(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 April 2012 (12.04.2012)

PCT

(10) International Publication Number
WO 2012/048271 A1

(51) International Patent Classification:
A01N 43/04 (2006.01) *A61K 31/70* (2006.01)

(21) International Application Number:
PCT/US2011/055447

(22) International Filing Date:
7 October 2011 (07.10.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/391,105 8 October 2010 (08.10.2010) US
61/405,655 22 October 2010 (22.10.2010) US

(71) Applicant *(for all designated States except US)*: **THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS** [US/US]; 352 Henry Admin Bldg., 506 S. Wright Street, Urbana, Illinois 61801 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **MORRISSEY, James H.** [US/US]; 2605 Willoughby Road, Champaign, Illinois 61822 (US). **CHOI, Sharon H.** [US/US]; 1807B Glenwood Oaks Court, Urbana, Illinois 61801 (US).

(74) Agents: **FERBER, Donna M.** et al.; Greenlee Sullivan, P.C., 4875 Pearl East Circle, Suite 200, Boulder, Colorado 80301 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2012/048271 A1

(54) Title: PHOSPHORAMIDATE DERIVATIZATION OF INORGANIC POLYPHOSPHATES AND METHODS

(57) Abstract: Provided are methods for making phosphoramidate-modified polyphosphate molecules using a reactant with a primary amine group and polyphosphate (optionally in the presence of divalent cations), in the presence of a zero-length linking group, where the modifying moiety is linked via the nitrogen of a primary amine group to a terminal phosphorus of a polyphosphate moiety. The phosphoramidate derivatized polyphosphate molecules are useful in methods to stimulate or accelerate coagulation in a human or animal subject where there is coagulation deficiency due to medication or genetic deficiency and/or where there is trauma, surgery or other event or risk of bleeding, and to inhibit tumor growth, survival or angiogenesis in a patient in need thereof.

Phosphoramidate Derivatization of Inorganic Polyphosphates and Methods

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of United States Provisional Application No.61/391,105, filed October 8, 2010 and of United States Provisional Application No. 61/405,655 filed October 17, 2010, both of which are incorporated by reference to the extent there is no inconsistency with the present disclosure.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under Grant R01 HL47014 from the National Heart, Lung and Blood Institute and Grant R01 GM75937 from the National Institute of General Medical Sciences. The government has certain rights in the invention.

BACKGROUND

[0003] 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 (positive) 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.

[0004] Inorganic polyphosphate (polyP, sometimes also abbreviated as $polyP_n$, in which the subscript n refers to the number of phosphate groups or the average number of phosphate groups), 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). 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; US 2010-029257). Many technical obstacles remain for investigating the biological roles of polyP and there is a need in the art 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 such as bandages or collagen sponges, for targeting polyP to a site of interest or for joining to a detectable moiety (label). One of the few published methods for immobilizing polyP onto surfaces is via Lewis acid/base interactions between polyP and zirconia beads (12). Although this method has been used (13), it suffers from the 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 of protein interactions. The goal of the present study was therefore to develop conditions for routine covalent attachment of labels or other materials to the terminal phosphates of polyP.

[0005] EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide; sometimes also abbreviated as EDC) has also been used to couple primary amines to organic phosphates, including the 5' phosphates of oligonucleotides, via stable phosphoramidate linkages (14), but it is believed not to have been employed in reactions with inorganic phosphates such as polyphosphates. Herein is disclosed that the terminal phosphates of (inorganic) polyP can be made to enter into covalent phosphoramidate linkages with primary amine-containing compounds via EDAC or other zero-length cross linking reagent (Scheme 1). This finding essentially opens up the entire armamentarium of protein chemistry to modifying polyP, greatly facilitating investigations into polyP's biological activities as well as therapeutic applications and usefulness as a reagent. Herein, we demonstrate conditions under which polyP can be biotinylated, labeled with fluorophores, and immobilized onto solid supports. The latter has been used to quantify the binding affinities of blood clotting proteins for polyP, and to demonstrate that covalently immobilized polyP retains its ability to trigger blood clotting. Furthermore, it is shown that derivatizing the ends of polyP via phosphoramidate linkages protects it from exopolyphosphatase degradation.

[0006] Hemorrhage is a major complication of both naturally occurring clotting factor deficiencies such as hemophilia, and anticoagulant therapy. Hemorrhagic episodes can result in significant patient morbidity and in rare cases, mortality. Even in patients with well-controlled stable anticoagulant therapy,

emergent circumstances may arise that necessitate immediate reversal of anticoagulant status. Hemorrhage is also associated with traumatic injury and surgery.

[0007] Rapid normalization of abnormal coagulation has generally relied on either replacement of missing factors or administration of specific antidotes. A major limitation of this approach is that transfusion with human-derived products has the potential for transmission of infectious disease. Furthermore, most anticoagulant drugs, including most newly approved anticoagulant drugs, as well as some in development, lack effective antidotes. In vitro studies of the effects of recombinant factor VIIa (rFVIIa) and off-label use in vivo have indicated that rFVIIa might have a role in a general method of reversing anticoagulant therapy. Use of rFVIIa may be associated with thromboembolic adverse events. Currently, the primary factors limiting use of rFVIIa as a universal procoagulant agent are the potential liability associated with off-label use, and the extreme expense associated with this drug.

[0008] There is a long-felt need in the art for effective methods for controlling bleeding in a patient by decreasing the time for clot formation, where the bleeding is due to genetic or acquired deficiency in a clotting factor, surgery, traumatic injury or clotting insufficiency due to anticoagulant therapy of toxicity from a compound with anticoagulant activity. There is also a need for improved methods for detection of clots in the body and for rapid and accurate determination of clotting time in the clinical setting.

[0009] There is also a long-felt need in the art for covalently attaching polyP to surfaces and for covalently modifying polyP with a label, targeting agent or other moiety of interest to facilitate assays of polyP action or antagonists or agonists of its activity.

SUMMARY OF THE INVENTION

[0010] Provided herein are methods for the derivatization of inorganic polyphosphate molecules by creating phosphoramidate linkages between one or both terminal phosphorus moieties and primary amines prior to reaction therewith. Such linkages allow for covalent attachment of polyP to a variety of chemical and

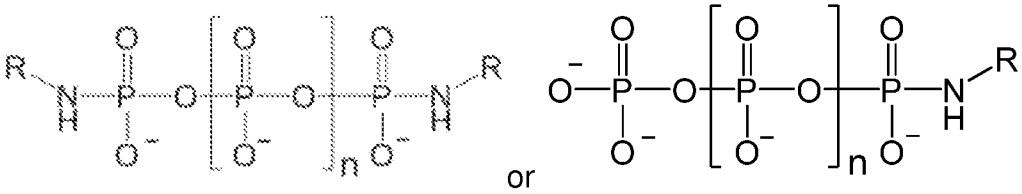
biological species as well as to solid supports including but not limited to bandages, chitosan materials, collagen sponges, gelatin scaffolds and assay devices. The present methods utilize a zero-length cross linking reagent (also termed a coupling agent in the art), for example, EDAC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide) to couple molecules of interest having primary amino groups (generally represented as R-NH_2, primary amines, in Scheme 1) to polyP molecules, especially those of n>25, or other size range of interest according to the desired usage, in a buffered solution of about pH 6-7.5, advantageously about 6-7, or about 6.5, and optionally in the presence of divalent cations. Other zero-length cross-linking reagents that can be used in place of EDAC to make phosphoramidate derivatized polyphosphate molecules include carbodiimide coupling reagents, including among others, N-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate (sometimes abbreviated as CMC); dicyclohexyl carbodiimide (sometimes abbreviated as DCC); diisopropyl carbodiimide (sometimes abbreviated as DIC), Bis(trimethylsilyl)carbodiimide, N,N'-bis(2-methylphenyl)carbodiimide, N-(tert-butyl)-N'-(2,6-dichlorophenyl)carbodiimide, N-(butyl)-N'-[1-(2-chlorphenyl)-1-methylethyl]carbodiimide, N-tert-butyl-N′-methylcarbodiimide, N-cyclohexyl-N′-isopropylcarbodiimide, N-tert-butyl-N′-ethylcarbodiimide, bis[[4-(2,2-dimethyl-1,3-dioxolyl)]methyl]carbodiimide, and N,N-dicyclopentylcarbodiimide.

[0011] In general any primary amine can be linked at a $PolyP_n$ terminus by the methods herein. The methods herein can be used as illustrated herein to link one or two primary amines which are the same or different to a $PolyP_n$ terminus. Specific primary amines that can be linked to $PolyP_n$ include among others, cystamine, glycine ethyl ester, ethylenediamine, protamine, spermine, spermidine, cadaverine, putrescine, polyamines in general, dendrimers, amino sugars such as glucosamine and amino sugar polymers such as chitosan, fluorescent dyes carrying at least one primary amine function, such as ethylenediamine derivatized dyes, cadaverine derivatized dyes, labels, probes or reporter molecules carrying at least one primary amine function, such as ethylenediamine derivatized labels or cadaverine derivatized labels, solids or surfaces carrying at least one primary amine functionality, polymers carrying at least one primary amine functionality, lipids or phospholipids with at least one primary amine group including but not limited to

phosphatidylethanolamine and phosphatidylserine, p-nitroaniline, antibodies, amino acids, peptides or proteins.

[0012] More specifically, the invention provides $PolyP_n$ labeled with one or two fluorescent dyes or other labels, such a biotin. $PolyP_n$ carrying one label can also be immobilized on or linked to a surface or solid. More specifically, primary amines useful for labeling of $PolyP_n$ include, among others, biotin, amine-PEG-functionalized biotin, ethylenediamine, cadaverine, amine-PEG-biotin reagents (e.g., EZ-link, Pierce), dansyl ethylenediamine, fluorescein ethylenediamine, Cascade Blue® ethylenediamine, Lissamine™ rhodamine B ethylenediamine, BODIPYL® FL EDA, Fluorescein cadaverine, Lucifer Yellow Cadaverine, Texas Red™ cadaverine, Oregon Green® 488 cadaverine, and various Alexa Fluor® cadaverine dyes including Alexa 647-cadaverine (Molecular Probes).

[0013] In an embodiment, the invention provides derivatized polyphosphates of formulas:



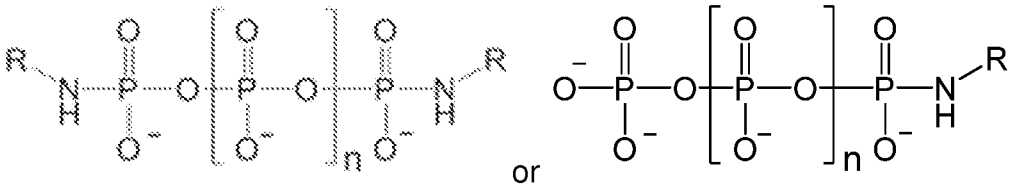
or

or salts or esters thereof, where n is an integer representing the number of repeating internal phosphate units or the average number of repeating internal phosphate units in a given polyP compound and R is a moiety carrying at least one amine group, particularly a primary amine group ($-NH_2$). In specific embodiments, R is a label, reporter, fluorescent dye, ethylenediamine, a polyamine such as spermidine or cadaverine or spermidine or a polyamine coupled to a moiety of interest such as a fluorophores or chromophore, a phospholipid with a primary amine group such as phosphatidylethanolamine or phosphatidylserine, biotin, glycine ethyl ester, an amino sugar or an amino sugar polymer such as glucosamine or chitosan, a surface or solid or combinations thereof. R may also include a linker to the functional species of interest, e.g., -Linker-dye, -Linker-biotin, or –Linker-solid where the linker is any of various linkers used in labeling or immobilization to surfaces, e.g., a $-(CH_2)_t-$ linker, an ether linker, an oligopeptide linker, a polyethylene glycol (PEG)-based linker or

the like. In specific embodiments, the linker is formally derived from an alkylene linker $–(CH_2)_t-$, by replacing one or more, preferably one, $–CH_2-$ groups with –O-, -NR_{10}-, -S-, -S-S-, -CO-, -NR_{10}-CO-, or -CO-NR_{10}- or by replacing one or more $–CH_2-CH_2-$ groups with
–CH=CH–.

[0014] Also provided are methods for reducing bleeding in a patient in need thereof, for example, due to trauma, surgery, anticoagulant therapy or deficiency in clotting factors. PolyP (see Schemes 1 and 2) covalently attached directly or via a linker moiety to a solid material or support such as a collagen, bandage, or chitosan material can be placed at the site of a bleed, and there is the advantage that cross-linked polyP cannot become readily dislodged from that material or support to cause coagulation at an undesired site in the body. In other embodiments, the phosphoramidate derivatized polyP can be administered via a pharmaceutical composition for topical application, together with a pharmaceutically acceptable salt, cation(s) and excipients, carriers and the like, appropriate for the intended site of use and patient, as readily understood in the art.

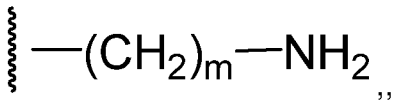
[0015] Alternatively, polyP derivatized with terminal spermidine, protamine, spermine or glucosamine (or other nontoxic primary amine-containing moiety prior to linkage to polyP) via a phosphoramidate linkage can be administered into circulation to promote clotting in a patient in need thereof. Optionally, the derivatized polyP is formulated into a pharmaceutical (therapeutic) composition with at least one pharmaceutically acceptable salt, for example, a cationic molecule or atom, or ester. The phosphoramidate-linked moiety provides at least some resistance to exopolyphosphatase degradation of the polyP portion of the molecule. For example:



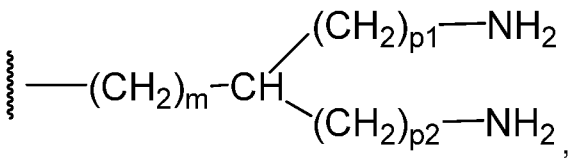
or salts or esters thereof, where n is an integer representing the number of repeating internal phosphate units or the average number of repeating internal phosphate units in a given polyP compound, where n is typically about 10 to about 5000, about 25 to about 5000, about 60 to about 100, at least about 250, about 300 to about 1000 or

greater, where n can be the average number of repeat units where the polyphosphate is a mixture having an average n, and

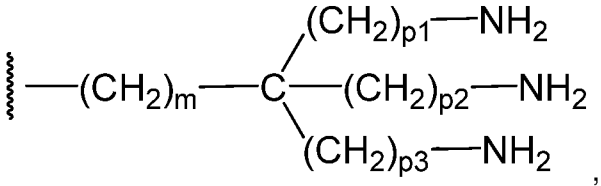
R is a moiety containing a primary amine including linear, branched, or cyclic moieties, for example



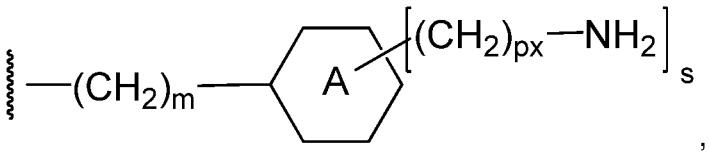
,,



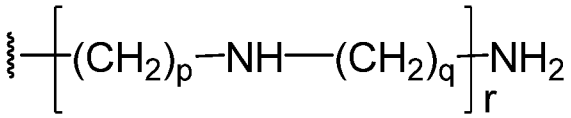
,



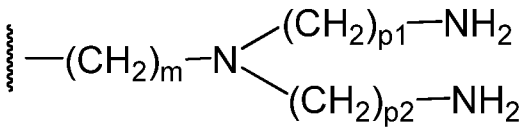
,



,



, or



m is an integer ranging from 1 to 12, and wherein R can be linear or branched chain or cyclic alkyl amino, aliphatic amino, or aromatic, alkene, alkyne, or a dendrimer or a complex combination of moieties with at least one primary amine group; p, q and r are integers ranging from 1-12 or 1-6, p1, p2, p3 and px are integers ranging from 1-12 or 1-6, the A ring is a carbocyclic, heterocyclic, aromatic or heteroaromatic ring having 5-8 ring atoms, and s is an integer ranging from 1 to the maximum number of positions on the ring for substitution with a bond to a carbon. Note that if the A ring is a phenyl ring, s can range from 1-5. With respect to the exemplified –R moieties,

one or more –CH_2- groups in the moieties can optionally be replaced with an –O-, -S-, -S-S-, -CO-, -$CONR_{10}$-,-NR_{10}-CO-, or -CO_2-, where R_{10} is hydrogen or an alkyl group having 1-6 carbon atoms. R can also be a polymer with at least one pendant primary amine group.

[0016] Preferred m are 1-3, preferred p and q are 1-4, preferred r is 1-3, preferred p1, p2, p3 and px are 1-4. Preferred n are 25 -1000. Preferred A rings are cyclohexyl and phenyl rings. Preferred s when A rings are cyclohexyl or phenyl are 1-3.

