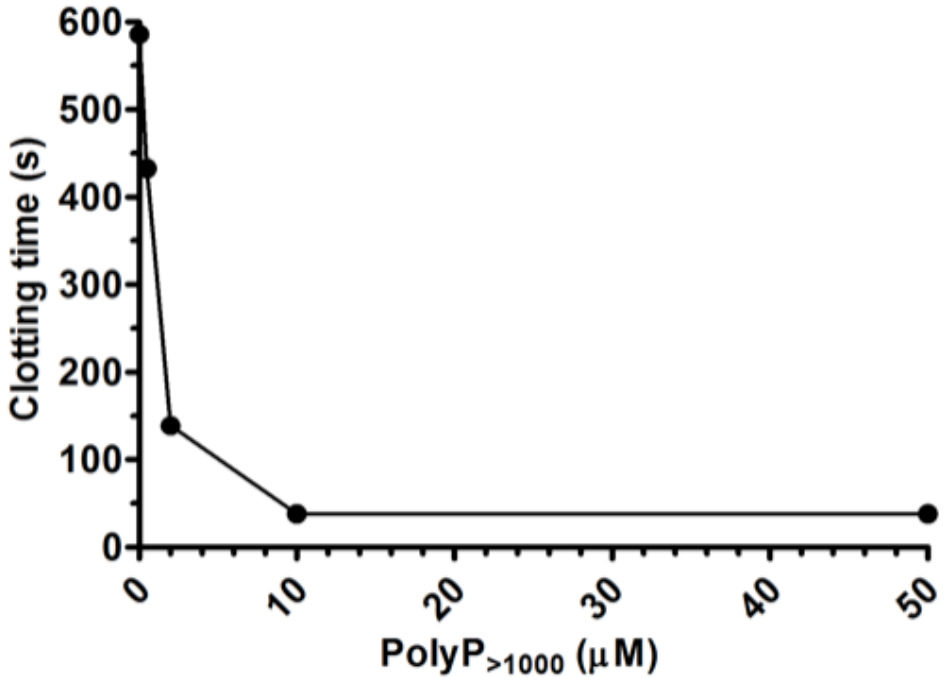
Supplemental Figure S6. PolyP$_{>1000}$ shortens the plasma clotting time. As described in the methods in the Supplemental Data section, clot turbidity assays were performed in untreated 96-well microplates with a range of polyP$_{>1000}$ co-incubated with plasma. The clotting time is dramatically shortened with low concentrations of polyP$_{>1000}$.



Supplemental Figure S6
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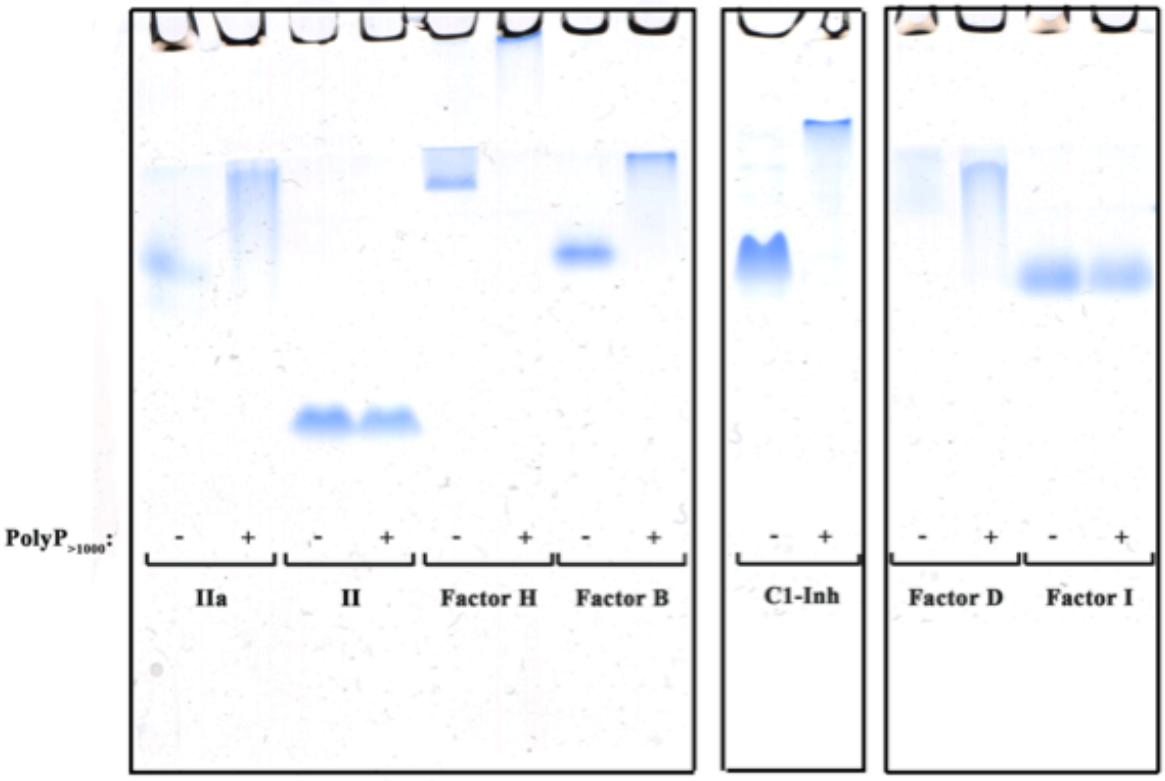
Supplemental Figure S7. The effect of polyP$_{>1000}$ on the electromobility of complement proteins. 2 μg of protein were incubated with or without 6 μg polyP$_{>1000}$ and resolved by native PAGE. The gel was stained with Coomassie blue for detection. PolyP binds to and causes a shift in the migration of thrombin (IIa), factor H, factor B and C1-esterase inhibitor (C1-Inh), but does not affect migration of prothrombin (II), factor D or factor I.



Supplemental Figure S7
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References for Supplementary Section

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