Phosphoramidate End-labeling of Inorganic Polyphosphates: Facile Manipulation of Polyphosphate for Investigating and Modulating its Biological Activities†

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Abstract

Polyphosphates, linear polymers of inorganic phosphates linked by phosphoanhydride bonds, are widely present among organisms and play diverse roles in biology, including functioning as potent natural modulators of the human blood clotting system. However, studies of protein-polyphosphate interactions are hampered by a dearth of methods for derivatizing polyphosphate or immobilizing it onto solid supports. We now report that EDAC (1-ethyl-3-[3-dimetyhlamino-propyl]carbodiimide) efficiently promotes the covalent attachment of a variety of primary amine-containing labels and probes to the terminal phosphates of polyphosphates via stable phosphoramidate linkages. Using ^{31}P NMR, we confirmed that EDAC-mediated reactions between primary amines and polyphosphate results in phosphoramidate linkages with the terminal phosphate groups. We show that polyphosphate can be biotinylated, labeled with fluorophores and immobilized onto solid supports; that immobilized polyphosphate can be readily used to quantify protein binding affinities; that covalently derivatized or immobilized polyphosphate retains its ability to trigger blood clotting; and that derivatizing the ends of polyphosphate with spermidine protects it from exopolyphosphatase degradation. Our findings open up essentially the entire armamentarium of protein chemistry to modifying polyphosphate, which should greatly facilitate studies of its biological roles.

Inorganic polyphosphate (polyP), a linear polymer of orthophosphate residues linked via phosphoanhydride bonds, is widely distributed in biology and plays important and diverse roles in nature (1,2). We recently showed that polyP is a potent modulator of the blood clotting cascade (3-5), and an expanding body of research is investigating its roles in other biological systems (6-11). Many technical obstacles remain, for investigating the biological roles of polyP and there is a real need for improved microscale methods for analyzing polyP. In particular, there is a dearth of approaches for covalently modifying polyP or attaching it to solid supports. One of the few published methods for immobilizing polyP onto surfaces is via Lewis acid/base interactions between polyP and zirconia beads (12). Although we have successfully used this method (13), it suffers from relatively high nonspecific binding of proteins to zirconia. Furthermore, this chemistry is not readily adaptable for attaching labels to polyP, or to immobilizing polyP onto the sorts of solid supports routinely used in analyses

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of protein interactions. The goal of the present study was therefore to develop conditions for routine covalent attachment of labels to the terminal phosphates of polyP.

The zero-length cross-linking reagent, EDAC (1-ethyl-3-[3-dimetyhlamino-propyl]carbodiimide), is widely used to couple primary amines to carboxylic acids via amide linkages. However, EDAC can also be used to couple primary amines to organic phosphates —including the 5' phosphates of oligonucleo-tides—via stable phosphoramidate linkages (14). We now report that the terminal phosphates of polyP can be made to enter into covalent phosphoramidate linkages with primary amine-containing compounds via EDAC (Scheme 1). This finding essentially opens up the entire armamentarium of protein chemistry to modifying polyP, greatly facilitating investigations into polyP's biological activities. In the present study, we demonstrate conditions under which polyP can be biotinylated, labeled with fluorophores, and immobilized onto solid supports. We use the latter to quantify the binding affinities of three blood clotting proteins for polyP, and to demonstrate that covalently immobilized polyP retains its ability to trigger blood clotting. Furthermore, we also show that derivatizing the ends of polyP via phosphoramidate linkages protects it from exopolyphosphatase degradation.

EXPERIMENTAL PROCEDURES

Materials

Amine Surface and Carbo-BIND (hydrazide) multiwell strips were from Corning (Corning, NY); Nunc Immobilizer Streptavidin multiwell strips and Covalink-NH plates were from Thermo-Fisher (Waltham, MA); polystyrene coagulometer cuvettes were from Diagnostica Stago (Parsippany, NJ); amine-PEG_2-biotin was from Pierce (Rockford, IL, USA); polyethylenimine, spermidine, streptavidin, benzamidine and EDAC were from Sigma-Aldrich (St. Louis, MO); Cascade Blue-ethylenediamine was from Invitrogen (Carlsbad, CA); factor XIa, kallikrein, and thrombin were from Enzyme Research Laboratories (South Bend, IN); calf intestinal alkaline phosphatase was from Promega (Madison, WI); phospholipids were from Avanti Polar Lipids (Alabaster, AL); Biacore CM5 sensorchips were from GE Healthcare (Piscataway, NJ); chromogenic substrates S-2366 and S-2322 were from diaPharma (West Chester, OH); recombinant factor VIIa, and substrates Spectrozyme TH and Spectrozyme fVIIa were from American Diagnostica (Stamford, CT); and Sepabeads EC-HA were kindly provided by Resindion SRL (Milan, Italy). $PolyP_5$, $polyP_{25}$ and $polyP_{45}$ (nominal mean polymer lengths, 5, 25 and 45, respectively, marketed as "sodium phosphate glass, types 5, 25 and 45"), and a heterodisperse preparation of very high MW polyP (marketed as "phosphate glass, water insoluble") were from Sigma-Aldrich, as were sodium monophosphate, pyrophosphate, and triphosphate. A water-soluble fraction of relatively high MW polyP (here termed $polyP_{HMW}$) was obtained from "water insoluble phosphate glass" by stirring it in 250 mM LiCl and processing as described (5). $PolyP_{14}$, $polyP_{60}$ and $polyP_{130}$ (polymer lengths, 14, 60 and 130, respectively) were kindly provided by Regenetiss, Inc. (Tokyo, Japan). PolyP concentrations are given throughout this paper in terms of phosphate monomer (monomer formula: $NaPO_3$).