[0017] Advantageously, the administered polyP can be linked to an amino group or other functional group of a targeting molecule such as an antibody, ligand or receptor protein or peptide targeted to a tumor via a zero-length cross linking reagent reaction as described herein, or a targeting molecule of interest can be linked to a functional group of the R moiety after the phosphoramidate derivatized polyP is created using methods well known in the art and according to the particular targeting molecule of interest and the particular R group derivatized to the polyP. Examples of targeting proteins and peptides include tumor specific antibodies including but not limited to CEA-specific antibody or binding portion thereof for a colon cancer tumor; the NGR-peptide (Gly-Asn-Gly-Arg-Ala-His-Ala) for targeting new blood vessels to inhibit angiogenesis in solid tumors (19), and various cancer-specific antibodies are known including but not limited to bevacizumab (for colon, rectal, certain lung and certain breast cancer tumors, targets vascular endothelial growth factor action); rituximab (CD20-specific, for certain lymphomas), cetuximab (colon, head and neck cancers, targets epidermal growth factor action), including those for breast cancer (HER2/neu-specific), ovarian cancer, melanoma, and all cancers (See, e.g., US Patent 7,053,188; US Published Applications US-2009-0042209; US-2008-030552, among other readily available patent and other publications. Disruption of tumor blood supply can be part of an anti-tumor therapy strategy. These targeting proteins, peptides or polypeptides which bind to a tumor antigen or other antigens of interest are attached via a phosphoramidate linkage or linkages to a polyP molecule as described herein for other moieties with primary amine groups. In other embodiments therapeutic radionuclides or other therapeutic agents can be attached to a targeting moiety or to via a primary amine to polyP.

[0018] Also provided herein are labeled derivatized polyP which are useful in methods for imaging in the body, due to the affinity of polyP for clotting proteins and fibrin clots. These methods allow determination of clot sites, especially those where clots are undesirable. For example, a radioactive material such as ^{35}S, ^{32}P, ^{14}C, ^{125}I, or other detectable material such as gadolinium can be incorporated in an amine-containing moiety (H_2NR) which is covalently bound to polyP via a reaction mediated by a zero-length cross linking reagent as described herein. Conventional imaging technology is used after administration, for example, intravenous administration, to determine clot location(s).

[0019] Phosphoramidate-derivatized polyP molecules are improved over unmodified polyP molecules with respect to exopolyphosphatase degradation, thus they can exhibit increased persistence in the body, in circulation or immobilized at a site of interest due to the protection from exopolyphosphatase degradation. Accordingly, the derivatized polyP can be incorporated into therapeutic compositions to decrease bleeding (by shortening clotting time) in a patient in need thereof or at risk of deleterious bleeding.

[0020] In an embodiment, the phosphoramidate derivatized polyP can be employed in materials and methods for removal of molecules which bind specifically or nonspecifically to it, where such molecules can be metal cations, including, but not limited to toxic metal cations such as lead or cadmium or chromium, a protein, a peptide, or a small molecule of less than about 1000 d. Advantageously, the polyP is covalently bound via phosphoramidate linkage using the methods described herein to an amine functionalized solid support, for example, in the form of a cartridge for flow-through of liquid or a microtiter or other plate or receptacle or a tube.

[0021] In a further embodiment, polyP covalently bound via phosphoramidate bonds to an amine functionalized tube or other device can inhibit certain enzymatic activity, for example, nuclease activity, by chelating divalent cations including magnesium and manganese from a solution therein.

[0022] In another embodiment, polyP immobilized via phosphoramidate linkage to an amine-functionalized surface (including but not limited to a Biacore

sensor chip, microarray chip, centrifuge tube, microwell plate, chromatography resin, optical tweezer-trapped polystyrene bead, flow chamber plate or coverslip, coagulometer cuvette or other material, especially for use at the point of care, can be employed in assays of coagulation, especially in an aPTT (activated partial thromboplastin time)-type assay for the contact pathway for clotting, example, in plasma or blood of a patient, especially a patient at risk for bleeding or suffering from trauma, undergoing surgery and the like. Comparison of clotting/coagulation time for the patient sample in the presence of polyP as compared to the clotting/coagulation times of normal plasmas allows the diagnosis or quantification of a deficiency in the patient's contact pathway proteins for clotting when the time is slower than for the normal reference. The immobilized polyP serves to activate the contact pathway for coagulation/clotting, and it has the advantage of being a defined component of the reaction. The results of an aPTT assay will show a longer time for clotting/coagulation where the patient is deficient in factors IX or XI, for example, and the medical practitioner can adjust procedures and therapy accordingly.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] Fig. 1 illustrates attachment of polyP to solid supports. (Panel; A) Dose response for covalently immobilizing polyP onto Amine Surface microplates. Varying polyPHMW concentrations were reacted overnight at 37ºC in microplate wells in the absence (○) or presence (●) of 100 mM EDAC, after which unbound polyP was removed by washing with 2 M LiCl. The amount of polyP bound to the plate is given as ng phosphate (quantified after acid hydrolysis). Data are mean + S.E.M. (n=3). (Panel B) Influence of polymer length on coupling efficiency. PolyP preparations of varying mean polymer lengths were reacted overnight at 37ºC 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 polyP5; 7 mM polyP14; 12.5 mM polyP25; 22.5 mM polyP45; 30 mM polyP60; and 65 mM polyP130. 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.

[0024] Fig. 2 shows one-dimensional ^{31}P NMR spectra of (Panel A) underivatized polyP45 and (Panel B) spermidine-labeled polyP45. Peaks corresponding to the external phosphates (α), penultimate phosphates (β), and internal phosphates (internal) are indicated.

[0025] Fig. 3 shows the binding of clotting proteases to immobilized polyP. (Panels A-C) PolyPHMW was coupled via EDAC to Amine Surface microplate wells, which were then used to quantify the binding of (Panel A) thrombin, (Panel B) factor XIa, (Panel C) kallikrein, or (Panel D) factor VIIa. (Panel E) Biotinylated polyPHMW 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. (Panel F) PolyPHMW 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).

[0026] Fig. 4 provides representative surface plasmon resonance (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).

[0027] Fig. 5 shows results of experiments with fluorescently labeled polyP. The primary amine-containing fluorophore, Cascade Blue ethylenediamine, was reacted with polyP45 in the presence of EDAC. PolyP was then purified, resolved by polyacrylamide gel electrophoresis, and the gels were either (Panel A) stained with

toluidine blue, or (Panel B) photographed under illumination by 365 nm UV light. Reaction conditions were: lane 1, 1 mM fluorophore + EDAC + polyP; lane 2, 0.5 mM fluorophore + EDAC + polyP; lane 3, 1 mM fluorophore + polyP but without EDAC.

[0028] Fig. 6 shows that phosphoramidate derivatization of the terminal phosphates of polyP confers resistance to exopolyphosphatase digestion. PolyP130 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 37ºC 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 of the remaining polyP. Data are mean + S.E.M. (n=3), although the error bars are smaller than the data points and therefore not visible.

[0029] Fig. 7 demonstrates that immobilized and derivatized polyP retains procoagulant activity. (Panel A) PolyPHMW was immobilized via EDAC onto polyethylenimine-coated polystyrene coagulometer cuvettes. Clotting was then initiated by incubating human plasma in the wells for 3 minutes at 37ºC, 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. (Panel B) Clotting assays were conducted as in panel A except that untreated cuvettes were employed and 20 μM polyPHMW-spermidine in solution was preincubated with plasma for 3 minutes at 37ºC, after which $CaCl_2$ was added and the time to clot formation recorded. Controls included polyPHMW 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).

[0030] Fig. 8 graphically illustrates the effects of various reaction parameters on EDAC-mediated immobilization of polyP to amine-derivative microplates. After removal of unreacted materials, thrombin binding to the wells was measured as amidolytic activity (indicated on the y-axis as “mOD/min”, which represents the rate of hydrolysis of a chromogenic thrombin substrate). Without wishing to be bound by any particular theory, it is believed that the decrease in phosphoramidate linked polyP at the higher pH in the presence of calcium cations is due to decreased solubility of the polyP.

[0031] Fig. 9 shows thrombin binding to biotinylated polyP bound to streptavidin-coated microplates. Long-chain polyP was reacted with amine-PEG-biotin in the presence of EDAC, purified using size exclusion chromatography, and bound to streptavidin-coated plates (Nunc). The wells were washed to remove unreacted materials and thrombin binding was measured as in Fig. 8 (biotinylated polyP, closed circles; unbiotinylated polyP, open circles).

[0032] Fig. 10 shows the effect of pH on the covalent binding of PolyP (mediated by EDAC) to amine-coated microtiter plate wells. Buffer concentrations are 0.1M. This demonstrates that the coupling reaction works over the pH range of 5.5-7.5, with the buffer appearing to affect the results to some extent.

[0033] Fig. 11 shows the effect of pH, metal ions and salts on the covalent binding of polyP to amine-coated microtiter plate wells. This graph indicates that the coupling reaction is roughly equivalent in either MES or imidazole buffer at pH 7.0 and that the presence of 1 mM CaCl2 slightly enhances the coupling reaction. Furthermore, the data shows that the presence of 1M LiCl interferes with the polyP derivatization reaction.

[0034] Fig. 12 shows that α-thrombin, β-thrombin, FXI, and FXIa bind with high affinity to immobilized polyP. Binding of α-thrombin, β -thrombin, FXI, or active-site-inhibited FXIa to polyP was quantified using surface plasmon resonance, with biotinylated polyP bound to streptavidin sensorchips, over which varying protein concentrations were flowed. Panels are representative sensorgrams for: (A) 2.5 to 20 nM α -thrombin; (B) 5 to 80 nM β-thrombin (C) 1.25 to 40 nM FXI; and (D) 2.5 to 20 nM active-site-inhibited FXIa. K_d values were derived as described herein below.

DETAILED DESCRIPTION

[0035] Abbreviations: EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; MES, 2-(N-morpholino)ethanesulfonic acid; polyP, polyphosphate; polyPHMW, high molecular weight polyphosphate, SPR, surface plasmon resonance; RU, resonance units; MW, molecular weight; 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, AEBSF.

[0036] A coagulation deficient patient is a patient whose plasma has a PT Test value or Dilute PT Test value which is at least 1.5 times greater than that of pooled normal plasma. Pooled normal plasma is plasma prepared by mixing equal amounts of citrate-anticoagulated plasma from at least 20 normal individuals. Coagulation deficiency can result from genetic deficiency, acquired deficiency, disease, or treatment with anticoagulants, for example.

[0037] Anticoagulants in the present context include, without limitation, heparin, low MW heparin, rivaroxaban, argatroban, Coumadin and warfarin. PolyP reverses the anticoagulant effects of heparin, low MW heparin, rivaroxaban and argatroban when administered in effective doses, and it reduces the anticoagulant effects of Coumadin and warfarin. Phosphoramidate-derivatized polyP acts in a similar fashion to polyP, but with at least some increased persistence in the patient due to the protection from exopolyphosphatase degradation.

[0038] Phosphoramidate-derivatized polyP, as used herein, is a phosphate polymer of greater than 5 phosphate units, advantageously 25-100, 45-100, 60-100, 100-1000, greater than about 250, or greater, 300-1000 or 300-5000, or greater or of a length or length range specified. Phosphoramidate linkage to a primary amine-containing moiety can be at one or both ends of a phosphate polymer. The primary amine compound is coupled using the methods described herein, and the compound can be an amine-functionalized solid support such as a functionalized microtiter plate or other assay device, a detectable compound such as a chromophore or fluorophores with primary amino groups prior to reaction, a biotin derivative containing a free primary amine functional group, an amino acid including but not limited to a naturally occurring amino acid or a synthetic amino acid, peptide or oligopeptide, protein, including but not limited to an antibody, receptor protein or specific binding protein or a fragment of a protein retaining a specific binding property of the entire protein, an amino sugar such as glucosamine, an amino sugar polymer such as chitosan, or a detectable peptide or detectable protein, or a compound with a functional group (such as a sulfhydryl, carboxyl or amino group) that remains after phosphoramidate linkage to the polymer, thus enabling further covalent modifications using methodology well known to the art.

[0039] PolyP$_n$ means a compound of the formula as shown in Scheme 1, where the value of n is equal to the number of PO_3 units in the molecule and n is at least 3. Polyphosphate (polyP) is a generic term for polyP$_n$, including mixtures, where n of each polyP$_n$ is at least 3. Also included are salts, esters, and anhydrides of polyphosphate, as well as cyclic polyphosphates. Concentrations of polyphosphate and any polyP$_n$ may be expressed as "phosphate equivalents", which means the concentration of PO_3 moieties (for example, 1 μM of polyP$_{75}$ polymer is the same as 75 μM phosphate equivalents of polyP$_{75}$). All amounts and concentrations of polyP and polyP$_n$ are expressed herein as phosphate equivalents. The polyP contains at least 3 PO_3 moieties. Preferably, polyP$_n$ wherein n is at least 25 may be used, for example, n=25-1000, more preferably, n=25-100 (including 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43 and 44 and so on), more preferably n is at least 45, including 45-100. (including 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100). Other size ranges are also set forth herein, and the size or size class is chosen according to the relevant biological activity.

[0040] Polyphosphate derivatives of this invention are those derivatized at one or both terminal ends with a primary amine, schematically RNH-PolyP$_n$-NHR or RNH-PolyP$_n$, where the primary amine-containing reactant is RNH_2. PolyP$_n$ can be a linear polymer or contain portions that are cyclic. Most generally, each R of the primary amine can be the same or different. In some embodiments, both R are the same. In other embodiments, the R groups at the two termini are different.

[0041] The size range of polyP is important in the biological activity exhibited. For example, factor V activation and/or abrogation of TFPI (tissue factor pathway inhibitor) is effected by shorter polyP molecules (about 30 to about 400, about 35 to about 300, or about 50 to about 200, or about 60 to about 100 P units), enhancement of fibrin polymerization and structure is effected by intermediate to long polyP (150 to about 500, or about 250 or greater P units), and factor XI activation is best with about 50 or greater units, especially about 150 or greater, while the contact pathway for coagulation is best activated by polyP of about 300 or greater, about 400 or greater, or advantageously about 500 or greater phosphate units. For reference, platelet polyP is typically in the range of about 60 to about 100

phosphate units per molecule. Note that bacterial polyP molecules are in the hundreds to thousands of phosphate units, and unicellular eukaryotes may have polyP in the hundreds of phosphate units long.

[0042] Because polyP shortens time to clot formation in plasma containing anticoagulants, and polyP normalizes clot dynamics in whole blood clotting as measured by thromboelastometry, a derivatized polyP as disclosed herein, which is significantly less susceptible to exopolyphosphatase degradation than underivatized polyP, is useful for administration to patients at risk for or experiencing hemorrhage as a result of imbalance in desirable clotting ability due to anticoagulant therapy, accidental administration or toxicity from an anticoagulant. The derivatized, immobilized or targeted polyP can have an extended persistence in circulation as compared to the natural form due to the protection from exopolyphosphatase degradation. Furthermore, derivatized polyP also shortens clotting times in plasma from individuals with factor deficiencies due to vitamin K antagonist therapy, and in plasma from individuals with naturally occurring hemophilia A or B. PolyP is additive with factor replacement or rFVIIa in normalizing clot time in hemophilia plasma.

[0043] Preferably, phosphoramidate-derivatized polyP or a pharmaceutically acceptable ester or salt thereof, as well known to the art, is administered intravenously as a solution including a pharmaceutically acceptable carrier, such as saline. Preferably, polyP is administered as an injection, for example intravenously, intraperitoneally, subcutaneously or intramuscularly. Administration over a longer period of time may be accomplished by implanting a controlled release device, by injection of the polyP in a controlled or extended release pharmaceutically acceptable carrier, or transdermally, for example with a transdermal patch. Preferably, the polyP and solutions of polyP are sterile. Advantageously, administration is by saline bolus or by continuous infusion.

[0044] Preferably, administration of phosphoramidate-derivatized polyP for reversing the effects of an anticoagulant, is carried out over a period of time sufficient for the body to clear the anticoagulant. For example, low MW heparins typically have a half-life in a patient of 4-13 hours, so administration of derivatized polyP for reversing the anticoagulant effects of low MW heparins should preferably be carried out for at least 4 hours. Rivaroxaban typically has a half-life in a patient of

7-10 hours, so administration of polyP for reversing the anticoagulant effect of rivaroxaban should preferably be carried out for at least 7 hours. PolyP or phosphoramidate-derivatized polyP also abrogates the anticoagulant activity of TFPI (tissue factor pathway inhibitor).

