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Synthesis and Evaluation of Chromogenic and Fluorogenic Substrates for High-Throughput Detection of Enzymes That Hydrolyze Inorganic Polyphosphate

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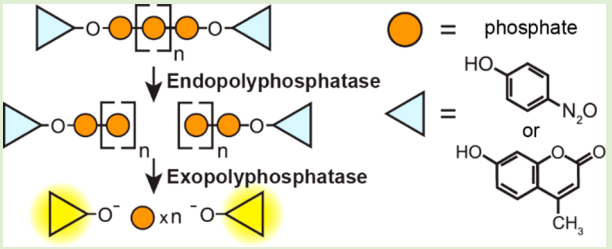
ABSTRACT: Inorganic polyphosphates, linear polymers of orthophosphate, occur naturally throughout biology and have many industrial applications. Their biodegradable nature makes them attractive for a multitude of uses, and it would be important to understand how polyphosphates are turned over enzymatically. Studies of inorganic polyphosphatases are, however, hampered by the lack of high-throughput methods for detecting and quantifying rates of polyphosphate degradation. We now report chromogenic and fluorogenic polyphosphate substrates that permit spectrophotometric monitoring of polyphosphate hydrolysis and allow for high-throughput analyses of both endopolyphosphatase and exopolyphosphatase activities, depending on assay configuration. These substrates contain 4-nitrophenol or 4-methylumbelliferone moieties that are covalently attached to the terminal phosphates of polyphosphate via phosphoester linkages formed during reactions mediated by EDAC (1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide). This report identifies Nudt2 as an inorganic polyphosphatase and also adds to the known coupling chemistry for polyphosphates, permitting facile covalent linkage of alcohols with the terminal phosphates of inorganic polyphosphate.



INTRODUCTION

Inorganic polyphosphates (polyP) are linear polymers of orthophosphate joined by high-energy phosphoanhydride bonds and can range in length from tens to thousands of phosphates. PolyP is widespread throughout biology and implicated in a multitude of physiologic processes in organisms from bacteria to man,[1−4] although many of its biological functions likely remain to be discovered and characterized. PolyP is also an industrial chemical with applications in areas such as water treatment, food processing, fertilizers, and flame retardants.[3] The biodegradable and versatile nature of polyP makes it an attractive material with many uses, and it would be desirable to understand how polyP is turned over. Known polyP-digesting enzymes include exopolyphosphatases which sequentially remove terminal phosphates from polyP, and endopolyphosphatases which hydrolyze internal phosphoanhydride bonds.[1] Although some of the enzymes responsible for degrading polyP have been identified in unicellular organisms, they remain relatively poorly studied in higher eukaryotes, with a few notable exceptions.[1,5] Two examples are mammalian alkaline phosphatase,[6] a highly potent exopolyphosphatase, and the human protein, h-prune, a short-chain exopolyphosphatase implicated as a regulator of metastasis.[7] Recent work has shown that polyP is secreted from activated human platelets[8] and mast cells[9] and that it is an important regulator of blood clotting[4] and complement.[10] PolyP is degraded in human plasma with a half-life of about 90 min,[11] which is no doubt important in controlling polyP's biological action, yet the mechanism of its degradation in vivo is currently unknown.

An impediment to identifying and studying the properties of polyP-degrading enzymes is the dearth of high-throughput means for detecting inorganic polyphosphatases and quantifying their activities. Many of the existing methods for quantifying enzymatic polyP degradation are cumbersome, of low sensitivity, or require the use of specialized equipment. The methods also typically rely on multiple steps including chromatography, gel electrophoresis, laborious physical extraction protocols coupled with chemical detection of liberated inorganic orthophosphate, or the use of radiolabeled polyP.[1] On the other hand, recently reported, more facile methods for detecting exopolyphosphatase activity include the continuous recording of released inorganic monophosphate, which was successfully employed to determine the kinetic parameters of the exopolyphosphatase, h-prune.[7] Detecting and quantifying the action of endopolyphosphatases remains substantially more time-consuming, however, as it typically involves resolving the digested polyP products using gel electrophoresis.[12] We therefore sought to develop chromogenic and fluorogenic polyP substrates that would allow polyP degradation to be followed spectrophotometrically and, in particular, a method that would allow high-throughput detection of endopolyphos-