Immobilization of polyP onto Polystyrene Microplate Wells and Coagulometer Cuvettes

A variety of reaction conditions were tested in order to optimize EDAC-mediated covalent coupling of $polyP_{HMW}$ to primary amines displayed on Amine Surface stripwells. Parameters varied included the concentrations of EDAC, polyP, divalent metal ions and 2-(N-morpholino)ethanesulfonic acid (MES); pH; coupling time; and the presence or absence of 0.1 M imidazole. Optimal coupling conditions for immobilizing polyP on Amine Surface stripwells were to treat each well at 37°C for 3 h to overnight with 200 μl of a freshly-made solution of 10 to 100 μM $polyP_{HMW}$ in 25 mM EDAC and 77 mM MES pH 6.5. Unreacted

polyP was then removed by two 10 min washes with 2 M LiCl followed by two 5 min water washes. When desired, immobilized polyP was quantified following hydrolysis in 1 M HCl at 100°C by malachite green assay. Briefly, 50 µL hydrolyzed phosphate sample was mixed with 100 µL malachite green reagent (0.1% malachite green, 4.2% ammonium molybdate, 4 M HCl) in Corning polypropylene multiwell plates and incubated for 20 min at room temperature, after which A_{660} was measured and phosphate concentrations determined by reference to a standard curve (5).

Optimal conditions for immobilizing $polyP_{HMW}$ onto polystyrene coagulometer cuvettes were to treat each well overnight at 37°C with 200 µl of 400 ng/mL polyethylenimine in 0.1 µM carbonate buffer pH 9.2, wash 5 times in purified water, then incubate each well for 4 h with 200 µl of a freshly made solution of 245 µM $polyP_{HMW}$ in 50 mM EDAC, 1 mM $CaCl_2$, and 77 mM MES pH 6.5. Wells were washed twice with 2 M LiCl, then twice with water.

Covalent Coupling of Biotin or Fluorophores to PolyP

For biotinylation of polyP, typical conditions were to incubate 10 mM $polyP_{HMW}$ overnight at 37°C with 0.5 mM amine-PEG_2-biotin, 100 mM EDAC, and 100 mM MES pH 6.5. For fluorescent labeling of polyP, typical reaction conditions were as for biotinylation except that 1 mM Cascade Blue-ethylenediamine replaced biotin and 1 mM $CaCl_2$ was added. Biotin-polyP and Cascade Blue-polyP adducts were purified by size-exclusion chromatography. PolyP and Cascade Blue-polyP preparations were resolved by polyacrylamide gel electrophoresis using 10% polyacrylamide gels in TBE (90 mM Tris, 90 mM borate, 2.7 mM EDTA, pH 8.3) and detected either by fluorescence (excitation at 365 nm) or by staining with toluidine blue as described (15).

Binding of Thrombin, Kallikrein, Factor XIa or Factor VIIa to Microplate-Immobilized PolyP

$PolyP_{HMW}$ was immobilized on Amine Surface stripwells using EDAC-mediated coupling as described above. Alternatively, biotin-$polyP_{HMW}$ was immobilized by incubating 67 µM biotin-$polyP_{HMW}$ overnight at 4°C in streptavidin stripwells. Following washing, wells were blocked for 3 h with 50 mM Tris-HCl pH 7.4, 0.05% Tween-20 (Tris-Tween) plus 5% bovine serum albumin. Wells were then incubated with various concentrations of factor XIa, kallikrein, thrombin or factor VIIa in Tris-Tween plus 0.6% bovine serum albumin, after which the wells were washed thrice with Tris-Tween. (In the case of factor VIIa, all solutions also contained 2.5 mM $CaCl_2$.) Bound factor XIa, kallikrein, thrombin or factor VIIa were detected by quantifying initial rates of hydrolysis of S-2366, S-2322, Spectrozyme TH or Spectrozyme fVIIa, respectively, and the single-site ligand binding equation was fitted to the data by nonlinear regression using Prism (GraphPad Software, La Jolla, CA).

Clotting Assays

Clotting times were quantified at 37°C on a Diagnostica Stago STart4 coagulometer by mixing, in coagulometer cuvettes, 50 µl prewarmed citrated human plasma (George King Biomedical, Overland Park, KS) with 50 µl prewarmed 20% phosphatidylserine/80% phosphatidylcholine vesicles (made by sonication) in imidazole buffer; incubating for 3 minutes; then initiating clotting by adding 50 µl prewarmed $CaCl_2$. Final concentrations were 33% plasma, 25 µM phospholipid, 41.7 mM imidazole pH 7.0, 8.33 mM $CaCl_2$ in 150 µl.

NMR Analyses

^{31}P NMR spectra of polyP preparations were acquired at 20°C as previously described (5), with a Varian Unity INOVA 600 spectrometer using a 5 mm Varian AutoTuneX ^{1}H/X PFG Z probe, 13.5 μs (90°) pulse excitation, 16 kHz spectral width, and 5 second recycle time. Chemical shifts were referenced to 0 ppm using an external phosphoric acid standard. Spectra were processed using 10 Hz line broadening.

Immobilization of PolyP onto Polymethacrylate Beads

PolyP was immobilized on primary amine-containing polymethacrylate beads (Sepabeads EC-HA) by gentle agitation of 100 mg (dry weight) of beads overnight at 37°C with 25 mM polyP$_{HMW}$ (or varying concentrations of other polyP polymer sizes) in 100 mM MES pH 6.5, 100 mM EDAC, and 1 mM CaCl$_2$, then washing with a solution of 2 M LiCl and 10 mM EDTA followed by water. Immobilized polyP was quantified by malachite green assay following hydrolysis in 1 M HCl at 100°C (5). The typical yield of bound polyP$_{HMW}$ was 11 μg polyP per mg dry weight of Sepabeads.

For binding assays, polyP$_{HMW}$-Sepabeads were blocked with 10% bovine serum albumin overnight at 4°C, washed twice with binding buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 0.1% bovine serum albumin), and incubated at room temperature for 30 min with thrombin, factor XIa, or kallikrein in binding buffer. The supernatants were collected by centrifugation using mini spin columns (Pierce), and beads were washed with binding buffer followed by elution buffer (50 mM Tris-HCl pH 7.5, 1 M NaCl, 0.1% bovine serum albumin). Enzymes were quantified by measuring initial rates of chromogenic substrate hydrolysis as described above.