[0045] The amount of phosphoramidate-derivatized polyP administered depends on the extent of coagulation deficiency, and typically is 0.1 to 100 mg per kg of body weight, such as 0.5 to 10 mg per kg of body, including 1, 2, 3, 4, 5, 6, 7, 8 and 9 mg per kg of body weight. Preferably, the total dose is diluted into 1 to 100 ml of a pharmaceutically acceptable carrier, such as saline. For extended administration, the polyP may be added to a bag of sterile saline (or other excipient suitable for intravenous administration), such as a 1 liter bag, at a concentration sufficient to maintain the same concentration in the blood of the patient as would result from the single injection of 0.1 to 100 mg per kg of body weight. The polyP may also be provided as a unit dosage, for example as a sterile solution pre-measured in a sealed container, such as a saline solution in a syringe, with sufficient polyP for a single administration to one patient. Another example of a unit dosage would be a vial with a rubber seal containing sterile dry polyP; a syringe may be used to add saline to the vial to dissolve the polyP, which may then be drawn into the syringe for administration of the single dose. Preferably, the amount of polyP administered is sufficient to reduce the PT Test value or Dilute PT Test value of the plasma of the patient to less than 1.5, more preferably 1.4 or less, most preferably 1.2 or less, such as 1 to 1.2, times the PT Test value or Dilute PT Test value of pooled normal plasma. In the case of chronic administration, preferably the PT Test value or Dilute PT Test value of the plasma of the patient is maintained to less than 1.5, more preferably 1.4 or less, most preferably 1.2 or less, such as 1 to 1.2, times the PT Test value or Dilute PT Test value of pooled normal plasma.

[0046] Prothrombin Time (PT) Test clotting assays, for determining the INR value of plasma of a patient, may be performed using an ST4 coagulometer (Diagnostica Stago, Parsippany, NJ). A 50 µL plasma sample is incubated in a cuvette for 2 minutes at 37°C., after which clotting is initiated by adding 100 µL pre-warmed (37°C.) thromboplastin reagent, and the time to clot formation is measured. PT Tests are typically performed in duplicate for each sample.

[0047] Using ^{31}P NMR, it was confirmed that EDAC-mediated reactions between primary amines and polyP results in phosphoramidate linkages with the terminal phosphate groups. PolyP can be biotinylated, labeled with fluorophores and immobilized onto solid supports; that immobilized polyP can be readily used to quantify protein binding affinities; that covalently derivatized or immobilized polyP retains its ability to trigger blood clotting; and that derivatizing the ends of polyP with spermidine protects it from exopolyphosphatase degradation. Thus, essentially the entire armamentarium of protein chemistry is now available for modifying polyP, which should greatly facilitate studies of its biological roles and enable assays, therapeutic compositions and reagents not previously possible. The zero-length cross-linking reagent, EDAC (1-ethyl-3-[3-dimethylamino-propyl]carbodiimide), is widely used to couple primary amines to carboxylic acids via amide linkages. It is soluble in aqueous solutions and promotes the reaction of amines/hydrazides with carboxylic acids and also mediates coupling of amines to organic phosphates via stable phosphoramidate linkages (for example, for covalently attaching labels or other moieties to the 5' ends of oligonucleotides). EDAC (1-ethyl-3-[3-dimethylamino-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. As noted herein, other zero-length cross linking reagents can be used instead of EDAC.

[0048] However, inorganic orthophosphate has little reactivity with EDAC, and in fact, the supplier's instructions for using EDAC to label the 5' phosphate groups of oligonucleotides with amines actually recommends the use of phosphate buffers for these reactions, provided the phosphate concentration is 10 mM or less. Because polyP is inorganic phosphate, it would have not been expected to react well with EDAC. However, reaction conditions are described herein which allow the use of EDAC to mediate the formation of stable phosphoramidate linkages between the terminal phosphates of polyP and the nitrogen of primary amine-containing compounds (see Scheme 1). This strategy allows the introduction of new reactive groups onto the ends of polyP, ands further labeling or coupling reactions (See Schemes 1 and 2). Two examples (out of many) include introducing free amines to the ends of polyP by reacting it with a large excess of ethylenediamine in the presence of EDAC (Scheme 2C) or free sulfhydryls to the ends of polyP by reacting

it with a large excess of cystamine in the presence of EDAC, followed by treatment of the purified cystamine-derivatized polyP with a strong reducing agent such as dithiothreitol or tris[2-carboxyethyl]phosphine to reduce the internal disulfide to free sulfhydryls (Scheme 2D). Once such reactive groups as free primary amines or sulfhydryls are present on the terminal phosphates of polyP, then there are myriad possibilities for further covalent reactions from protein chemistry. The ability to attach moieties such as biotin, fluorophores, peptides, proteins, and solid supports including beads and flow chamber surfaces to the terminal phosphates of polyP and the ability to couple polyP covalently to solid supports allows for dramatic improvements in methods for studying polyP interactions with physiological molecules, identifying antagonists or agonists, inhibitors or competitors, and in methods for activating the clotting system in order to control unwanted bleeding or to decrease the risk thereof.

[0049] EDAC-mediated reactions have been used to covalently immobilize polyP to amine-derivatized microtiter plates (Corning™ 96 well Stripwell™ plates, amine-coated chromatography resin and also to immobilize polyP onto microtiter plates that had been previously coated with polylysine or polyethyleneimine. It was found that under the conditions used, the binding of long chain polyP to amine-functionalized plates was strictly dependent on EDAC, ruling out the possibility that polyP is merely interacting noncovalently with the plate, for example through ionic interactions between the anionic phosphate polymer and the positively charged surface of the plate. (Any such noncovalently bound polyP was effectively removed by washing with high concentrations of LiCl or other salt solutions.) As an example of the utility of this approach, these wells were used to quantify thrombin binding to immobilized polyP (Fig. 3).

[0050] Various reaction conditions for forming EDAC-mediated phosphoramidate linkages have been examined. To date, the optimal conditions for EDAC-mediated reactions with amines are different for polyP than for oligonucleotides. First, EDAC-mediated coupling of amines to the 5' phosphates of oligonucleotides have been reported to be enhanced when imidazole is included in the reaction (via formation of a reactive phosphorimidazolide intermediate), but it was found in the present work that imidazole was without effect on the efficiency of

EDAC-mediated coupling of amines to polyP; EDAC was absolutely required. Fig. 8 shows that the maximum EDAC-mediated polyP immobilization on amine plates is reached when the reactions took place in 100 mM MES at a pH between 6.5 and 7 in the presence of divalent cations such as 1 mM calcium or magnesium.

[0051] The terminal phosphates of polyP were also derivatized with amine-PEG_2-biotin (Pierce, Rockford, IL). Long-chain polyP was reacted with amine-PEG_2-biotin in the presence of EDAC, purified using size exclusion chromatography, and bound to streptavidin-coated plates (Nunc). Immobilized polyP was indirectly detected by incubating the treated wells with thrombin, washing away unbound thrombin and then measuring the enzymatic activity of the bound thrombin. The results (Fig. 9) show that underivatized polyP and unbound thrombin were completely washed from the wells, whereas thrombin remained bound to the biotinylated polyP by virtue of the tight binding interactions between the biotin moieties on the polyP-biotin adducts and the streptavidin attached to the wells.

[0052] Labeling polyP with fluorophores, for example, permits detection of derivatized polyP molecules in a variety of settings. Accordingly, the primary amine-containing fluorescent dye Cascade blue ethylenediamine (Invitrogen, Carlsbad, CA) was reacted with polyP45 (mean polymer length 45, Sigma Chemical Corporation, St. Louis, MO) in the presence and absence of EDAC. After reaction, polyP was purified using size exclusion chromatography and then resolved by electrophoresis on a 10% polyacrylamide gel. Part of the gel was stained with toluidine blue (a general stain for polyP) and the rest of the gel was photographed with illumination at 365 nm (Fig. 5). The dye which was covalently bound to polyP was intensely fluorescent but polyP from the reaction mixture without EDAC was not fluorescent.

[0053] Some polyP preparations from biological sources are naturally resistant to exopolyphosphatase degradation, believed to be so due to an as-yet unidentified terminal modification. Although the distribution of endo- and/or exopolyphosphatases in human blood is not known, the goal of extending polyP plasma half-life by terminal modification was pursued. Thus, $polyP_{45}$ was reacted with glucosamine in the presence or absence of EDAC and the reaction product was purified. Then the preparations were digested with calf intestinal alkaline phosphatase (a very active exopolyphosphatase). A substantial fraction of the

product of the glucosamine/polyP/EDAC reaction was resistant to alkaline phosphatase digestion.

[0054] The glucosamine-derivatized polyP was used in ^{31}P NMR studies to obtain direct evidence for the phosphoramidate linkages with the terminal phosphate residues of polyP and to estimate the extent of end-labeling. The characteristic ^{31}P chemical shift of the terminal phosphates (easily resolved from the ^{31}P signal of the internal phosphates) should be altered by the presence of the P-N bond. Therefore, glucosamine-derivatized polyP was prepared and purified and ^{31}P-NMR spectra calibrated against phosphoric acid were collected. As expected, there was a large signal for internal phosphates of polyP at about -21 ppm, and a signal for the terminal phosphates (α) at about -9 ppm. In this sample, there was a novel peak at -5.9 ppm that was tentatively identified as the 31P signal from the phosphorous atoms in phosphoramidate linkages with glucosamine, a chemical shift that is consistent with literature reports for similar phosphoramidates. It is noted that the peak at -5.9 ppm, is stronger than the peak at -9 ppm, consistent with labeling the majority of the terminal phosphates of polyP with glucosamine. NMR spectra of polyP not reacted with glucosamine or EDAC exhibited a peak of terminal phosphates around -9 ppm but there was no peak at -5.9 ppm. Similar results for spermidine-derivatized polyP are shown in Fig. 2.

[0055] **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, it was 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. (Noncovalently bound polyP was quantitatively removed from these plates by washing the wells with 2 M LiCl.) Fig. 1A shows the results of a typical optimization study, in this case optimization of the polyP concentration. Substantial covalent attachment of polyP to the amine-derivatized polystyrene surface resulted when reactions were carried out in the presence of 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% of the offered polyP, 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), indicating that this reaction is much more efficient with primary amines.

[0056] Additional studies were undertaken to optimize the reaction conditions for covalently linking polyP to primary amines on Amine Surface microplates. 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 hours to overnight or about 15-20 hrs. Inclusion of 1 mM Ca^{2+}, Mg^{2+}, or Mn^{2+} in the reaction mixture increased the immobilization of polyP by about 1.5- to 2-fold relative to reactions in the absence of divalent metal ions.

[0057] EDAC-mediated formation of phosphoramidate linkages between primary amines and the 5' phosphates of oligonucleotides (i.e., organic phosphates) is more efficient in the presence of imidazole, due to the formation of reactive phosphorimidazolide intermediates (14). However, in the present context, the efficiency of EDAC-mediated immobilization of inorganic polyP onto Amine Surface microplates was unaffected by the presence of up to 100 mM imidazole.

[0058] The effect of polyP polymer length on efficiency of EDAC-mediated coupling to primary amines was investigated, using amine-containing polymethacrylate chromatography beads (Sepabeads EC-HA). PolyP preparations of varying polymer lengths (holding the concentration of ends at a constant 1 mM) were covalently linked to the beads, and then the extent of covalent attachment of polyP was quantified. The results (Fig. 1B) show that pentaphosphates and shorter molecules coupled poorly to the beads, while 14mers and longer coupled relatively efficiently. Even at 50 mM, monophosphate still coupled very 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.

[0059] Reaction conditions for EDAC-mediated immobilization of polyP to amine-derivatized microplates were studied. Long-chain polyP was reacted with amine-derivatized microplates with EDAC in 0.1 M MES buffer at various pH values

with or without 1 mM $CaCl_2$. After incubation at 37 °C overnight, the wells were washed with 2 M LiCl to remove unreacted reagents. Thrombin binding to the immobilized polyP was quantified by measuring amidolytic activity, which served as an indirect measure of the amount of polyP immobilized on the microplates. See Fig. 8.

[0060] **^{31}P NMR Spectroscopy of a PolyP-Spermidine Adduct**. NMR was used to obtain evidence for phosphoramidate linkages with the terminal phosphates of polyP. Fig. 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 Fig. 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 (Fig. 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.

[0061] **Binding Affinities of Blood Clotting Proteases for Immobilized PolyP**. It was previously demonstrated that thrombin binds to polyP with relatively high affinity, via its anion-binding exosite II (13). PolyP was shown to be 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, it was used to quantify the binding of thrombin, factor XIa, kallikrein and factor VIIa to polyP. In Fig. 3A-D, polyP was immobilized by EDAC-mediated covalent coupling to amine-derivatized polystyrene microplate wells. This immobilized polyP 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 (Fig. 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 (Fig. 3E), very similar to that obtained when polyP was covalently linked to amine-derivatized wells (Fig. 3A). Thrombin binding, for

example, was dependent on the presence of EDAC in the polyP derivatization or immobilization reactions.

[0062] 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 (Fig. 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. This demonstrates the utility of using polyP-derivatized beads to identify and isolate polyP binding proteins by pull-down assays, etc.

[0063] Initial SPR analyses of thrombin binding to polyP were also performed by first immobilizing biotin-polyPHMW onto streptavidin sensorchips and then flowing varying concentrations of thrombin over the surface. The results (Fig. 4) demonstrate the utility of immobilizing biotin-polyP onto streptavidin-derivatized sensorchips in SPR methods for studying the kinetics of protein-polyP binding interactions Fig. 12 shows the results obtained using biotinylated polyP bound via streptavidin. Binding interactions between polyP and thrombin, FXI and FXIa utilizing SPR in which biotinylated polyP was immobilized on sensorchips and the proteins were flowed over the surface. FXI, FXIa, α-thrombin and β-thrombin all bound tightly to immobilized polyP (Figure 12), yielding the following association (k_{ass}) and dissociation (k_{diss}) rate constants and K_d values: α-thrombin, $k_{ass} = 5.12 \times 10^6\ M^{-1}\ s^{-1}$, $k_{diss} = 7.71 \times 10^{-2}\ s^{-1}$, $K_d = 15.1$ nM; β-thrombin, $k_{ass} = 3.85 \times 10^6\ M^{-1}\ s^{-1}$, $k_{diss} = 3.12 \times 10^{-2}\ s^{-1}$, $K_d = 8.1$ nM; FXI, $k_{ass} = 1.64 \times 10^6\ M^{-1}\ s^{-1}$, $k_{diss} = 1.05 \times 10^{-2}\ s^{-1}$, $K_d = 6.4$ nM; and FXIa, $k_{ass} = 1.92 \times 10^6\ M^{-1}\ s^{-1}$, $k_{diss} = 2.91 \times 10^{-3}\ s^{-1}$, $K_d = 1.5$ nM.

[0064] **Fluorescently Labeled PolyP**. End-labeling of polyP with fluorophores is highly advantageous for detecting polyP binding to proteins, cells and tissues, and for following polyP in vivo. Accordingly, the primary amine-containing fluorescent dye, Cascade Blue-ethylenediamine, was reacted with $polyP_{45}$ in the presence or absence of EDAC, and the derivatized polyP was purified and resolved by polyacrylamide gel electrophoresis (Fig. 5). PolyP that had been reacted with Cascade Blue-ethylenediamine in the presence of EDAC was intensely fluorescent

(Fig. 5B, lanes 1 and 2), whereas polyP incubated with the dye but without EDAC had no detectable fluorescence (Fig. 5B, lane 3).

[0065] 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 led to an investigation of whether attaching primary amine-containing compounds to the terminal phosphates of polyP via phosphoramidate linkages protected polyP from exopolyphosphatase degradation. Accordingly, polyP130 was reacted with spermidine in the presence of EDAC, the polyP isolated, and then over-digested with excess calf intestinal alkaline phosphatase (a very active exopolyphosphatase (16)). Fig. 6 shows that the polyP-spermidine adduct was highly resistant to phosphatase degradation, while the underivatized polyP was rapidly digested to completion.

[0066] Immobilized and Derivatized PolyP Retains Procoagulant Activity. The effect of immobilizing or end-labeling polyP on its procoagulant activity was studied. 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 (Fig. 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 (Fig. 7B).

[0067] Previously, studies of protein-polyP interactions were hampered by a paucity of methods for derivatizing and immobilizing polyP. Herein it is demonstrated 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. The reaction conditions were optimized and NMR evidence for the presence of phosphoramidate linkages with the terminal phosphates of polyp is provided. As examples of the utility of this approach, K_d values for the binding of polyP to the blood clotting proteases, thrombin, factor XIa and kallikrein were quantified.

Relatively low nonspecific background was observed using primary amine-containing solid supports, making this a very attractive method for immobilizing polyP. The utility of using biotinylated polyP in SPR studies to measure protein binding to polyP was also demonstrated.

[0068] Carbodiimide-mediated crosslinking of polyP to labels, probes and solid supports greatly facilitates 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 also allows 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 large excess of polyamine such as ethylenediamine, putrescine, spermine, protamine, 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 is coupling of a disulfide-containing primary amine such as cystamine to the ends of polyP; following reduction, this provides free sulfhydryls covalently tethered to the ends of polyP for subsequent reaction with maleimide- or iodoacetate-derivatives of biotin, fluorescent dyes or other labels.

[0069] Modifying the ends of polyP by covalently attaching spermidine protected polyP from exopolyphosphatase degradation, suggesting that such end-labeled polyP derivatives are more stable in biological systems. These end-labeled polyP adducts are also useful in detecting the presence of endo- versus exopolyphosphatase enzyme activities, since the derivatized polyP preparations are sensitive to digestion by the former but not the latter.