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phatase activity. Ideally, we would covalently attach chromogenic or fluorogenic dyes to the terminal phosphates of polyP. Chromogenic and fluorogenic substrates are available for a number of hydrolases and are readily adaptable to high-throughput assays in multiwell formats. We previously showed that primary amines can be covalently coupled via phosphoramidate linkages to the terminal phosphates of polyP in a reaction promoted by the zero-length cross-linking reagent, 1-ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDAC).[13] In the present study, we now show that EDAC can also be used to promote the efficient formation of phosphoester linkages with the terminal phosphates of polyP, and we apply this chemistry to create chromogenic or fluorogenic polyphosphatase substrates in which polyP is end-labeled with either 4-nitrophenol (NOL) or 4-methylumbelliferone (MU). We also show that these polyP derivatives can be used to detect the action of endo- and exopolyphosphatases, depending on assay configuration.

EXPERIMENTAL SECTION

Materials. Specified reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. NOL was recrystallized using hot water and ethanol. All experiments in this report used a polyP preparation (Natriumpolyphosphat P70) that was a kind gift from BK Giulini GmbH (Ludwigshafen, Germany). The polymer lengths of this preparation ranged from about 20 to 100 phosphates, with a mean length of approximately 45 to 50. PolyP concentrations were quantified using malachite green after acid hydrolysis[14] and are reported here in terms of phosphate monomer (monomer formula: $NaPO_3$). PolyP was end-labeled with spermidine via phosphoramidate linkages as described.[13]

Methods. *EDAC-Mediated End-Labeling of PolyP by Esterification with Methanol.* A mixture of 5.9 mM polyP, 150 mM freshly dissolved EDAC, and 6.4 M methanol in 100 mM MES buffer pH 6.5 was incubated for either 5 h at 37 °C or 1 h at 65 °C, after which the reaction mixtures were cooled on ice. Reaction volumes varied from 0.35 to 6.5 mL. PolyP-methanol was purified by acetone precipitation; briefly, NaCl was added to the reaction mixture (to 535 mM) followed by two reaction volumes of acetone, with mixing after each addition. The mixture was then centrifuged at 11,000 × *g* for 7 min at room temperature, after which the supernatant was discarded. The polyP pellet was washed twice by adding acetone to the tube followed by centrifugation. Pellets were then dried and redissolved in water.

Prior to NMR analyses, polyP-methanol was further purified by adsorption to a suspension of silica particles ("glass milk"). Glass milk was produced by a modification of the method of Vogelstein and Gillespie,[15] in which 250 mL silica (325 mesh) was stirred in 400 mL water for 1 h, then allowed to settle for 1 h to remove large particles. The supernatant was then centrifuged for 4000 × *g* for 15 min after which the pellet was collected and resuspended in 200 mL 50% nitric acid. This was then stirred and heated to close to boiling, after which it was cooled to room temperature. The silica fines were then collected by centrifugation and washed five times with water by resuspension and centrifugation. The final pellet of washed silica fines was resuspended as a 50% slurry by volume (glass milk). PolyP was purified by binding to, and elution from, glass milk as described,[14] except that the solutions were kept chilled throughout, and the polyP was eluted with 95 °C water instead of buffer.

EDAC-Mediated End-Labeling of PolyP by Esterification with NOL. A mixture of 5.9 mM polyP, 150 mM freshly dissolved EDAC, and 200 to 525 mM NOL was incubated for 1 h at 65 °C. Reaction volumes varied from 0.35 to 40 mL, and mixtures were agitated throughout, since the NOL concentrations exceeded solubility limits even in hot water. Completed reactions were cooled on ice and polyP was isolated by acetone precipitation. Because some free NOL coprecipitated with polyP in the first acetone precipitation, three full cycles of acetone precipitation were employed in which the collected polyP pellets were completely resuspended in water and reprecipitated by addition of NaCl and acetone followed by centrifugation. PolyP-NOL was then further purified using Bio-Gel P-6 desalting columns (Bio-Rad; Hercules, CA). The polyP-containing column fractions were identified by toluidine blue staining,[16] pooled, and lyophilized.

EDAC-Mediated End-Labeling of PolyP by Esterification with MU. A mixture of 5.5 mM polyP, 150 mM freshly dissolved EDAC, and 280 mM MU in a reaction volume of 1 mL was incubated for 1 h at 65 °C with agitation because the concentrations of MU used exceeded solubility limits. The reactions were then cooled on ice and polyP-MU was isolated using acetone precipitation as described above for the preparation of polyP-NOL.