Surface Plasmon Resonance (SPR) Studies

SPR analyses were conducted at 25°C using a Biacore 3000 instrument (Biacore, Columbia, MD). Streptavidin was covalently bound to CM5 sensorchips by the standard amine coupling method; after blocking and washing, biotin-polyP$_{HMW}$ was flowed over the surface until the signal reached 400 resonance units (RUs). Varying concentrations of thrombin in 50 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM benzamidine, 0.005% surfactant P20 were then flowed over the chip surface at 50 l/min using a 2 min association phase and 3 min dissociation phase, with background subtraction using a reference cell without polyP. Sensorchips were regenerated by washing with 1 M NaCl between runs.

Preparation and Digestion of Spermidine-PolyP Adducts

5 mM polyP$_{130}$ was incubated for 6 h at 37°C with 70 mM spermidine, 100 mM MES pH 6.5, 300 mM EDAC, after which polyP was purified by size-exclusion chromatography in the presence of 1 M LiCl followed by acetone precipitation as previously described (5). To examine resistance to exopolyphosphatase digestion, 12 μM spermidinepolyP adduct was digested at 37°C with 5 U/ml calf intestinal alkaline phosphatase in 50 mM Tris-HCl pH 7.4, 1 mM MgCl$_2$, 0.1 mM ZnCl$_2$. Timed samples were removed and free monophosphate was quantified by malachite green assay (5). At the end of the experiment, an aliquot of the reaction was hydrolyzed for 1 h at 100°C in 1 M HCl and monophosphate was quantified.

RESULTS

Covalent Immobilization of PolyP onto Amine-Derivatized Polystyrene Microplates and Chromatography Beads

To optimize the reaction conditions for EDAC-mediated formation of phosphoramidate linkages between primary amines and the terminal phosphates of polyP, we found it

convenient to employ Amine Surface microplates as the source of primary amine. The degree of immobilization of polyP onto this surface was then used as the readout for optimizing conditions. (We found that noncovalently bound polyP was quantitatively removed from these plates by washing the wells with 2 M LiCl.) Figure 1A shows the results of a typical optimization study, in this case to optimize the polyP concentration. We obtained substantial covalent attachment of polyP to the aminederivatized polystyrene surface when reactions were carried out in the presence EDAC (but not in its absence), with maximal coupling at ≥2 μg/ml polyP. At 1 and 2 μg/ml polyP, the efficiency of coupling to the surface was 49% and 27%, respectively. When polyP was reacted with EDAC in secondary amine-modified (Covalink NH) or hydrazide-modified (Carbo-Bind) microplates under the same conditions, little or no bound polyP was detected over background (data not shown), suggesting that this reaction is much more efficient with primary amines.

Additional studies were undertaken to optimize the reaction conditions for covalently linking polyP to primary amines on Amine Surface microplates (data not shown, but the findings summarized in this paragraph): Optimal polyP immobilization was obtained when the EDAC concentration was 25 to 300 mM, when the pH was 6 to 7 (using 25 to 100 mM MES buffer), and when the reaction was allowed to proceed for 2 h to overnight. We also found that inclusion of 1 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} increased the immobilization of polyP by about 1.5- to 2-fold relative to reactions in the absence of divalent metal ions.

EDAC-mediated formation of phosphoramidate linkages between primary amines and the 5' phosphates of oligonucleotides is reported to be more efficient in the presence of imidazole, due to the formation of reactive phosphorimidazolide intermediates (14). We found, however, that the efficiency of EDAC-mediated immobilization of polyP onto Amine Surface microplates was unaffected by the presence of up to 100 mM imidazole (not shown).

We also investigated the effect of polyP polymer length on efficiency of EDAC-mediated coupling to primary amines, using amine-containing polymethacrylate chromatography beads (Sepabeads EC-HA). To do this, we coupled polyP preparations of varying polymer lengths (holding the concentration of ends at a constant 1 mM) to the beads, then quantified the extent of covalent attachment of polyP. The results (Figure 1B) show that pentaphosphates and shorter coupled poorly to the beads, while 14mers and longer coupled relatively efficiently. Even at 50 mM, monophosphate still coupled inefficiently to the beads, demonstrating that the terminal phosphates of polyP are much more efficiently coupled to amines by EDAC than are small inorganic phosphates.

^{31}P NMR Spectroscopy of a PolyP-Spermidine Adduct

NMR was used to obtain evidence for phosphoramidate linkages with the terminal phosphates of polyP. Figure 2 shows representative ^{31}P NMR spectra of underivatized polyP$_{45}$ and of spermidine-labeled polyP$_{45}$. For underivatized polyP, the ^{31}P signal for the terminal phosphates at approximately -5 ppm (α peak in Figure 2A) was well resolved from the much larger peak for internal phosphates at about -21 ppm. (In this particular spectrum, the penultimate phosphate residues—β peak—were also clearly resolved, although this is not always the case). For spermidine-derivatized polyP (Figure 2B), the signal at -5 ppm was greatly reduced and a new peak at about -0.5 ppm appeared, which we attribute to the presence of the P-N bond in the phosphoramidate-linked spermidine-polyP adduct.

Binding Affinities of Blood Clotting Proteases for Immobilized PolyP

We previously demonstrated that thrombin binds to polyP with relatively high affinity, via its anion-binding exosite II (13). We also showed that polyP is a potent triggering agent for

the contact pathway of blood clotting (3), and that it binds to prekallikrein and factors XI and XII (4). As an example of the utility of immobilized polyP, we used it to quantify the binding of thrombin, factor XIa, kallikrein and factor VIIa to polyP. In Figure 3A-D, polyP was immobilized by EDAC-mediated covalent coupling to amine-derivatized polystyrene microplate wells. This was successfully used to quantify the binding affinities of thrombin, factor XIa and kallikrein for polyP, yielding K_d values of 66, 32 and 92 nM, respectively. Factor VIIa, on the other hand, did not bind to immobilized polyP (Figure 3D). Alternatively, biotinylated polyP was immobilized via capture on streptavidin-coated microplate wells, and this presentation of polyP was also used to quantify thrombin binding. It yielded a K_d value of 56 nM (Figure 3E), very similar to that obtained when polyP was covalently linked to amine-derivatized wells (Figure 3A).