[0070] Previously, soluble polyP was shown to act as a general hemostatic agent, shortening the clotting time of plasma from patients with hemophilia and other deficiencies in the clotting system and reversing the effect of several anticoagulant drugs (17). Phosphoramidate-derivatized polyP preparations can be administered either therapeutically during a bleeding episode or it can be administered prophylactically before surgery or other activities or procedures where a coagulation deficient or other patient would be at risk of deleterious bleeding. Phosphoramidate

linkage to a nontoxic material such as glucosamine or a polyamine is useful; such materials may have a longer half-life in circulation (or localized site) than do unmodified polyP. In addition, the choice of length of the phosphoramidate-derivatized polyP depends on the therapeutic effect needed. For example, stimulation of clotting and decrease of clotting time via enhancement of factor V activation or abrogation of TFPI is best achieved with shorter polyP derivatives, activation of the contact pathway is best with larger molecules, and strengthening of fibrin clots is advantageously achieved with intermediate phosphoramidate-modified polyP, for example with modified polyP, as discussed above. See, for example Choi et al. (2010) and US 2010/0297257, US 2010/0284998.

[0071] Phosphoramidate-derivatized polyP or a pharmaceutically acceptable salt or ester thereof can be administered intravenously, or intramuscularly, intraperitoneally, subcutaneously, as a solution with a pharmaceutically acceptable carrier such as sterile saline. Alternatively, for example, where extended delivery is desired, it can be provided to a patient via an implanted controlled release device or locally covalently attached to an applied or implanted solid material such as chitosan, a collagen sponge, gelatin scaffold, bandage or other material with at least one primary amine group.

[0072] The dose of phosphoramidate-derivatized polyP or a pharmaceutically acceptable salt or ester thereof administered depends on the extent of coagulation deficiency as well as the nature of the bleeding event, surgery or other traumatic event. Typical ranges include 0.1 to 100 mg per kg body weight, 0.5 to 10 mg per kg body weight, and interval values including and between 1 and 10 mg per kg body weight. Advantageously, the dose can be added to sterile saline, for example to a 1 liter bag, at ta concentration sufficient to achieve the desired dosage with the administration of the appropriate volume. Standard clotting testing methods are used to assess the patient and to monitor the patient after administration has begun. In addition, the nature and extent of coagulation deficiency affects the choice of polymer length of the derivatized polyP to be used.

[0073] Herein, it is shown 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, bandages, gelatin scaffolds, chitosan supports, collagen sponges, etc., to create improved topical hemostatic agents to control bleeding.

[0074] The invention may be further understood by the following non-limiting examples.

[0075] **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); polyethylenimine, spermidine, streptavidin, benzamidine, 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 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); purified β-thrombin, FXI, and FXIa were obtained from Hematologic Technologies (Burlington, VT), purified α-thrombin was available 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). PolyP5, polyP25 and polyP45 (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 polyPHMW) was obtained from “water insoluble phosphate glass” by stirring it in 250 mM LiCl and processing as described (5). PolyP14, polyP60 and polyP130 (polymer lengths, 14, 60 and 130, respectively) were

obtained from Regenetiss, Inc. (Tokyo, Japan). PolyP concentrations are given throughout this paper in terms of phosphate monomer (monomer formula: $NaPO_3$).

[0076] 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 polyPHMW 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 hours to overnight with 200 µl of a freshly-made solution of 10 to 100 µM polyPHMW in 25 mM EDAC and 77 mM MES pH 6.5. Unreacted polyP was then removed by two 10 minute washes with 2 M LiCl followed by two 5 minute 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 minutes at room temperature, after which A_{660} was measured and phosphate concentrations determined by reference to a standard curve (5).

[0077] Optimal conditions for immobilizing polyPHMW onto polystyrene coagulometer cuvettes were to treat each well overnight at 37°C with 200 µl of 400 ng/mL polyethylenimine in 0.1 M carbonate buffer pH 9.2, wash 5 times in purified water, then incubate each well for 4 hours with 200 µl of a freshly made solution of 245 µM polyPHMW 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.

[0078] Covalent Coupling of Biotin or Fluorophores to PolyP. For biotinylation of polyP, typical conditions were to incubate 10 mM polyPHMW 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).

[0079] Binding of Thrombin, Kallikrein, Factor XIa or Factor VIIa to Microplate-Immobilized PolyP. PolyPHMW was immobilized on Amine Surface stripwells using EDAC-mediated coupling as described above. Alternatively, biotin-polyPHMW was immobilized by incubating 67 µM biotin-polyPHMW overnight at 4°C in streptavidin stripwells. Following washing with 2M LiCl to remove unreacted materials, wells were blocked for 3 hours 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). Thrombin binding was measured as enzymatic activity.

[0080] Clotting Assays. Clotting times were quantified at 37°C on a Diagnostica Stago STart4 coagulometer by mixing, in coagulometer cuvettes, 50 µl prewarmed citrated pooled 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.

[0081] 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 1H/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.

[0082] **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 polyPHMW (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 polyPHMW was 11 µg polyP per mg dry weight of Sepabeads.

[0083] For binding assays, polyPHMW-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 minutes 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.

[0084] **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-polyPHMW was flowed over the surface until the signal reached 400 resonance units (RUs). Varying concentrations of α-thrombin, β-thrombin, Factor XI or Factor XIa in 50 mM Tris-HCl pH 7.4, 50 mM NaCl, and 0.005% surfactant P20 were flowed over the chip surfaces at 50 µl/min using a 2 minute association phase and 3 (Fig. 4) or 5 (Fig. 12) minute dissociation phase, with background subtraction using a streptavidin-coated reference cell without polyP. The running buffer for α-thrombin or β-thrombin contained 5 mM Benzamidine, while FXI and FXIa were pretreated with 4 mM AEBSF for 30 minutes prior to use to block the active sites of FXIa. Sensorchips were regenerated by washing with 1 M NaCl between runs. Binding kinetics were analyzed according to the 1:1 Langmuir binding model. K_d values were calculated from the quotient of the derived dissociation (k_{diss}) and associate (k_{ass}) constants.

[0085] SPR resonance studies as described above can be adapted to the identification of compounds which inhibit or compete with the binding of α- or β-thrombin or other clotting or other protein or other molecule which interacts (especially specifically) with polyP by substituting the protein or molecule interest for the thrombin, and comparing interactions in the presence and absence of a potential inhibitor or competitor of the interaction of the protein or molecule of interest with the derivatized polyP. Inhibition or competition is recognized by less binding with the derivatized polyP in the presence of the potential inhibitor or competitor than in the absence.

[0086] **Preparation and Digestion of Spermidine-PolyP Adducts**. 5 mM polyP130 was incubated for 6 hours 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 spermidine-polyP 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 hour at 100°C in 1 M HCl and monophosphate was quantified.

[0087] All references cited herein are hereby incorporated by reference to the extent there is no inconsistency with the present disclosure. All references throughout this application, for example patent documents including issued or granted patents or equivalents; patent application publications; and non-patent literature documents or other source material; are hereby incorporated by reference herein in their entireties, as though individually incorporated by reference, to the extent each reference is at least partially not inconsistent with the disclosure in this application (for example, a reference that is partially inconsistent is incorporated by reference except for the partially inconsistent portion of the reference).

[0088] All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. References cited herein are incorporated by reference herein in their entirety to indicate the state of the art, in some cases as of their filing date, and it is

intended that this information can be employed herein, if needed, to exclude (for example, to disclaim) specific embodiments that are in the prior art. For example, when a compound is claimed, it should be understood that compounds known in the prior art, including certain compounds disclosed in the references disclosed herein (particularly in referenced patent documents), are not intended to be included in the claim.

[0089] When a group of substituents is disclosed herein, it is understood that all individual members of those groups and all subgroups, including any isomers and enantiomers of the group members, and classes of compounds that can be formed using the substituents are disclosed separately. When a compound is claimed, it should be understood that compounds known in the art including the compounds disclosed in the references disclosed herein are not intended to be included. When a Markush group or other grouping is used herein, all individual members of the group and all combinations and subcombinations possible of the group are intended to be individually included in the disclosure.

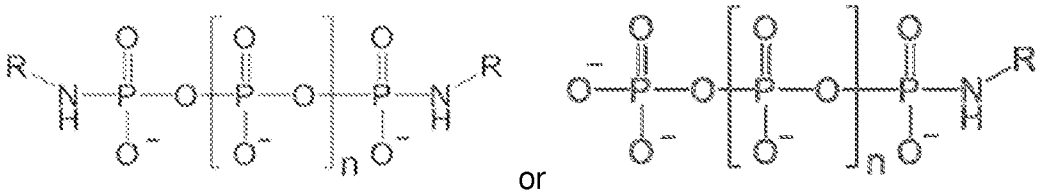
[0090] Every formulation or combination of components described or exemplified can be used to practice the invention, unless otherwise stated. Specific names of compounds are intended to be exemplary, as it is known that one of ordinary skill in the art can name the same compounds differently. When a compound is described herein such that a particular isomer or enantiomer of the compound is not specified, for example, in a formula or in a chemical name, that description is intended to include each isomers and enantiomer of the compound described individual or in any combination. One of ordinary skill in the art will appreciate that methods, device elements, starting materials, synthetic methods, and reagents or reactants other than those specifically exemplified can be employed in the practice of the invention without resort to undue experimentation. All art-known functional equivalents, of any such methods, device elements, starting materials, synthetic methods, and reagents and reactants other than those specifically disclosed herein are intended to be included in this invention. Whenever a range is given in the specification, for example, a temperature range, a time range, or a composition range, all intermediate ranges and subranges, as well as all individual values included in the ranges given are intended to be included in the disclosure.

[0091] As used herein, “comprising” is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any element, step, or ingredient not specified in the claim element. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claim. Any recitation herein of the term “comprising”, particularly in a description of components of a composition or in a description of elements of a device, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or elements. The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein.

[0092] The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the appended claims.

[0093] In general the terms and phrases used herein have their art-recognized meaning, which can be found by reference to standard texts, journal references and contexts known to those skilled in the art. The following definitions are provided to clarify their specific use in the context of the invention.

[0094] Phosphoramidate-derivatized polyphosphate, as used herein, is a polyphosphate molecule covalently linked by a phosphoramidate bond to a moiety, which prior to the covalent linkage, comprised at least one primary amine group. Phosphoramidate-derivatized polyphosphate can be represented by the alternatives



or

where R is as defined hereinabove in broad as well as more specific terms. Advantageously, where the phosphoramidate derivatized polyphosphate is to be used in humans or animals, the R moiety is not toxic when incorporated in the derivatized molecule and is of relatively and tolerably low toxicity when and it is released from the derivatized molecule. Phosphoramidate bond formation between the polyphosphate and the RNH2 is mediated by a zero-length cross linking reagent (or coupling agent or coupling reagent) as taught herein.

[0095] A phosphoramidate derivatized polyphosphate molecule can be immobilized by directly reacting a polyphosphate with an amine functionalized solid support so as to create a phosphoramidate bond between the polyphosphate and a primary amine moiety of or on the support, or it may be indirectly bound covalently or noncovalently to a solid support, via an amide linkage or a specific binding interaction between a functional group bound to the polyphosphate and a functional group bound to the solid support. Examples of noncovalent interactions include the streptavidin-biotin binding pair or polyhistidine binding to a nitrilotriacetate material bound to a solid support.

[0096] As used herein, a solid support is a material with sufficient structure so as to be not an emulsion, in solution or a soft gel. It can be a bead, fiber, plate or other material of nanoscale or microscale for use in analytic assays, or for use in for use in preparative or adsorptive applications, including chromatography, or for placement in a body or in contact with a wound, surgical incision or at other site of bleeding due to trauma or other injury, such as a spontaneous injury causing or at risk for causing bleeding in a patient, or it can be a chip including but not limited to a sensorchip such as a Biacore sensorchip. The solid support material can have at least one primary amine or it can be coated with a primary amine-containing material for use in the phosphoramidate derivatization of polyP as described herein.

[0097] In the context of this disclosure, a solid support can be a chip such as a sensorchip, for example, a Biacore chip for SPR, a microtiter plate (microwell plate) or well, a tube or other container. Advantageously the solid support is amine-functionalized (contains accessible primary amino groups) so as to allow the formation of a phosphoramidate bond between polyphosphate and the support surface, mediated by a zero-length cross linking reagent reaction as taught herein. The amine functionalized material can be, but is not limited to, a plastic, a polystyrene, coagulometer cuvette, a chitosan or a gelatin material.

[0098] The contact pathway for blood or plasma coagulation (clotting) is that related to the activation of factor IX. Polyphosphate, or phosphoramidate derivatized polyphosphate activates this pathway in plasma or blood, advantageously where the number of phosphate units is between about 25 and about 1000 or more, advantageously greater than about 250, or from about 250 or about 300 to about 1000 or up to about 5000, or greater than about 300.

[0099] A deficiency in clotting/coagulation in a sample from a patient, advantageously plasma or blood, is reflected in an increased clotting time, or in a more fragile fibrin clot than is observed for a reference normal sample of plasma or blood.

[00100] The exact formulation, route of administration and dosage of a phosphoramidate-derivatized polyP preparation can be chosen by the individual physician in view of the patient's condition and cause of need for polyP (see e.g. Fingl et. al., in The Pharmacological Basis of Therapeutics, 1975, Ch. 1 p. 1, for formulation advice).

[00101] It should be noted that the attending physician, veterinarian or other human or animal health care professional would know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions, or to successful treatment. Conversely, the attending physician would also know to adjust treatment to higher levels if the clinical response were not adequate (precluding toxicity). The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated and to the route of administration. The severity of the condition may, for example, be evaluated, in part,

by standard prognostic evaluation methods. Further, the dose and perhaps dose frequency, will also vary according to the age, body weight, and response of the individual patient. A program comparable to that discussed above also may be used in veterinary medicine.

[00102] Depending on the specific condition being treated and the targeting method selected, such agents may be formulated and administered systemically or locally. Techniques for formulation and administration may be found in Alfonso and Gennaro (1995). Suitable routes may include, for example, topical, internal oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, or intramedullary injections, as well as intrathecal, intravenous, or intraperitoneal injections.

[00103] For injection, the agents herein may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

[00104] For use to stop bleeding of an injury, other trauma or due to surgical incision, phosphoramidate-derivatized, especially immobilized, polyP can be administered to the site from which bleeding occurs. The immobilization via the terminal phosphoramidate modification with a linker such as EDAC and reaction with an amine on a solid surface allows precise targeting of the coagulation-promoting activity to the site of interest. Alternatively a phosphoramidate derivatized polyphosphate can be formulated for topical application in a gel, cream, or ointment or other material, as appropriate to the intended use and as readily apparent to one of skill in the art. Pharmaceutically acceptable salts or esters or excipients and the like are known to the art,

[00105] The phrase "pharmaceutically acceptable salt(s)", as used herein, means those salts of compounds of the invention that are safe and effective for use in mammals and that do not significantly diminish the desired biological activity of the phosphoramidate derivatized polyphosphate. Pharmaceutically acceptable salts include salts of acidic or basic groups present in compounds of the invention.

Pharmaceutically acceptable Certain can form pharmaceutically acceptable salts with various amino acids. Suitable base salts include, but are not limited to, aluminum, calcium, lithium, magnesium, potassium, sodium, zinc, and diethanolamine salts. For a review on pharmaceutically acceptable salts; see, for example, Berge et al., 66 J. Pharm. Sci I. 1-19 (1977).

[00106] Use of pharmaceutically acceptable carriers to formulate the compounds herein disclosed into dosages suitable for systemic administration is within the scope of the invention. With proper choice of carrier and suitable manufacturing practice, the compositions provided and/or enabled herein, in particular those formulated as solutions, may be administered parenterally, such as by intravenous injection. Appropriate compounds can be formulated readily using pharmaceutically acceptable carriers well known in the art into dosages suitable for oral administration. Such carriers enable the compounds of the invention to be formulated as tablets, pills, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated.

[00107] Agents intended to be administered intracellularly may be administered using techniques well known to those of ordinary skill in the art. For example, such agents may be encapsulated into liposomes, then administered as described above. Liposomes are spherical lipid bilayers with aqueous interiors. All molecules present in an aqueous solution at the time of liposome formation are incorporated into the aqueous interior. The liposomal contents are both protected from the external microenvironment and, because liposomes fuse with cell membranes, are efficiently delivered into the cell cytoplasm. Additionally, due to their hydrophobicity, small organic molecules may be directly administered intracellularly.

[00108] Pharmaceutical compositions suitable for use in the present invention include compositions wherein the active ingredients are contained in an effective amount to achieve the intended purpose. Determination of the effective amounts is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

[00109] In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising

excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. The preparations formulated for oral administration may be in the form of tablets, dragees, capsules, or solutions, including those formulated for delayed release or only to be released when the pharmaceutical reaches the small or large intestine.