NMR Analyses. Purified polyP and polyP derivatives were dissolved in water containing 10% (v/v) D_2O. All solution NMR spectra were collected on a Varian Unity INOVA 600 MHz proton frequency spectrometer with a 5 mm Varian AutoTuneX 1H/X PFG Z probe at 23 °C. 1D ^{31}P and ^{13}C spectra were acquired with a 2 s recycle delay. 1D ^{1}H spectra were acquired with a 1 s recycle delay, and solvent suppression was done by presaturation. 2D $^{1}H-^{13}C$ Heteronuclear Single Quantum Coherence (HSQC) spectra were acquired with 2048 and 160 points in the ^{1}H and ^{13}C dimensions, respectively. ^{1}H and ^{13}C spectra were referenced with external tetramethylsilane at 0 ppm, and ^{31}P spectra were referenced with external phosphoric acid at 0 ppm. 1D spectra were processed with MNOVA (MestreLab Research), and 2D spectra with NMRPipe.[17] polyP: ^{31}P NMR (90% H_2O 10% D_2O, 243 MHz) δ: −7.01 (s), −21.14, −21.65. polyP-methanol: ^{31}P NMR (90% H_2O 10% D_2O, 243 MHz) δ: −9.36 (d, $J_{P,P}$ = 17.8 Hz), −21.67. ^{1}H NMR (90% H_2O 10% D_2O, 600 MHz) δ: 3.51 (d, $J_{H,P}$ = 11.42 Hz). ^{13}C NMR (90% H_2O 10% D_2O, 151 MHz) δ: 53.7. polyP-NOL: ^{31}P NMR (90% H_2O 10% D_2O, 243 MHz) δ: −10.43, −16.81(d, $J_{P,P}$ = 17.7 Hz), −21.43, −21.61.

Gel Electrophoresis of PolyP. PolyP preparations were resolved on urea-containing 15% polyacrylamide gels and visualized using DAPI negative staining as described.[18]

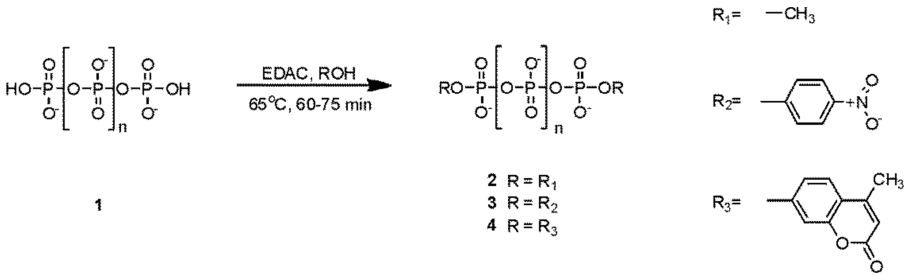
Alkaline Phosphatase Digestion of PolyP. Protection against exopolyphosphatase-mediated degradation was employed to determine the extent to which polyP molecules were doubly end-labeled, as previously described.[13] Such digestions used calf intestinal alkaline phosphatase (CIAP, Promega; Madison, WI), a highly active exopolyphosphatase.[6] Typical reactions included 250 μM polyP and 20 units/mL CIAP; the liberated monophosphate was quantified using malachite green analysis.[14]

PolyP-NOL preparations often varied in the extent to which both ends of polyP were derivatized. To rid these preparations of singly labeled polyP, some were digested to completion with recombinant shrimp alkaline phosphatase (SAP, New England BioLabs; Ipswich, MA) by incubating 50 mM derivatized polyP with 50 U/mL SAP for 2 h at 37 °C in the manufacturer's buffer. SAP then was inactivated by heating at 65 °C (7 min), after which the remaining polyP was repurified by acetone precipitation. These preparations were termed SAP-treated polyP-NOL.