In another experiment, thrombin, factor XIa, and kallikrein were incubated with polyP-derivatized, primary amine-containing chromatography beads, after which recovery of the enzyme was quantified in the flow-through and high-salt eluates (Figure 3F). These proteins bound quantitatively to polyP-derivatized beads and were eluted quantitatively by high salt concentration. There was negligible background binding to beads that had been treated with polyP in the absence of EDAC, or with EDAC in the absence of polyP (not shown). This demonstrates the utility of using polyP-derivatized beads to identify and isolate polyP binding proteins by pull-down assays, etc.

We also performed initial SPR analyses of thrombin binding to polyP by first immobilizing biotinpolyP$_{HMW}$ onto streptavidin sensorchips and then flowing varying concentrations of thrombin over the surface. The results (Figure 4) demonstrate the utility of immobilizing biotin-polyP onto streptavidinderivatized sensorchips in order to use SPR to study the kinetics of protein-polyP binding interactions.

Fluorescently Labeled PolyP

End-labeling of polyP with fluorophores would be highly advantageous for detecting polyP binding to proteins, cells and tissues, and for following polyP *in vivo*. Accordingly, we reacted the primary amine-containing fluorescent dye, Cascade Blue-ethylenediamine, with polyP$_{45}$ in the presence or absence of EDAC, purified the polyP and resolved it by polyacrylamide gel electrophoresis (Figure 5). PolyP that had been reacted with Cascade Blue-ethylenediamine in the presence of EDAC was intensely fluorescent (Figure 5B, lanes 1 and 2), whereas polyP incubated with the dye but without EDAC had no detectable fluorescence (Figure 5B, lane 3).

Derivatizing the Terminal Phosphates of PolyP Confers Resistance to Exopolyphosphatase Digestion

Some polyP preparations isolated from biological sources are reported to be naturally resistant to exopolyphosphatase degradation, apparently due to an unidentified modification of the terminal phosphates (1). This prompted us to investigate the possibility that attaching primary amine-containing compounds to the terminal phosphates of polyP via phosphoramidate linkages might protect polyP from exopolyphosphatase degradation. Accordingly, we reacted polyP$_{130}$ with spermidine in the presence of EDAC, isolated the polyP, and then over-digested it with excess calf intestinal alkaline phosphatase (a very active exopolyphosphatase (16)). As can be seen in Figure 6, the polyP-spermidine adduct was highly resistant to phosphatase degradation, while underivatized polyP was rapidly digested to completion.

Immobilized and Derivatized PolyP Retains Procoagulant Activity

We investigated whether immobilizing or end-labeling polyP would interfere with its procoagulant activity. EDAC was employed to covalently react long-chain polyP with polyethylenimine that had been coated onto polystyrene coagulometer cuvettes, after which the cuvettes were employed in plasma clotting assays. Immobilized polyP dramatically shortened the plasma clotting time, demonstrating that it retains significant ability to activate the contact pathway of blood clotting (Figure 7A). Similarly, in solution, 20 μM spermidine-labeled polyP was as active in triggering the clotting of human plasma as was 20 μM underivatized polyP (Figure 7B).

DISCUSSION

Studies of protein-polyP interactions have been hampered by a paucity of methods for derivatizing and immobilizing polyP. Here, we demonstrate that polyP preparations of varying chain lengths can be efficiently derivatized using the water-soluble carbodiimide, EDAC, to create phosphoramidate linkages between the terminal phosphates of polyP and several primary amines. We optimized the reaction conditions and provided NMR evidence for the presence of phosphoramidate linkages with the terminal phosphates of polyP. As examples of the utility of this approach, we quantified K_d values for the binding of polyP to the blood clotting proteases, thrombin, factor XIa and kallikrein. Relatively low nonspecific background was observed using primary amine-containing solid supports, making this a very attractive method for immobilizing polyP. We also demonstrated the utility of using biotinylated polyP in SPR studies to measure protein binding to polyP.

Carbodiimide-mediated crosslinking of polyP to labels, probes and solid supports should greatly facilitate studies on the ever-expanding role of polyP in important biological processes, including blood clotting. In addition to the examples provided in this study, the ability to covalently couple amine-containing compounds will also allow other types of labeling reactions with polyP, opening up essentially the entire armamentarium of protein chemistry. For example, polyP that has been end-labeled with a polyamine such as ethylenediamine, cadaverine or spermidine will have free primary amino groups available for further reactions, including coupling to succinimidyl ester derivatives of solid supports, biotin, fluorescent dyes or other probes, which are often more readily available commercially than are the same compounds with primary amines. Another example would be to couple a disulfide-containing primary amine such as cystamine to the ends of polyP; following reduction, this will provide free sulfhydryls tethered to the ends of polyP for reaction with maleimide- or iodoacetate-derivatives of biotin, fluorescent dyes or other labels.

We also found that modifying the ends of polyP by covalently attaching spermidine protected polyP from exopolyphosphatase degradation, suggesting that such end-labeled polyP derivatives may be more stable in biological systems. These end-labeled polyP adducts may also be useful in detecting the presence of endo- versus exopolyphosphatase enzyme activities, since the derivatized polyP preparations should be sensitive to digestion by the former but not the latter.

Previously, we demonstrated that soluble polyP can act as a general hemostatic agent, shortening the clotting time of plasma from patients with hemophilia and reversing the effect of several anticoagulant drugs (17). In this study, we found that covalently attaching amine-containing compounds to the terminal phosphates of polyP did not interfere with polyP's procoagulant activity, and polyP retained potent clotting activity when covalently attached to solid supports. This latter finding opens the possibility of covalently immobilizing polyP

onto wound dressings, collagen sponges, etc., to create improved topical hemostatic agents to control bleeding.