[00110] The pharmaceutical compositions provided or enabled herein may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levitating, emulsifying, encapsulating, entrapping, immobilizing or lyophilizing processes.

[00111] Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

[00112] Pharmaceutical preparations for oral use can be obtained by combining the active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores, especially with enteric coatings to reduce potential hydrolysis and/or enzymatic degradation in the gut. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

[00113] Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

[00114] Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

[00115] Although the description herein contains certain specific information and examples, these should not be construed as limiting the scope of the invention, but as merely providing illustrations of some of the embodiments of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

REFERENCES

1. Rao, N. N., Gomez-Garcia, M. R., and Kornberg, A. (2009) Inorganic polyphosphate: essential for growth and survival. Annu. Rev. Biochem. 78, 605-647.

2. Docampo, R., de Souza, W., Miranda, K., Rohloff, P., and Moreno, S. N. (2005) Acidocalcisomes - conserved from bacteria to man. Nat. Rev. Microbiol. 3, 251-261.

3. Smith, S. A., Mutch, N. J., Baskar, D., Rohloff, P., Docampo, R., and Morrissey, J. H. (2006) Polyphosphate modulates blood coagulation and fibrinolysis. Proc. Natl. Acad. Sci. U. S. A. 103, 903-908.

4. Muller, F., Mutch, N. J., Schenk, W. A., Smith, S. A., Esterl, L., Spronk, H. M., Schmidbauer, S., Gahl, W. A., Morrissey, J. H., and Renne, T. (2009) Platelet polyphosphates are proinflammatory and procoagulant mediators in vivo. Cell 139, 1143-1156.

5. Smith, S. A., Choi, S. H., Davis-Harrison, R., Huyck, J., Boettcher, J., Rienstra, C. M., and Morrissey, J. H. (2010) Polyphosphate exerts differential effects on blood clotting, depending on polymer size. Blood, 116, 4353-4359.

6. Kim, D., and Cavanaugh, E. J. (2007) Requirement of a soluble intracellular factor for activation of transient receptor potential A1 by pungent chemicals: role of inorganic polyphosphates. J. Neurosci. 27, 6500-6509.

7. Han, K. Y., Hong, B. S., Yoon, Y. J., Yoon, C. M., Kim, Y. K., Kwon, Y. G., and Gho, Y. S. (2007) Polyphosphate blocks tumour metastasis via anti-angiogenic activity. Biochem. J. 406, 49-55.

8. Hernandez-Ruiz, L., Gonzalez-Garcia, I., Castro, C., Brieva, J. A., and Ruiz, F. A. (2006) Inorganic polyphosphate and specific induction of apoptosis in human plasma cells. Haematologica 91, 1180-1186.

9. Pavlov, E., Aschar-Sobbi, R., Campanella, M., Turner, R. J., Gomez-Garcia, M. R., and Abramov, A. Y. (2010) Inorganic polyphosphate and energy metabolism in mammalian cells. J. Biol. Chem. 285, 9420-9428.

10. Morita, K., Doi, K., Kubo, T., Takeshita, R., Kato, S., Shiba, T., and Akagawa, Y. (2010) Enhanced initial bone regeneration with inorganic polyphosphate-adsorbed hydroxyapatite. Acta Biomater. 6, 2808-2815.

11. de Jesus, T. C., Tonelli, R. R., Nardelli, S. C., da Silva Augusto, L., Motta, M. C., Girard-Dias, W., Miranda, K., Ulrich, P., Jimenez, V., Barquilla, A., Navarro, M., Docampo, R., and Schenkman, S. (2010) Target of rapamycin (TOR)-like 1 kinase is involved in the control of polyphosphate levels and acidocalcisome maintenance in *Trypanosoma brucei*. J. Biol. Chem. 285, 24131-24140.

12. Lorenz, B., Marme, S., Muller, W. E., Unger, K., and Schroder, H. C. (1994) Preparation and use of polyphosphate-modified zirconia for purification of nucleic acids and proteins. Anal. Biochem. 216, 118-126.

13. Mutch, N. J., Myles, T., Leung, L. L., and Morrissey, J. H. (2009) Polyphosphate binds with high affinity to exosite II of thrombin. J. Thromb. Haemost. 8, 548-555.

14. Hermanson, G. T. (2008) Bioconjugate techniques, 2nd ed. ed., Academic, London.

15. Smith, S. A., and Morrissey, J. H. (2007) Sensitive fluorescence detection of polyphosphate in polyacrylamide gels using 4',6-diamidino-2-phenylindol. Electrophoresis 28, 3461-3465.

16. Lorenz, B., and Schroder, H. C. (2001) Mammalian intestinal alkaline phosphatase acts as highly active exopolyphosphatase. Biochim. Biophys. Acta 1547, 254-261.

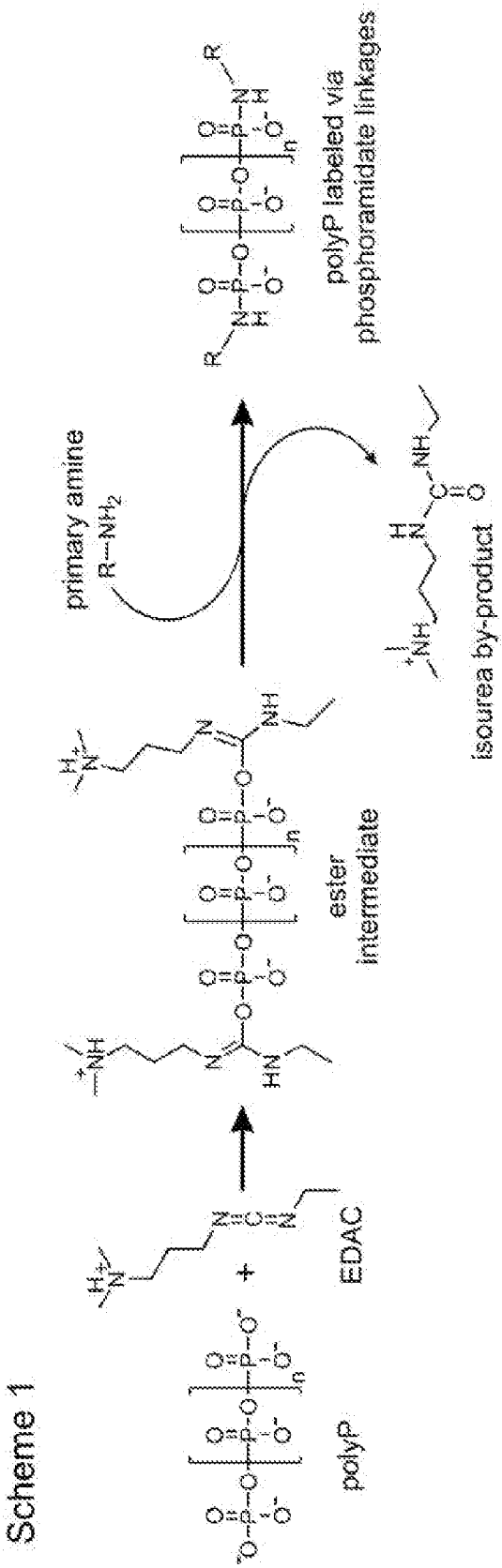
17. Smith, S. A., and Morrissey, J. H. (2008) Polyphosphate as a general procoagulant agent. J. Thromb. Haemost. 6, 1750-1756.

18. Choi, S.H., Collins, J.N.R., Smith, S.A., Davis-Harrison, R. L., Rienstra, C.M. and Morrissey, J.H. (2010) Phosphoramidate end labeling of inorganic polyphosphates: Facile manipulation of polyphosphate for investigating and modulating its biological activities. Biochemistry 49, 9935-9941.

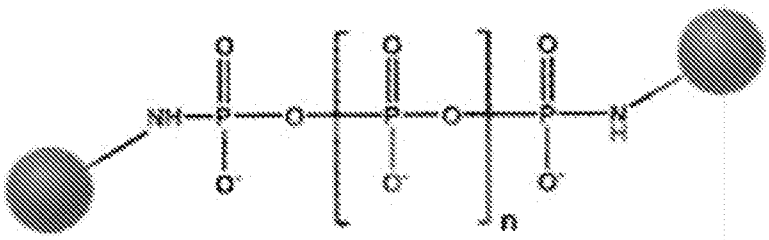
19. Bieker, R., Kessler, T., Schwoppe, C., Padro, R., Persigehl, T., Bremer, C., Dreischaluck, J., Kolkmeyer, A., Jeindel, W., Mesteres, R.M. and Berdel, W.E. (2009) Infarction of tumor vessels by NGR-peptide-directed targeting of tissue factor: experimental results and first-in-man experience. Blood 113, 5019-5027.

20. WO 2006/096345; WO 2009/061697; WO 2009/046194

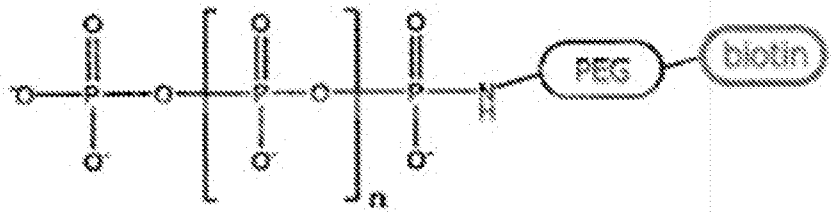
Scheme 1


polyP
EDAC
ester intermediate
primary amine
R—NH2
isourea by-product
polyP labeled via phosphoramidate linkages

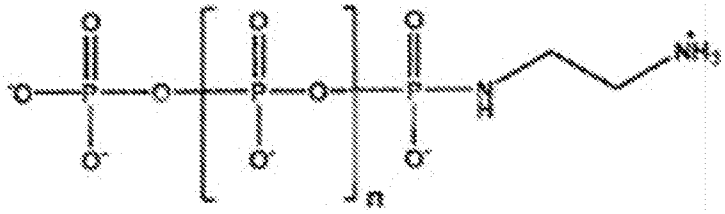
Scheme 2A



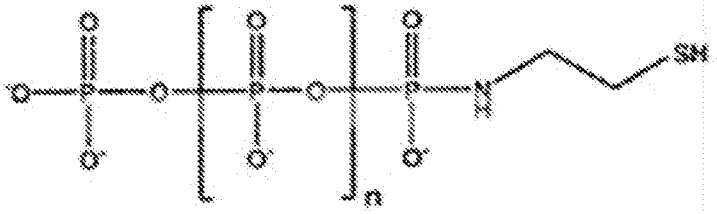
Scheme 2B



Scheme 2C

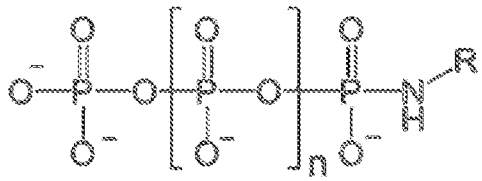
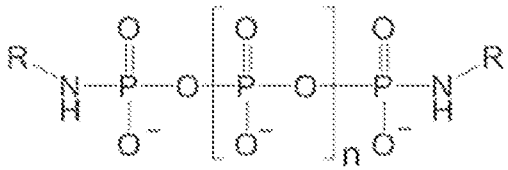


Scheme 2D

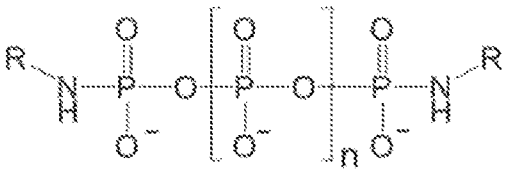
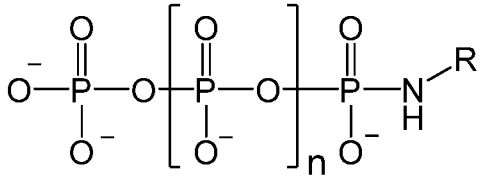


We claim:

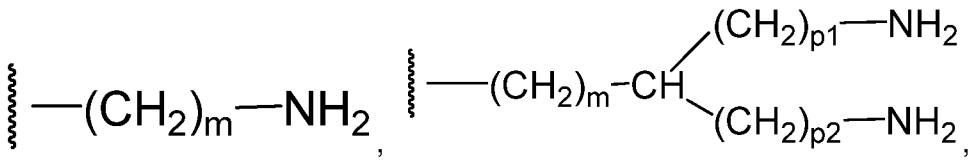
1. A phosphoramidate derivatized polyphosphate of the formula

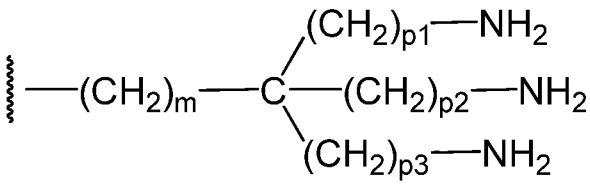

. or  .

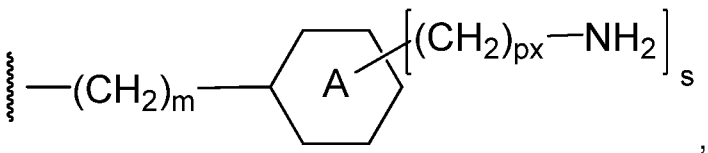
2. A phosphoramidate derivatized polyphosphate of claim 1 of formula:

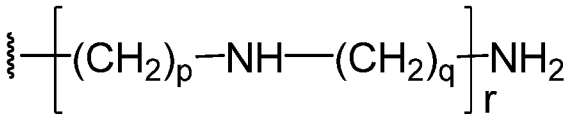

or 

or salts or esters thereof, wherein n is an integer representing the number of repeating internal phosphate units or the average number of repeating internal phosphate units in a given compound and R is derived from any amine, particularly a primary amine, optionally, each R is independently selected from an alkyl amine, an alkyl diamine, a polyamine group, a dye, a label, biotin, a protein or peptide, a solid or a surface or wherein R is a moiety containing a primary amine including linear, branched, or cyclic moieties, optionally R is

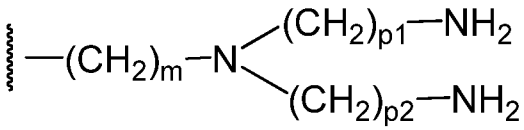








, or



m is an integer ranging from 1 to 12, p, q and r are integers ranging from 1-12 or 1-6, p1, p2, p3 and px are integers ranging from 1-12 or 1-6, the A ring is a carbocyclic, heterocyclic, aromatic or heteroaromatic ring having 5-8 ring atoms, and s is an integer ranging from 1 to the maximum number of positions on the ring for substitution with a bond to a carbon, and wherein if the A ring is a phenyl ring, s can range from 1-5; and wherein one or or more $–CH_2-$ groups can optionally be replaced with –O-, -S-, -S-S-, -CO-, $-CONR_{10}-$, $-NR_{10}-CO-$, or $-CO_2-$, where R_{10} is hydrogen or an alkyl group having 1-6 carbon atoms, wherein R is a polymer with at least one pendant primary amine group.

3. The phosphoramidate derivatized polyphosphate of claim 1, wherein R is a glucosaminyl moiety, a chitosan moiety, a gelatin scaffold moiety, or an amine-functionalized biotin moiety.

4. The phosphoramidate derivatized polyphosphate of claim 9 wherein the polyphosphate is covalently bound to a primary amine-functionalized polystyrene or a primary amine-functionalized chip, optionally a microtiter plate.

5. A method of derivatizing polyphosphate, said method comprising the step of

contacting a polyphosphate with a molecule comprising at least one primary amine in the presence of a zero-length cross-linking reagent, and optionally a divalent cation, under conditions wherein one or more phosphoramidate linkages are formed between the polyphosphate and the primary amine.

6. The method of claim 5, wherein the polyphosphate comprises from 3 to 5000, 25 to 1000, 25-5000, from 200-5000,from 25 to 500, or 25 to 130, 25 to 100, 60 to 100, or 5, 45, 67, or 130 or more phosphate moieties.