Endopolyphosphatase Digestion of PolyP. Certain nudix hydrolases were examined for endopolyphosphatase activity, typically in a two-stage assay. In the first stage, polyP-MU or SAP-treated polyP-NOL was incubated with endoacting enzyme (Nudt2 or Nudt3, Fitzgerald Industries International; Acton, MA) in the appropriate buffer at 37 °C, after which the reactions were chilled on ice. Buffer conditions were the following: for Nudt2, 8 mM SAP-treated polyP-NOL or 2 mM polyP-MU, 50 mM HEPES pH 7.4, and 5 mM $MgCl_2$; for Nudt3: 5.5 mM SAP-treated polyP-NOL or 2 mM polyP-MU, 25 mM HEPES pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol.

For the second stage, a solution of CIAP in 100 mM Tris-HCl pH 8.8, 0.2 mM $ZnCl_2$ was prepared and warmed to 37 °C in 96-well polystyrene plates (Corning; Tewksbury, MA). The second stage was initiated by pipetting 100 μL of the chilled nudix-polyP reaction into prewarmed wells containing 100 μL CIAP solution, after which the rate of dye release was monitored spectrophotometrically at 37 °C. For polyP-NOL substrate, absorbance at 400 nm was measured using a SpectraMax M2 microplate reader (Molecular Devices; Sunnyvale, CA); for polyP-MU substrate, fluorescence was quantified in

Scheme 1. EDAC-Mediated Esterification of the Terminal Phosphates of PolyP[a]



[a]Compounds: **1**, polyP; **2**, polyP-methanol; **3**, polyP-NOL; **4**, polyP-MU.

fluorescence mode using excitation at 360 nm, emission at 450 nm, and a 435 nm cutoff filter.

RESULTS

EDAC-Mediated Esterification of the Terminal Phosphates of PolyP with Methanol. EDAC has been used to promote the formation of ester linkages between alcohols and carboxylates,[19] as well as phosphoester linkages between alcohols and certain organic phosphates.[20] We therefore examined whether EDAC could promote the formation of ester linkages between alcohols and the terminal phosphate groups of inorganic polyP (Scheme 1).

As proof of principle, and to identify reaction conditions more readily, we examined EDAC-mediated esterification of polyP with methanol. The reaction resulted in a product that was protected from exopolyphosphatase (CIAP) digestion to an extent comparable to polyP that had been end-labeled with spermidine via phosphoramidate linkages (Table 1).

Table 1. Resistance of PolyP Derivatives to Hydrolysis by Alkaline Phosphatase (CIAP)

reactant	end label	% hydrolysis		
(none)	–	96.0	±	16.7
spermidine	$NH_2(CH_2)_3NH(CH_2)_4NH$–	20.8	±	2.1
methanol	H_3CO–	19.8	±	2.0
NOL	$O_2NC_6H_4O$–	27.0	±	2.0
MU	$C_{10}H_7O_3$–	3.9	±	1.6

We used solution-state NMR to further verify the product's identity. In the ^{31}P spectra, unmodified polyP displayed a relatively broad alpha (terminal) phosphorus peak at about 7 ppm (Figure 1A); the broadness likely reflects exchange of protonation states of the phosphate group.

Methylated polyP displayed an alpha peak shifted to 9.4 ppm (Figure 1B), which was also much sharper than the alpha peak of underivatized polyP. The methylated polyP alpha peak sharpness likely is caused by attenuation of the exchange broadening. This shifted alpha peak displayed as a doublet (Figure 1C) owing to the ^{31}P–^{31}P *J*-coupling between the alpha- and beta-phosphorus atoms. We next utilized the $^3J_{H-P}$ coupling between the methyl protons and alpha-phosphorus atom to confirm end modification and map connectivity. When proton decoupling was turned off, each peak of the alpha phosphorus doublet signal was split into a quartet pattern (Figure 1D), indicating the presence of three neighboring protons. To assign the chemical shift of the methyl protons, we acquired a 1D ^{1}H spectrum with heteronuclear decoupling irradiation at the alpha phosphorus frequency. The resulting spectrum, when compared to the spectrum without decoupling (compare Figure 1E and F), shows that the peak at around 3.5 ppm is converted from a doublet to a singlet. This transformation is consistent with a proton signal being coupled to a single neighboring phosphorus atom. To identify the carbon signal of the methyl group, we used ^{13}C-enriched (20%) methanol to synthesize methylated polyP. The resulting 1D ^{13}C spectrum (Figure 1H) shows significant enhancement of the carbon peak at ~53.6 ppm, which was almost unobservable in natural abundance methyl-polyP preparation (Figure 1G). To confirm that coupling existed between the carbon peak and the previously identified methyl proton signal, we performed 2D ^{1}H–^{13}C HSQC on the ^{13}C-enriched sample, with clearly observable correlation between the two signals (Figure 1I).