Acknowledgments

Abbreviations

EDAC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
MES	2-(N-morpholino)ethanesulfonic acid
polyP	polyphosphate
polyP$_{HMW}$	high molecular weight polyphosphate
SPR	surface plasmon resonance
RU	resonance units

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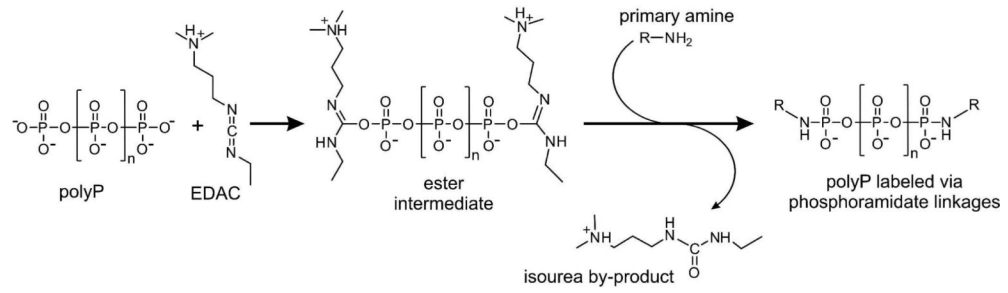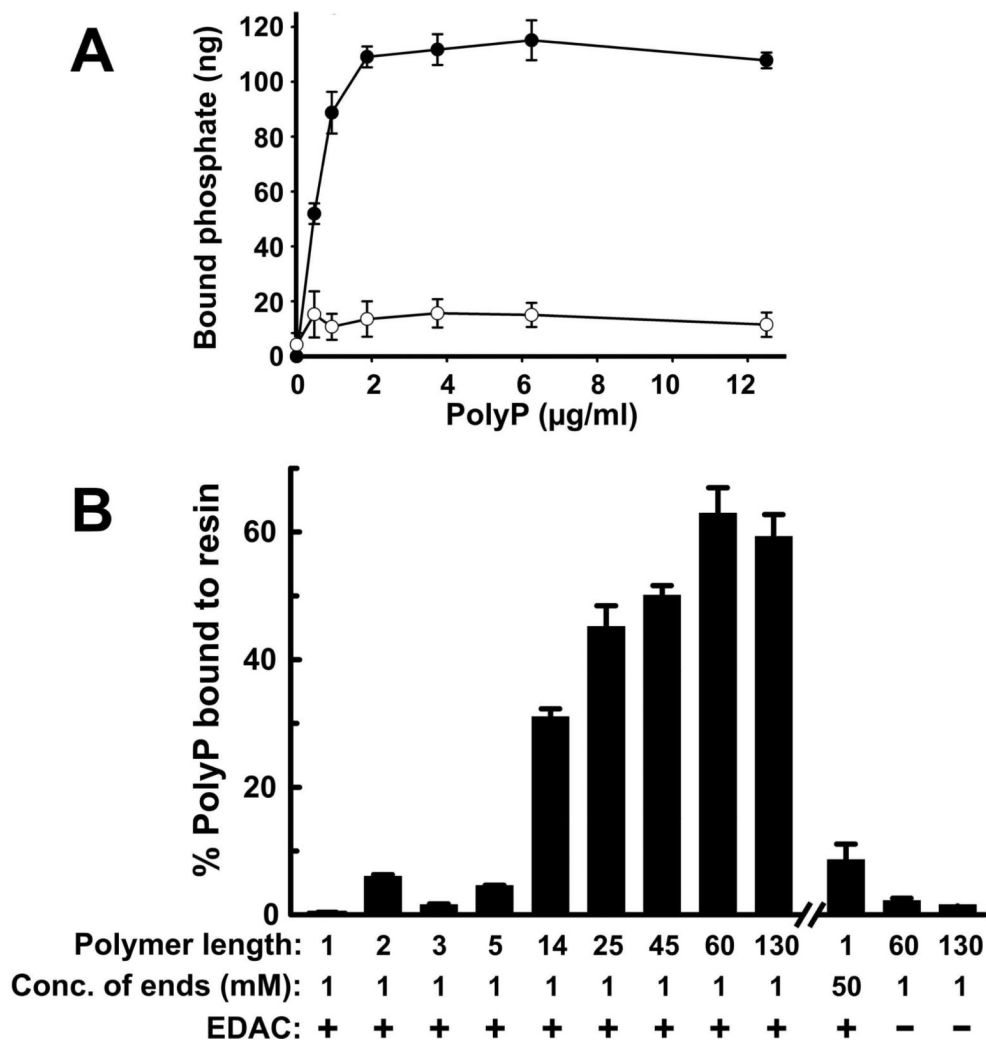
Scheme 1.