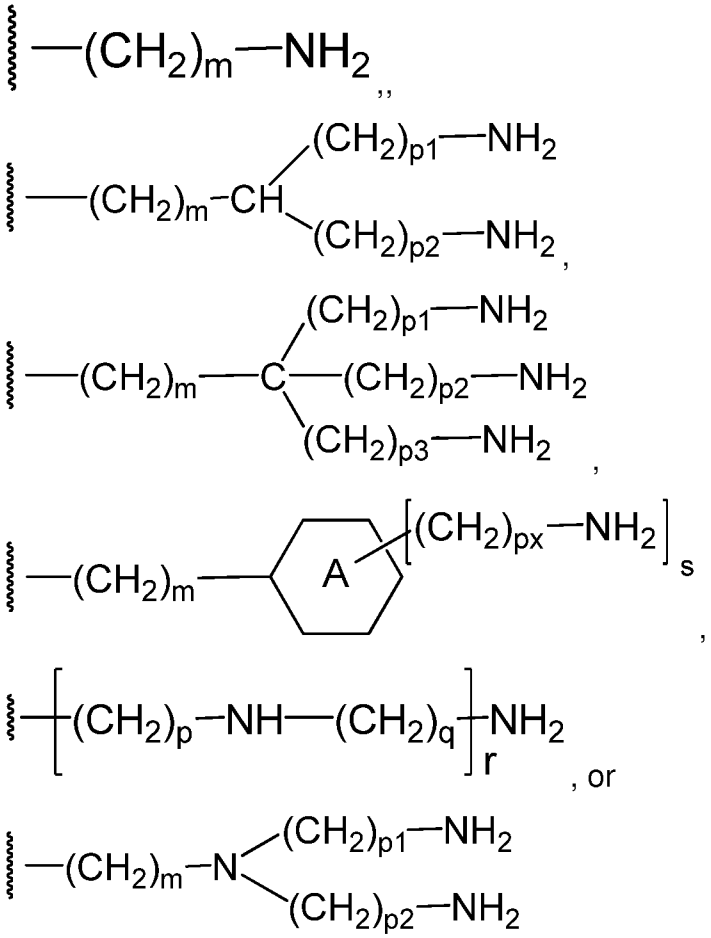
7. The method of claim 5 or 6, wherein said divalent cation is calcium, magnesium or manganese, optionally at a concentration of from about 0.1 to about 100, about 0.5 to about 10, or about 1 mM.

8. The method of any of claims 5 to 7, wherein the contacting step is in a buffer, optionally wherein the pH of the buffer has a pH of from about 5 to about 8, about 6 to about 7.5, or about 6.5.

9. The method of claim 5, wherein the zero-length cross linking reagent is a carbodiimide coupling reagent optionally selected from the group consisting of one of 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide (EDAC), N-cyclohexyl-3-(2-morpholinoethyl)carbodiimide methyl-p-toluenesulfonate (CMC); dicyclohexyl carbodiimide (DCC); diisopropyl carbodiimide (DIC), bis(trimethylsilyl)carbodiimide, N,N'-bis(2-methylphenyl)carbodiimide, N-(tert-butyl)-N'-(2,6-dichlorophenyl)-carbodiimide, N-(butyl)-N'-[1-(2-chlorphenyl)-1-methylethyl]carbodiimide, N-tert-butyl-N -methylcarbodiimide , N-cyclohexyl-N′-isopropylcarbodiimide, N-tert-butyl-N′-ethylcarbodiimide, bis[[4-(2,2-dimethyl-1,3-dioxolyl)]methyl]carbodiimide, and N,N-dicyclopentylcarbodiimide.

10. The method of any of claims 5 to 8, wherein the zero-length cross linking reagent, optionally EDAC, is at a concentration of from about 0.5 to about 500, from about 1 to about 100, or about 50 mM.

11. The method of claim 5, wherein the molecule comprising at least one primary amine is an amino sugar optionally glucosamine, an amino sugar polymer, optionally chitosan; cystamine, a polyamine, optionally spermine, spermidine, protamine, putrescine or cadaverine; an amino acid; a peptide; a protein; a dendrimer, phosphatidylethanolamine, phosphatidylserine, p-nitroaniline, glycine ethyl ester, chromophore; a fluorophore; or an amine-functionalized solid material, optionally a microtiter plate, methacrylate beads or other material, chromatography beads or resin r coagulometer cuvette; collagen or collagen sponge, gelatin scaffold, or bandage or other wound dressing or wherein the primary amine is of the formula R-NH2, wherein R is a moiety containing a primary amine including linear, branched, or cyclic moieties, or



m is an integer ranging from 1 to 12, p, q and r are integers ranging from 1-12 or 1-6, p1, p2, p3 and px are integers ranging from 1-12 or 1-6, the A ring is a carbocyclic, heterocyclic, aromatic or heteroaromatic ring having 5-8 ring atoms, and s is an integer ranging from 1 to the maximum number of positions on the ring for substitution with a bond to a carbon, and wherein if the A ring is a phenyl ring, s can range from 1-5; and wherein one or more $–CH_2-$ groups can optionally be replaced with –O-, -S-, -S-S-, -CO-, $-CONR_{10}-$,$-NR_{10}-CO-$, or $-CO_2-$, where R_{10} is hydrogen or an alkyl group having 1-6 carbon atoms, or wherein R is a polymer with at least one pendant primary amine group.

12. Use of phosphoramidate derivatized polyphosphate or a pharmaceutically acceptable salt thereof, wherein said polyphosphate moiety comprises ≥ 25, ≥30, ≥150, ≥250, ≥300, ≥400, ≥500 phosphate residues, optionally 25-5000, 60-100, 35-300, 140-500, 300-1000, 300-5000, 250-5000, in the formulation of a medicament, together with a pharmaceutically acceptable carrier, to promote or accelerate blood

coagulation, to enhance clot structure or to inhibit tumor growth, survival or angiogenesis in a patient in need thereof.

13. The use of claim 12, wherein the patient suffers from hemophilia, thrombocytopenia, a deficiency in factor IX or other hereditary or acquired bleeding disorder.

14. The use of claim 12, wherein the patient is having a bleeding episode, is undergoing or is about to undergo a surgical procedure, or has suffered trauma.

15. The use of claim 12, wherein the patient has been administered an anticoagulant, wherein the anticoagulant is optionally is heparin, low MW heparin, rivaroxaban, argatroban, Coumadin or warfarin.

16. The use of claim 12, wherein said phosphoramidate derivatized polyphosphate is covalently bound to a solid support having at least one primary amine group, optionally chitosan, a bandage, a gelatin scaffold, a wound dressing, collagen or a collagen sponge.

17. A method for assessing clotting activity or clotting time in the presence of phosphoramidate-derivatized polyphosphate, said method comprising the steps of:

contacting a blood or plasma sample of a patient with phosphoramidate-derivatized polyphosphate under conditions allowing activation of at least one clotting pathway;

assessing clotting time; and

determining if patient from whom the blood or plasma sample is taken has a deficiency in clotting activity, said deficiency being determined by a longer clotting time than for a normal comparison sample of blood or plasma.

18. The method of claim 17, wherein the phosphoramidate-derivatized polyphosphate is a polyphosphate covalently linked to an amine functionalized surface of a solid support or is indirectly bound, optionally a sensorship, microwell plate, coagulometer cuvette, tube or other solid surface or solid support.

19. A method for removing a molecule of interest from a liquid sample, said method comprising the step of contacting the sample comprising the molecule of interest with

phosphoramidate-derivatized polyphosphate, optionally wherein the phosphoramidate-derivatized polyphosphate is immobilized to a solid support, and wherein the molecule of interest is a protein, peptide, a cationic molecule or a metal cation, whereby the molecule of interest is bound to the phosphoramidate derivatized polyphosphate..

20. The method of claim 19, wherein the metal cation is a toxic metal cation, optionally lead, cadmium or chromium.

21. A method for inhibiting nuclease activity in a sample, said method comprising the step of contacting the sample with polyphosphate covalently bound via phosphoramidate linkage to a solid material, optionally wherein the solid material is a container for holding said sample.

22. A method for identifying an inhibitor or competitor of binding of a molecule of interest to polyphosphate, said method comprising the steps of:

(a) contacting a test composition with phosphoramidate derivatized polyphosphate and the molecule of interest, and in parallel contacting a reference composition not comprising an inhibitor or competitor of binding of the molecule of interest to polyphosphate;

(b) assessing binding of the molecule of interest in the presence of the test composition and assessing binding of the molecule of interest in the presence the reference composition; and

(c) identifying the test composition as comprising an inhibitor or competitor of binding of the molecule of interest to polyphosphate when binding is less in the presence of the test composition than in the presence of the reference composition.

23. The method of claim 22, wherein the molecule of interest is a protein, optionally thrombin, factor IX, factor IXa or kallikrein.

24. The method of claim 22 or 23, wherein the phosphoramidate derivatized polyphosphate is attached to a solid support.

Figure 1A

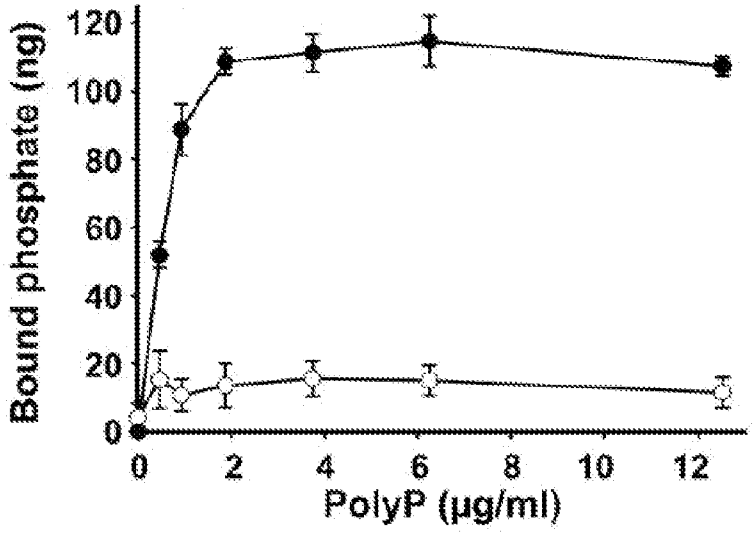


Figure 1B

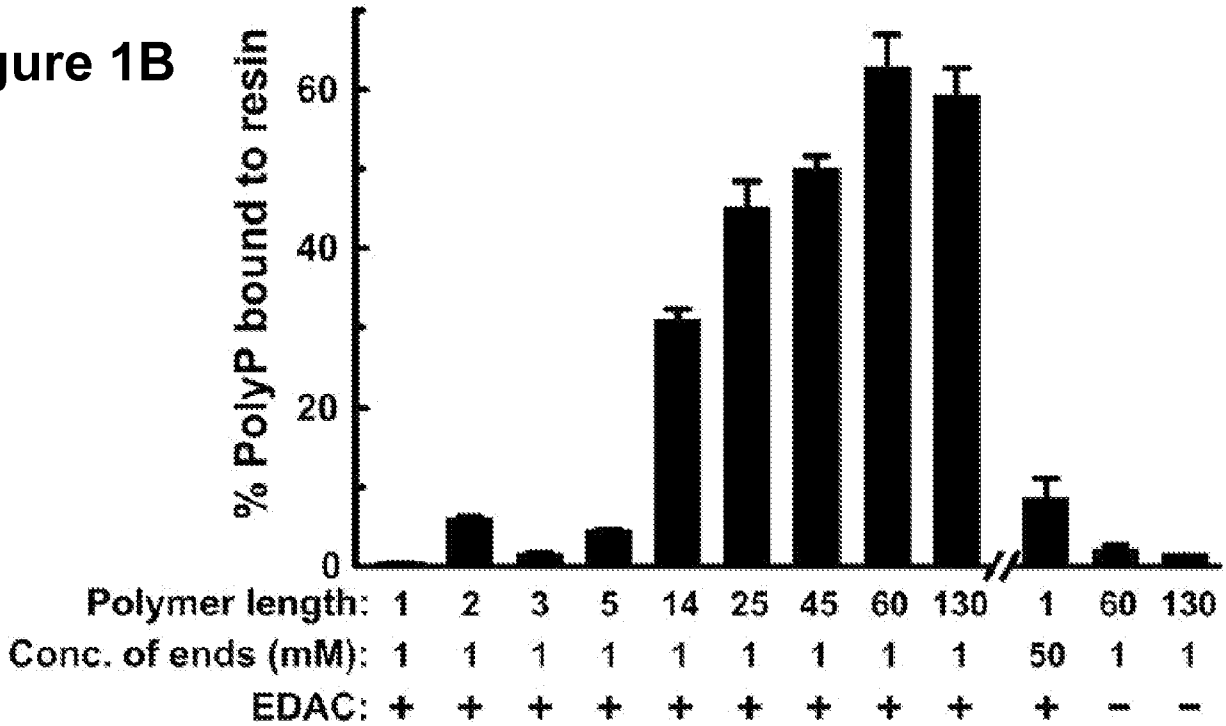


Figure 2A

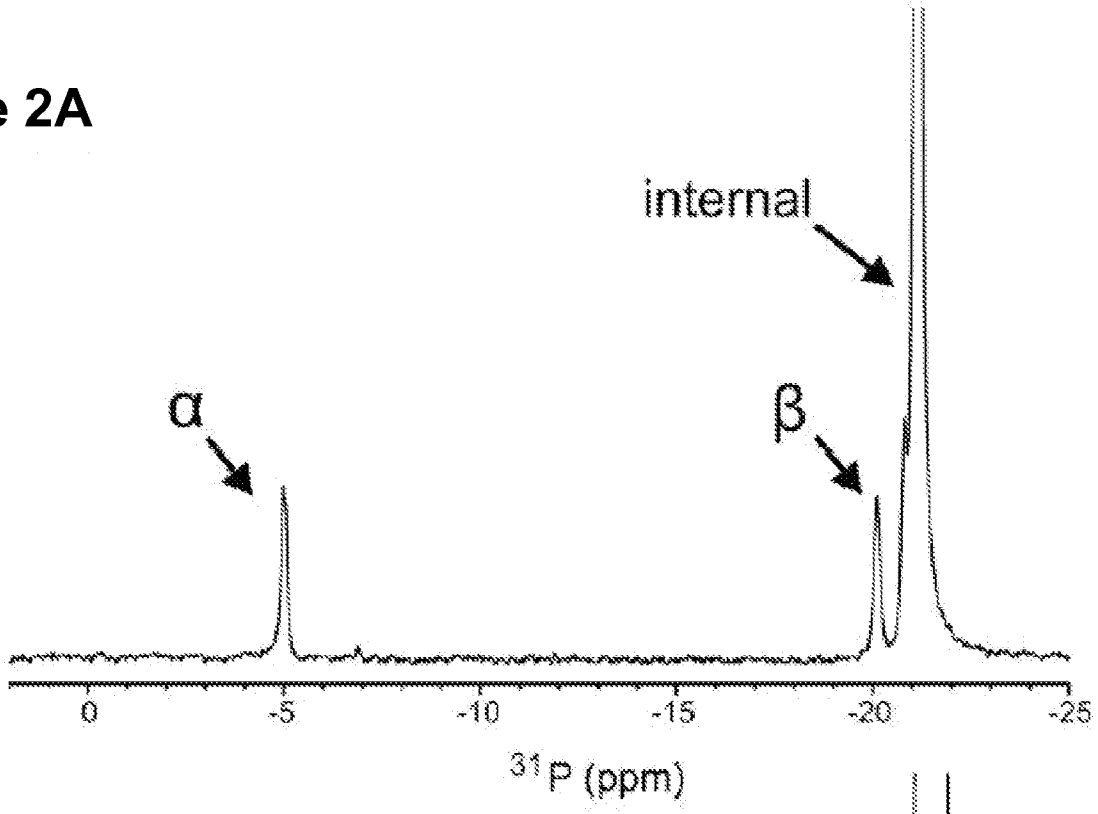
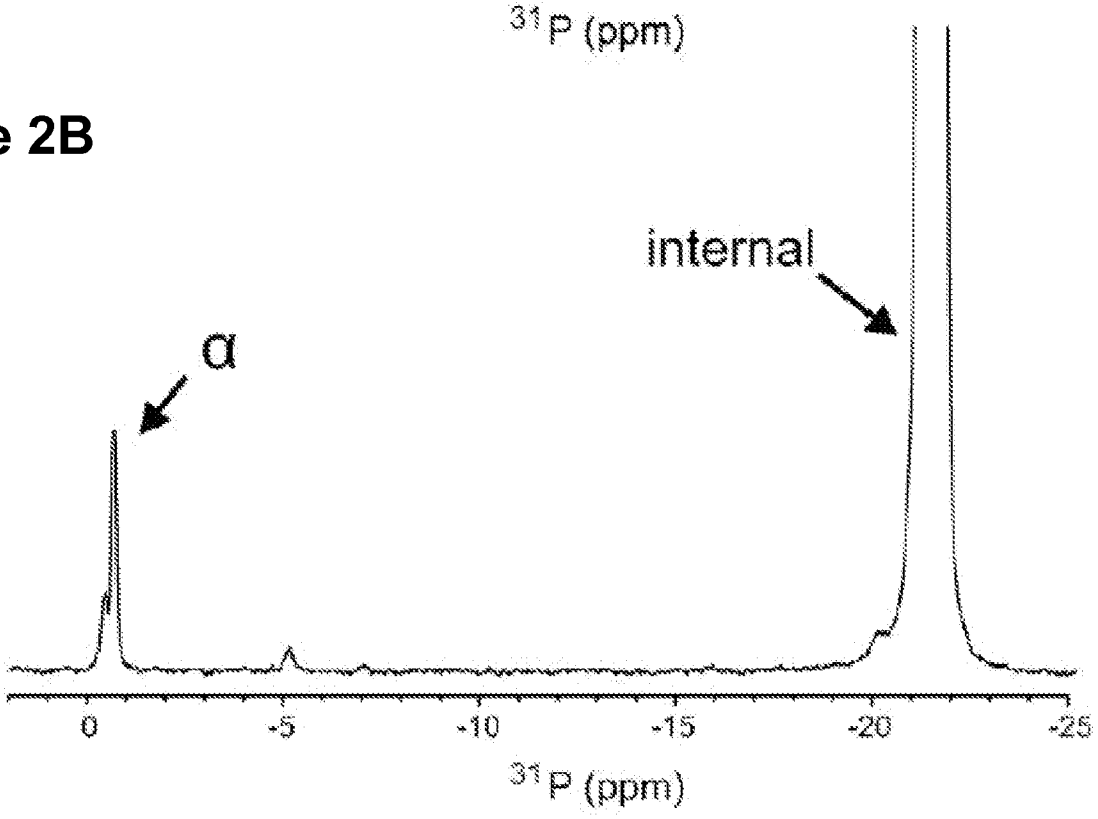