Figure 1.
Attachment of polyP to solid supports. (A) Dose response for covalently immobilizing polyP onto Amine Surface microplates. Varying $polyP_{HMW}$ concentrations were reacted overnight at 37°C in microplate wells in the absence (○) or presence (•) of 100 mM EDAC, after which unbound polyP was removed by washing with 2 M LiCl. Data are mean ± S.E.M. (n=3). (B) Influence of polymer length on coupling efficiency. PolyP preparations of varying mean polymer lengths were reacted overnight at 37°C with Sepabeads EC-HA and 100 mM EDAC with 1 mM $CaCl_2$, after which unbound polyP was removed by washing with 2 M LiCl and 10 mM EDTA. PolyP concentrations (given in terms of phosphate) were adjusted to yield a constant 1 mM ends: 1 mM monophosphate and pyrophosphate; 1.5 mM triphosphate; 2.5 mM $polyP_5$; 7 mM $polyP_{14}$; 12.5 mM $polyP_{25}$; 22.5 mM $polyP_{45}$; 30 mM $polyP_{60}$; and 65 mM $polyP_{130}$. Controls plotted on the right include 50 mM monophosphate as well as reactions without EDAC. Data are expressed as mean percent recoveries of offered polyP (± S.E.M.; n=3 to 5). In both panels, bound polyP was quantified as monophosphate following acid hydrolysis.
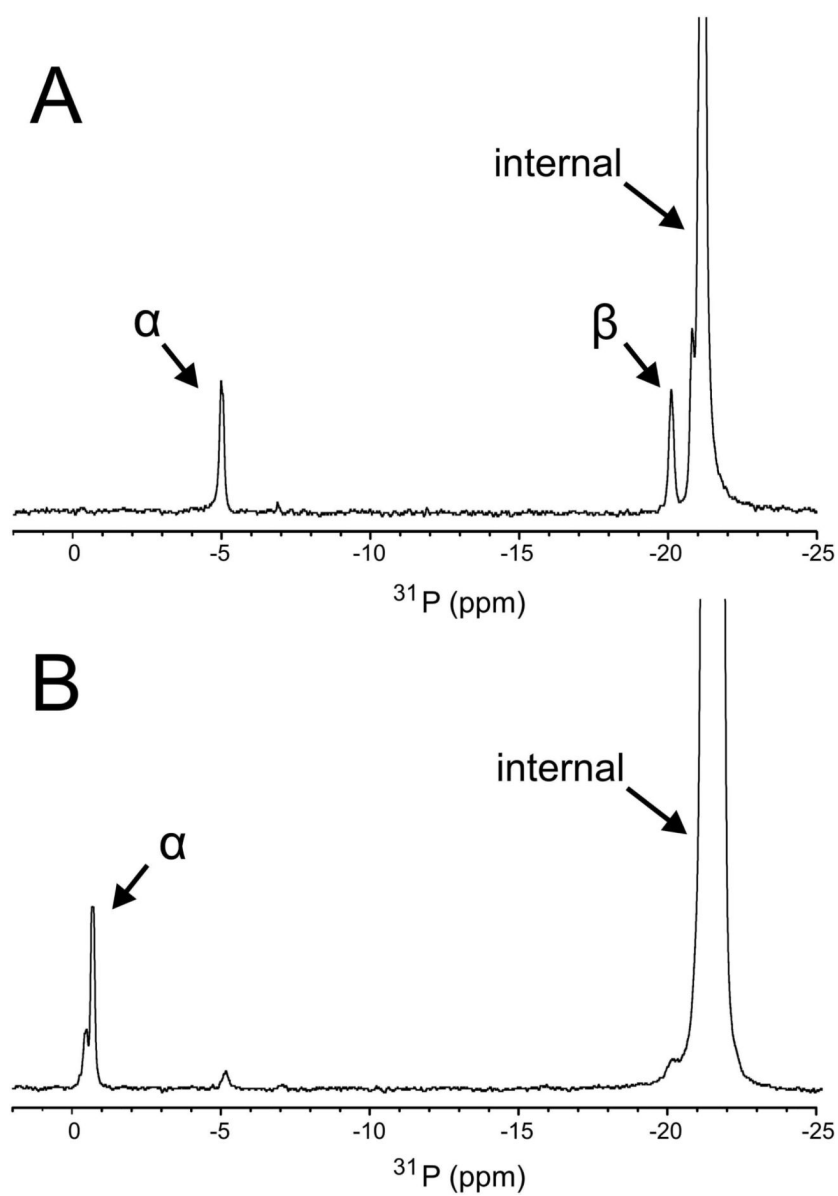
Figure 2.
One-dimensional ^{31}P NMR spectra of (A) underivatized $polyP_{45}$ and (B) spermidine-labeled $polyP_{45}$. Peaks corresponding to the external phosphates (α), penultimate phosphates (β), and internal phosphates (*internal*) are indicated.
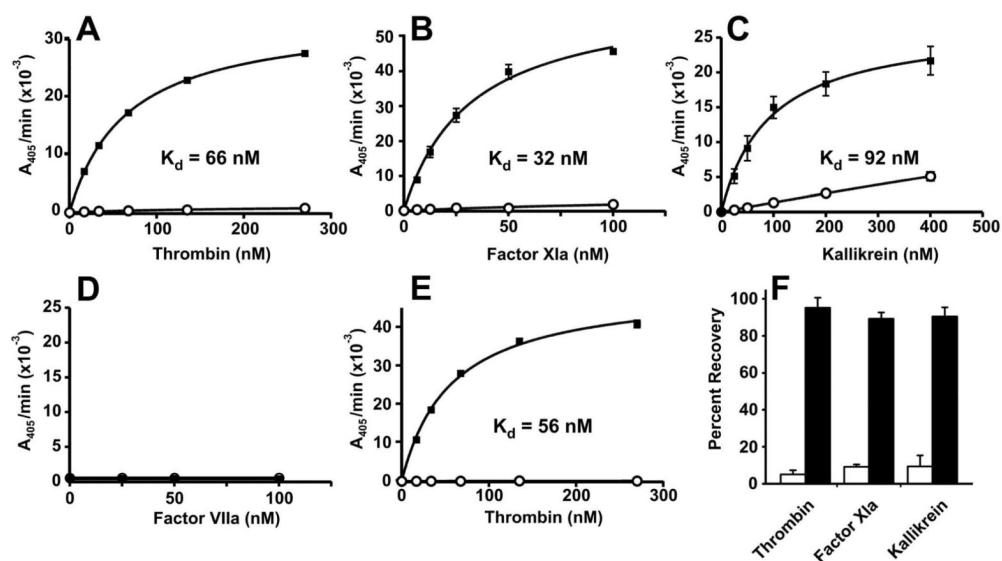
Figure 3.