Figure 2B



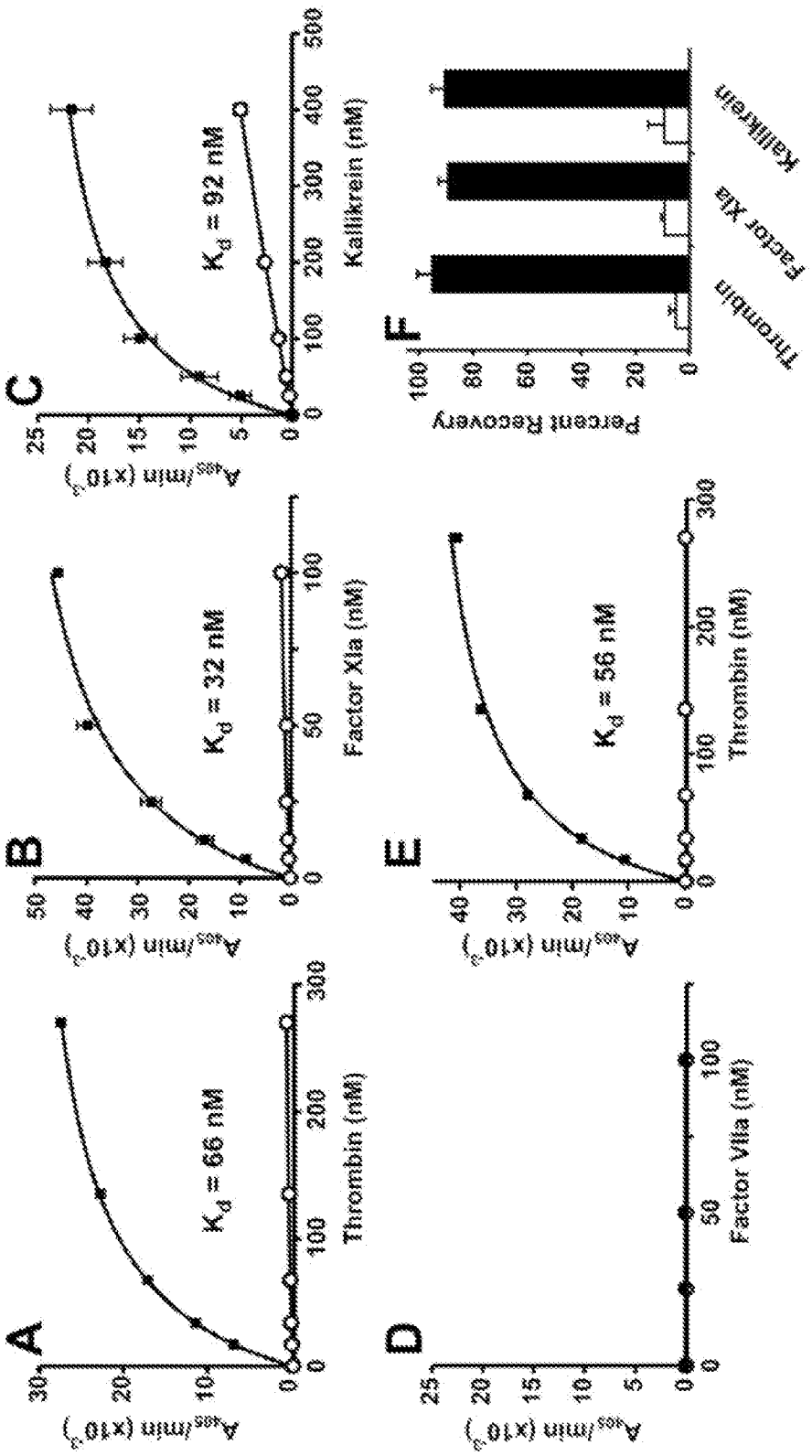

A
B
C
D
E
F
K_d = 66 nM
K_d = 32 nM
K_d = 92 nM
K_d = 56 nM
Thrombin (nM)
Factor Xia (nM)
Kallikrein (nM)
Factor VIIa (nM)
Thrombin (nM)
Percent Recovery
Thrombin
Factor Xia
Kallikrein

Figure 3

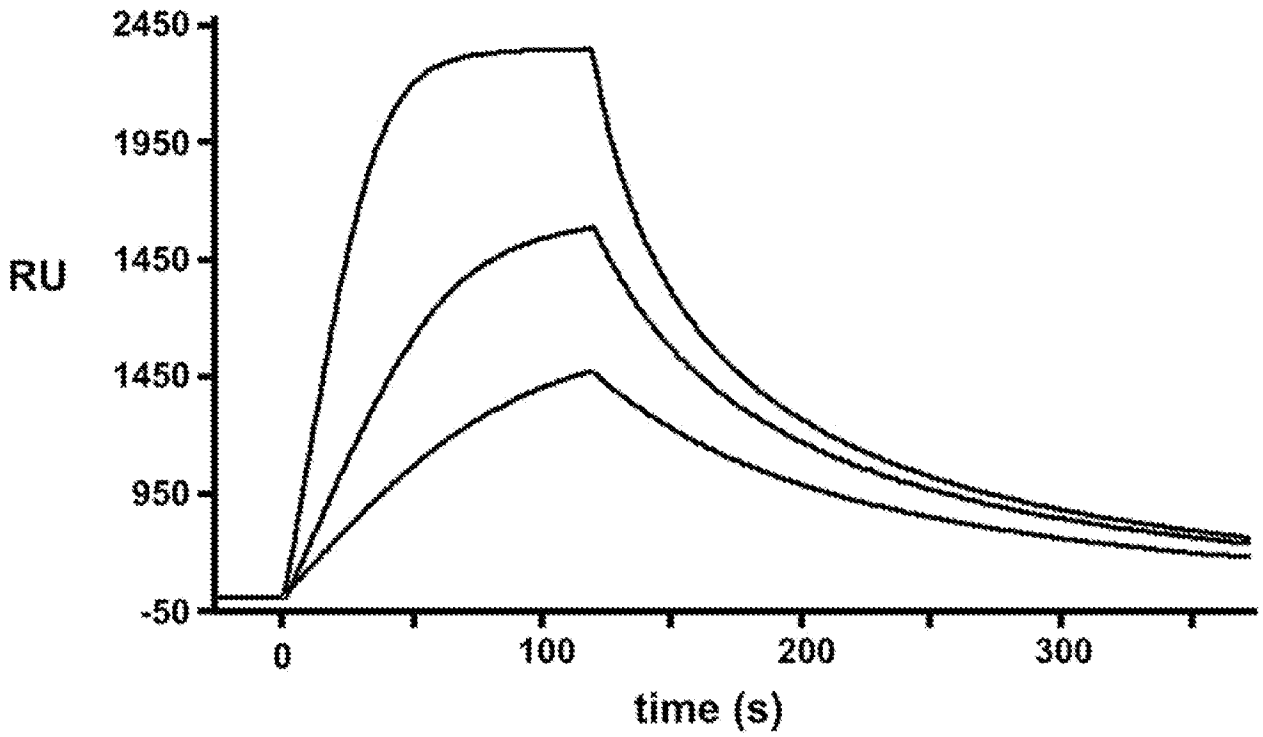


Figure 4

Figure 5A

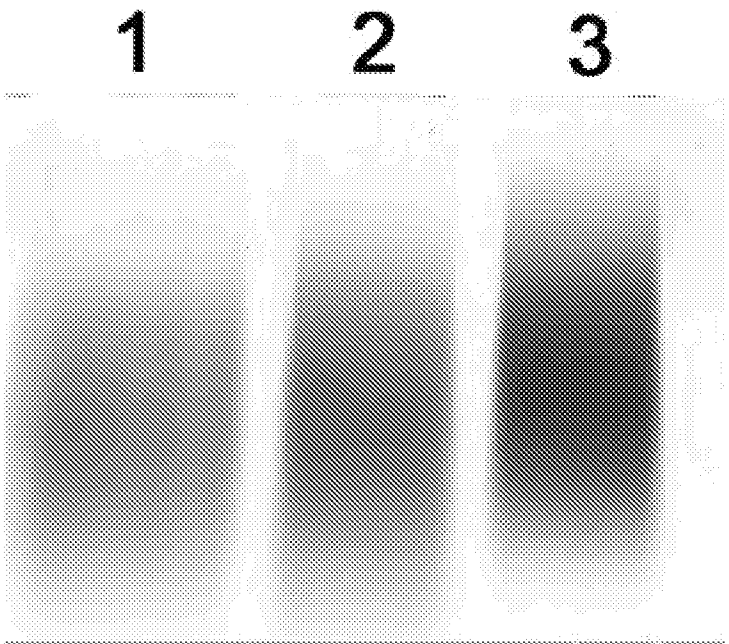
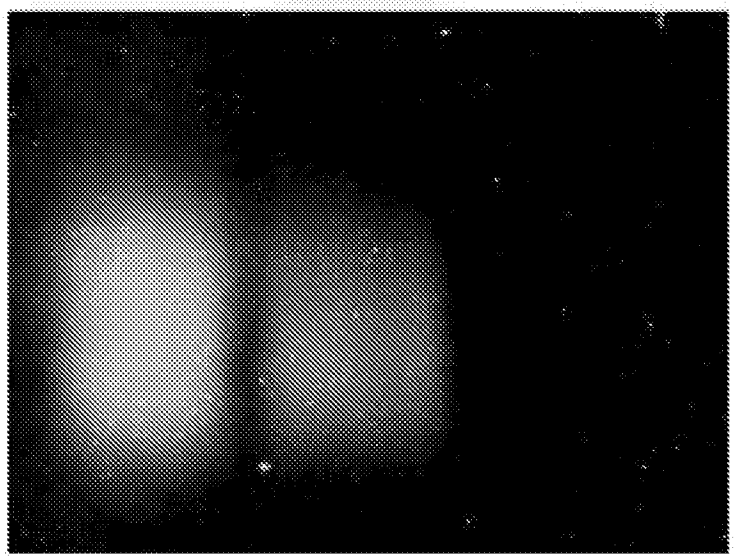


Figure 5B



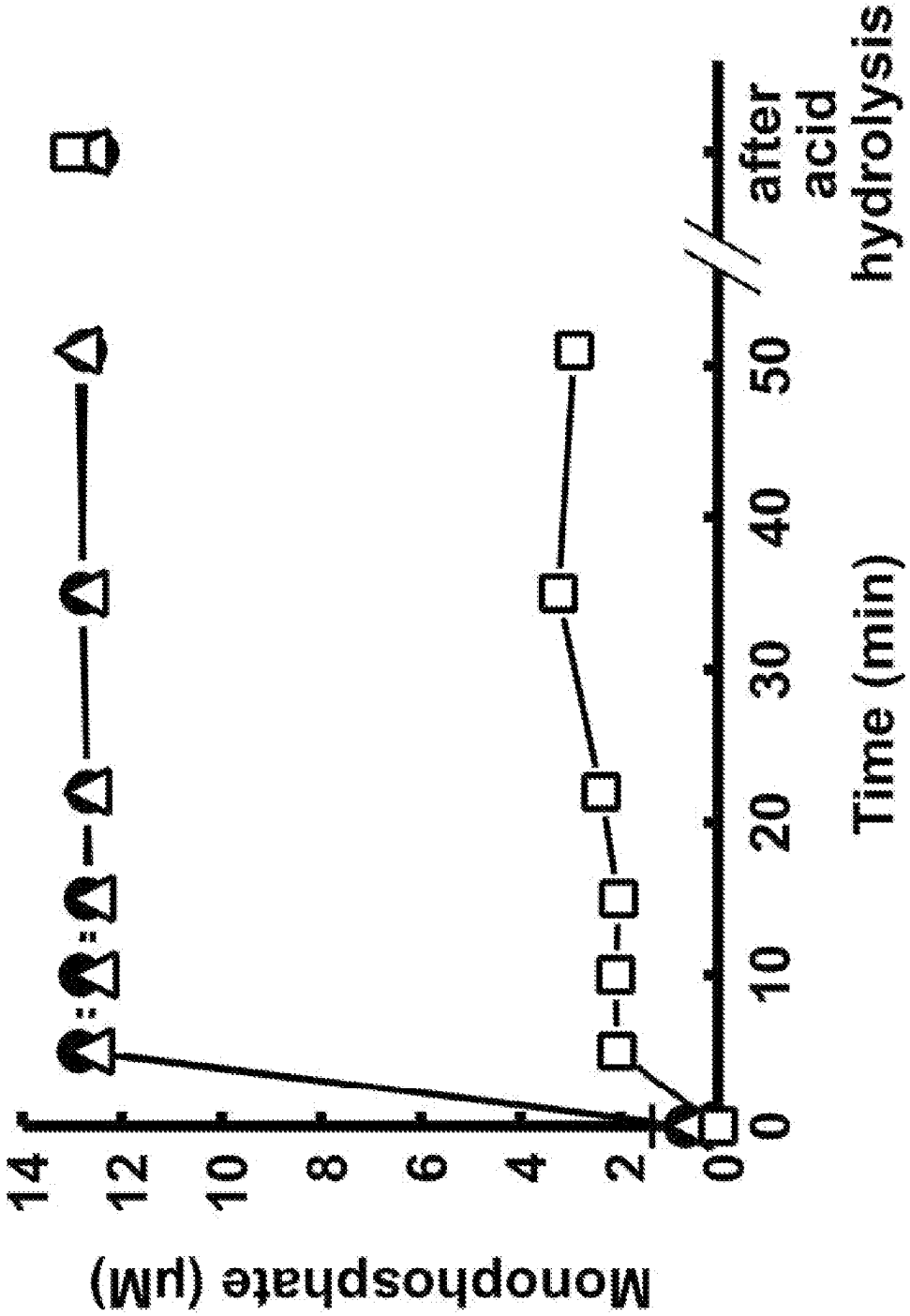

Monophosphate (μM)
14
12
10
8
6
4
2
0
0
10
20
30
40
50
Time (min)
after acid hydrolysis