Binding of clotting proteases to immobilized polyP. (A-C) PolyP$_{HMW}$ was coupled via EDAC to Amine Surface microplate wells, which were then used to quantify the binding of (A) thrombin, (B) factor XIa, (C) kallikrein, or (D) factor VIIa. (E) Biotinylated polyP$_{HMW}$ was immobilized on streptavidin-coated microplate wells, which were then used to quantify thrombin binding. For panels A-E, solid squares are binding data after background subtraction, while open circles are background binding (from wells without polyP); lines represent the single-site ligand binding equation fitted to the binding data, yielding the indicated K_d values. (F) PolyP$_{HMW}$ was coupled via EDAC to primary amine-containing Sepabeads. Thrombin (27 pmol), factor XIa (10 pmol), or kallikrein (10 pmol) were incubated with polyP-Sepabeads, after which the beads were collected and washed by centrifugation. Enzyme recovery was quantified in the flow-through (open bars) and high-salt eluates (closed bars), with recoveries calculated as percent of the starting material. Data are mean ± S.E.M. (*n*=3).
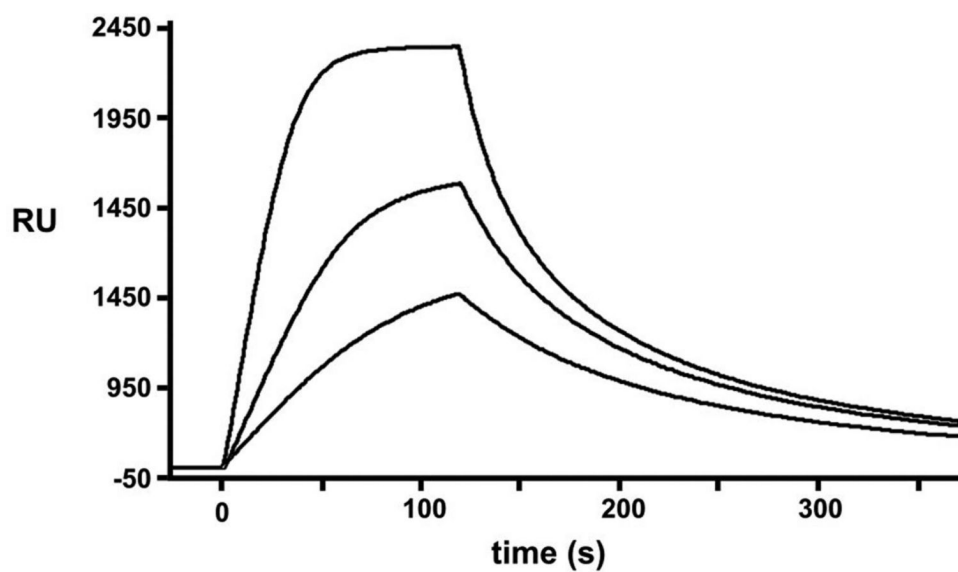
Figure 4.
Representative SPR sensorgrams of thrombin binding to polyP. Biotin-polyPHMW was immobilized onto a streptavidin-derivatized sensorchip, after which varying concentrations of thrombin were flowed over the chip surface and RU values were measured using a Biacore 3000 instrument. Thrombin concentrations were 20 nM (lower curve), 60 nM (middle curve), and 120 nM (upper curve).
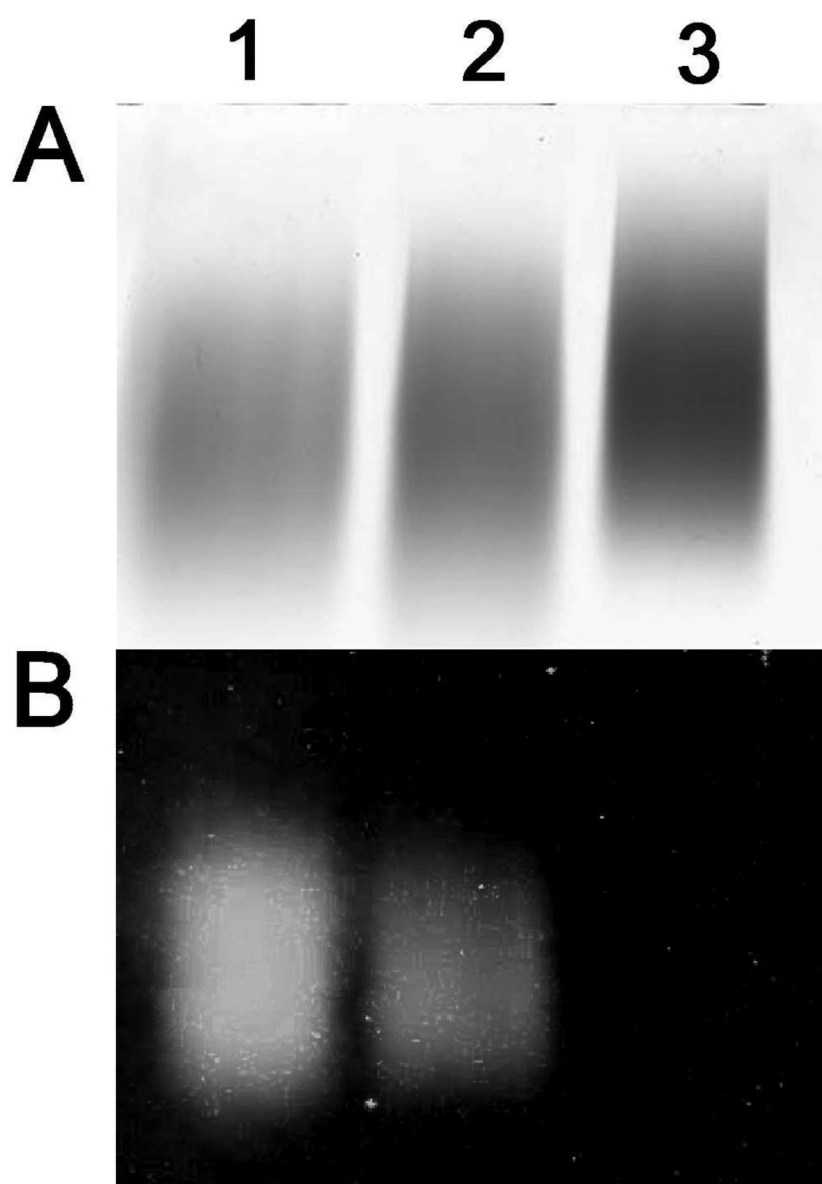
Figure 5.
Fluorescently labeled polyP. The primary amine-containing fluorophore, Cascade Blue ethylenediamine, was reacted with $polyP_{45}$ in the presence of EDAC. PolyP was then purified, resolved by polyacrylamide gel electrophoresis, and the gels were either (A) stained with toluidine blue, or (B) photographed under illumination by 365 nm UV light. Reaction conditions were: lane 1, 1 mM fluorophore + EDAC; lane 2, 0.5 mM fluorophore + EDAC; lane 3, 1 mM fluorophore without EDAC.
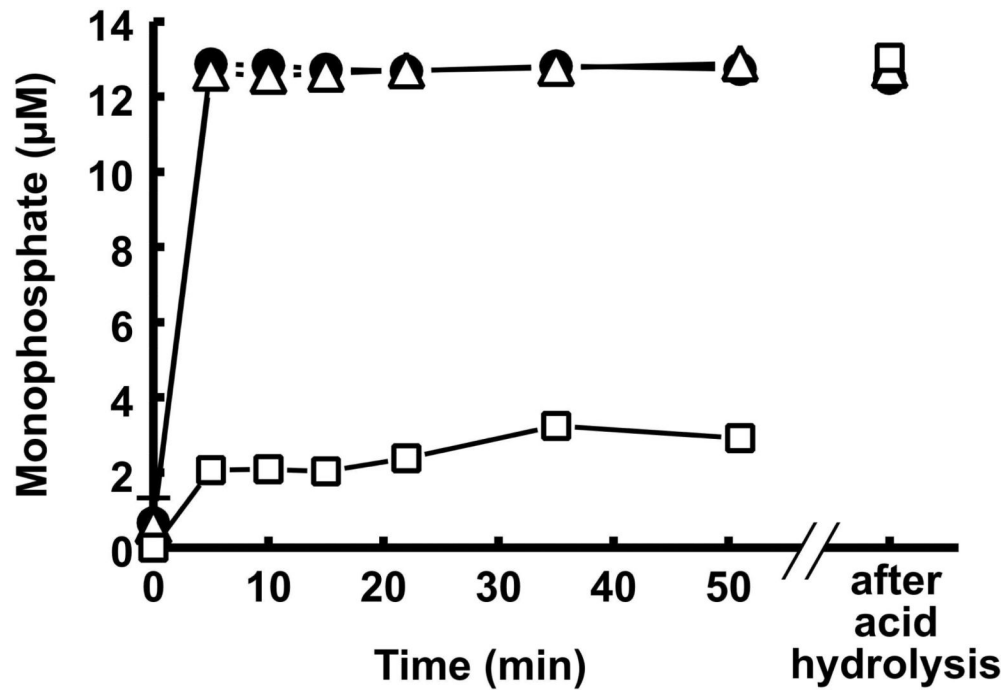
Figure 6.
Phosphoramidate derivatization of the terminal phosphates of polyP confers resistance to exopolyphopshatase digestion. PolyP$_{130}$ was either untreated (•) or reacted with spermidine in the presence (□) or absence (δ) of EDAC, after which the polyP was purified and then digested at 37°C with 5 U/ml alkaline phosphatase. Levels of free monophosphate were quantified in timed samples, and at the end of the experiment, monophosphate was quantified following complete acid hydrolysis. Data are mean ± S.E.M. (*n*=3), although the error bars are smaller than the data points and therefore not visible.
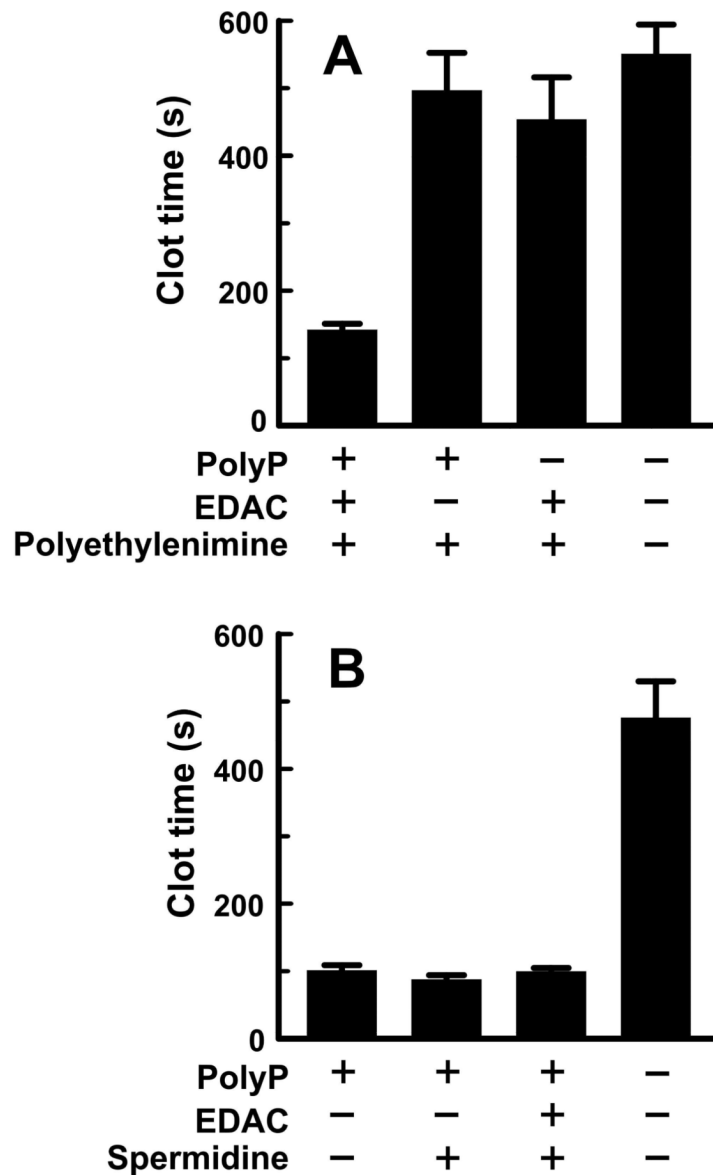
Figure 7.
Immobilized and derivatized polyP retains procoagulant activity. (A) PolyP$_{HMW}$ was immobilized via EDAC onto polyethylenimine-coated polystyrene coagulometer cuvettes. Clotting was then initiated by incubating human plasma in the wells for 3 min at 37°C, after which $CaCl_2$ was added and the time to clot formation recorded. Control cuvettes included those untreated with polyethylenimine, EDAC and/or polyP, as indicated. (B) Clotting assays were conducted as in panel A except that untreated cuvettes were employed and 20 μM polyP$_{HMW}$-spermidine in solution was preincubated with plasma for 3 min at 37°C, after which $CaCl_2$ was added and the time to clot formation recorded. Controls included polyP$_{HMW}$ that had been reacted without EDAC and/or spermidine, and also wells that received plasma but no polyP, as indicated. Data are mean ± S.E.M. (*n*=3).