Figure 6

Figure 7A

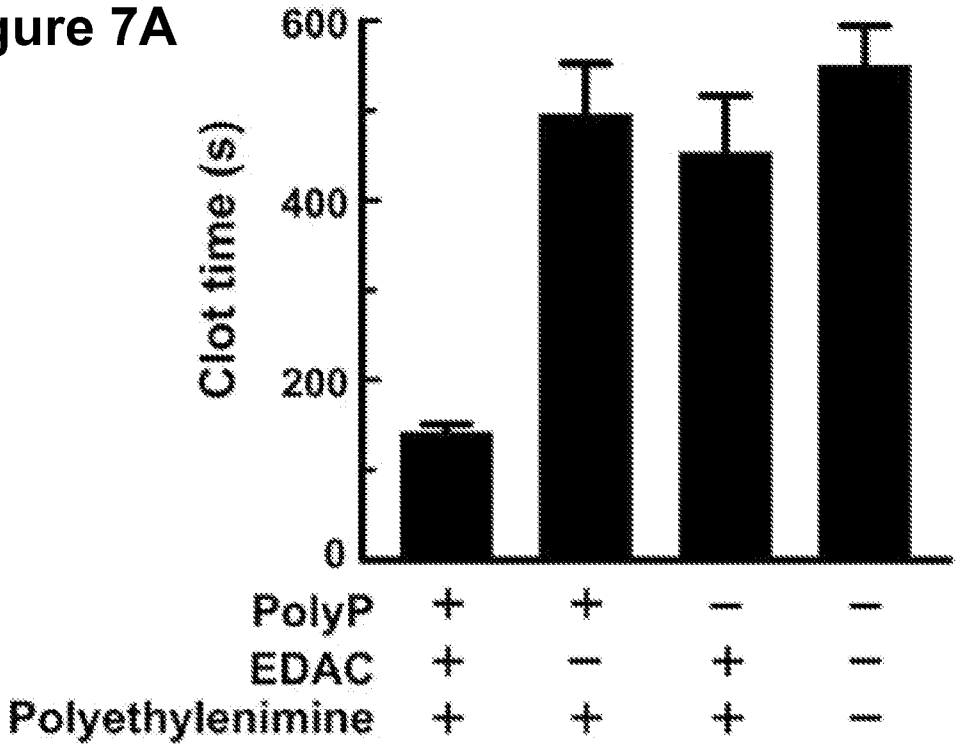
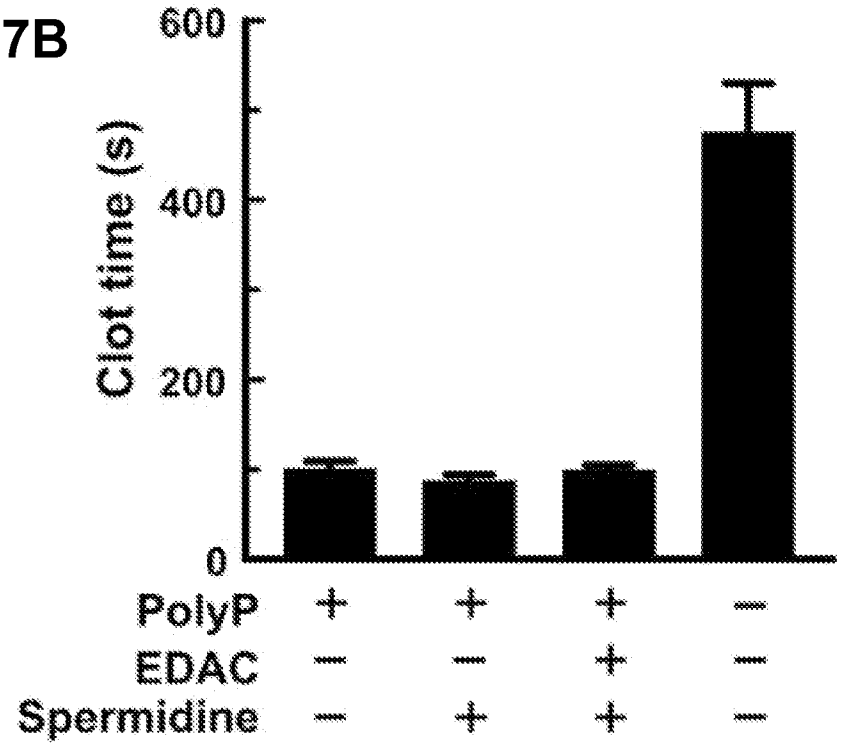


Figure 7B



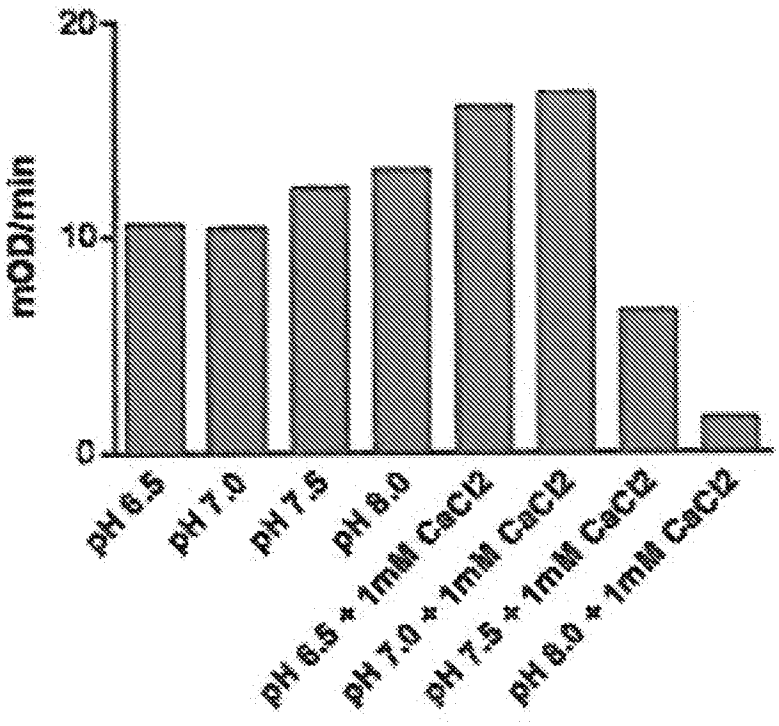

mOD/min
20
10
0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
pH 6.5 + 1mM CaCl2
pH 7.0 + 1mM CaCl2
pH 7.5 + 1mM CaCl2
pH 8.0 + 1mM CaCl2

Figure 8

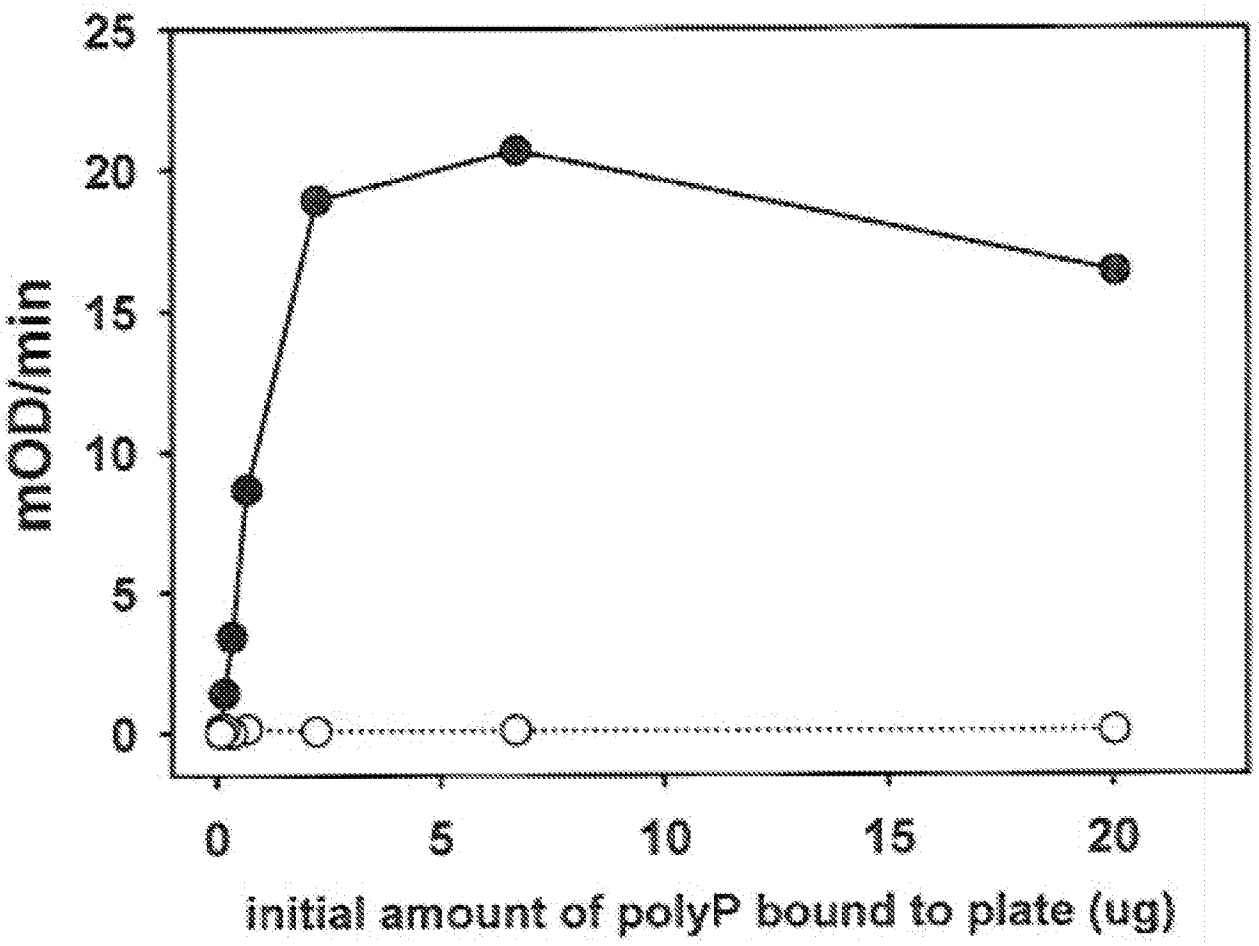

25
20
15
10
5
0
mOD/min
0
5
10
15
20
initial amount of polyP bound to plate (ug)

Figure 9

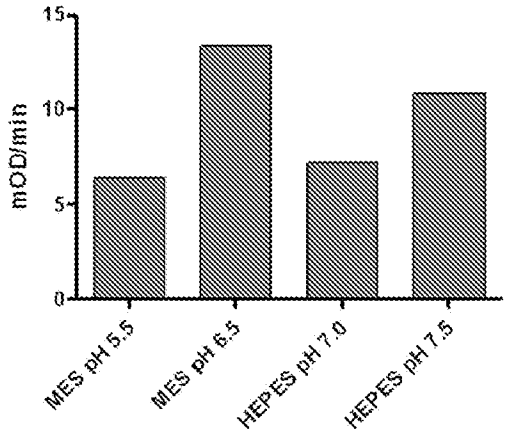


Figure 10

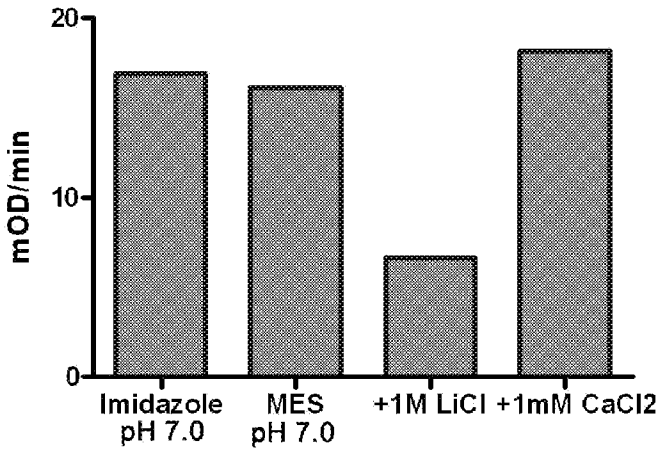


Figure 11

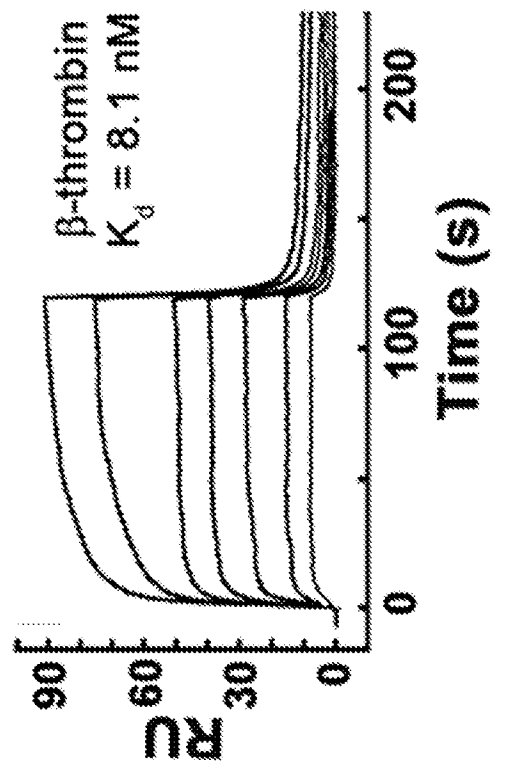


Figure 12A

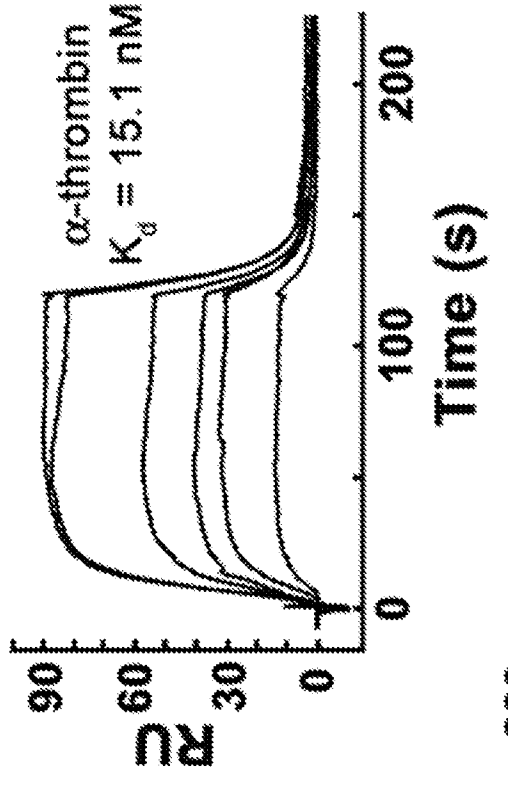


Figure 12B

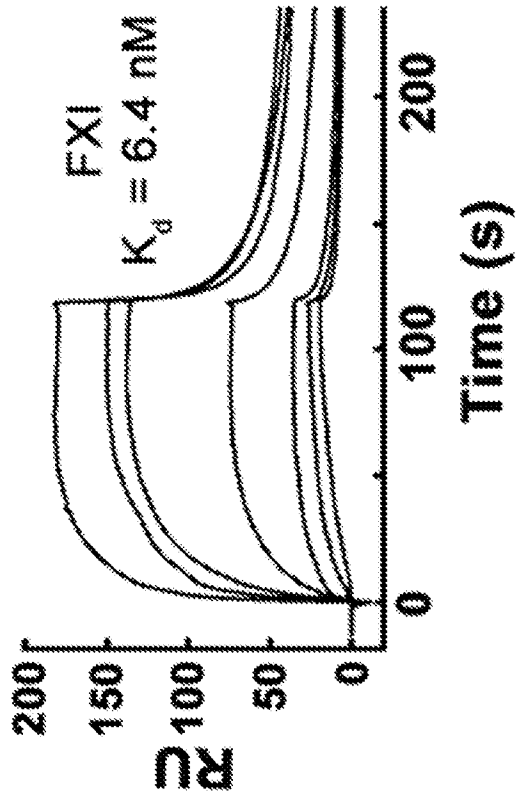


Figure 12C

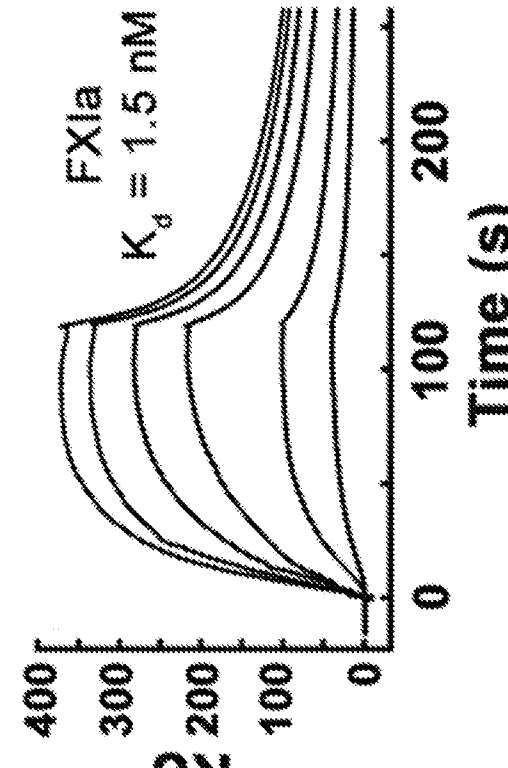


Figure 12D

INTERNATIONAL SEARCH REPORT

PCT/US2011/055447 08.03.2012

International application No.
PCT/US 11/55447

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 43/04; A61K 31/70 (2012.01)
USPC - 514/48-49
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 514/48-49

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 514/25, 47, 51, 94-99 (see search terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB) Terms - polyphosphate phosphoramidate EDAC EDC support chip plate titer linker clot
Google - phosphoramidate (EDAC OR EDC) solid-support clotting (chip OR plate) primary-amine; phosphoramidate polyphosphates (EDAC OR EDC) solid-support clotting

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 2006/0287271 A1 (FISCHER et al.) 21 December 2006 (21.12.2006) para [0075], [0082]	1-2, 5-7, 9 --- 3-4, 11-24
Y	US 2007/0244068 A1 (DOUGLASS, III et al.) 18 October 2007 (18.10.2007) para [0016]	3
Y	US 6,013,789 A (RAMPAL) 11 January 2000 (11.01.2000) col 3, ln 45-57; col 9, ln 10-19; FIG. 1	4, 11, 16-24
Y	WO 2009/046194 A2 (SMITH et al.) 09 April 2009 (09.04.2009) pg 8, para 1; pg 8, para 3; pg 13, para 2; pg 14, para 4	12-18
A	US 2010/0048500 A1 (CHENG et al.) 25 February 2010 (25.02.2010) entire document	1-7, 9, 11-24
A	US 2004/0241716 A1 (KUMAR et al.) 02 December 2004 (02.12.2004) entire document	1-7, 9, 11-24

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
09 February 2012 (09.02.2012)	08 MAR 2012

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

International application No.
PCT/US 11/55447

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8, 10
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.