PolyP End-Labeled with NOL or MU. NOL is used often in making chromogenic substrates, as its absorption spectrum shifts dramatically when ester-linked to carboxylates or a single phosphate. MU is also extensively employed in synthesizing fluorogenic substrates because its fluorescence is quenched when ester-linked to carboxylates. Reacting polyP with NOL and EDAC resulted in a polyP preparation that was nearly as resistant to CIAP digestion as was polyP end-labeled with spermidine or methanol (Table 1). Reacting polyP with MU and EDAC resulted in a product with even greater CIAP resistance (Table 1) than that of polyP derivatized with spermidine or methanol. Absorption spectra of polyP-NOL before and after hydrolysis reveal an absorption maximum of 285 nm before hydrolysis (indicating covalent coupling of the NOL dye to phosphate) and 398 nm after hydrolysis (characteristic of free NOL; Figure 2C).

Analyzing polyP-NOL by 1D ^{31}P NMR produced an alpha peak shifted from ~7 ppm in underivatized polyP (Figure 1A) to a doublet at ~17 ppm in polyP-NOL (Figure 3A), consistent with covalent modification of the terminal phosphates. Resolving polyP and polyP-NOL by gel electrophoresis indicated little change in the distribution of polymer lengths after reacting the polyP with NOL and EDAC (Figure 3B).

Exopolyphosphatase (CIAP) Digestion of PolyP-NOL. End-labeling efficiency was calculated after complete acid hydrolysis of polyP-NOL and quantification and comparison of liberated NOL and monophosphate ratios (and assuming a mean polymer length of 50 phosphates). Using a polyP-NOL preparation in which approximately 40% of the polyP molecules were singly end-labeled, we added CIAP and monitored A_{400} versus time at 37 °C (Figure 3C). These results show that singly labeled polyP-NOL can be used to follow the progress of exopolyphosphatase digestion. The curvilinear progress curves probably reflect the fact that the substrate is ~50 phosphates long but the chromophore is

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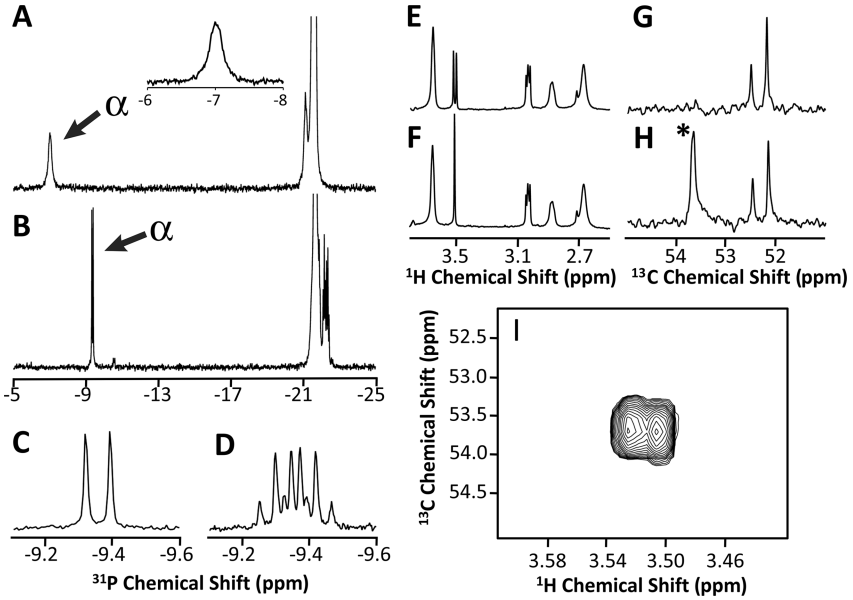

Figure 1. NMR analyses of end-methylated polyP. 1D ^{31}P spectra of (A) unmodified polyP and (B) polyP following reaction with methanol and EDAC. The terminal phosphate (alpha) peaks are indicated by arrows, and the inset in panel A is an expanded view of the alpha peak region of this spectrum. (C) The expanded view shows that the sharp alpha peak in panel B is resolved as a doublet. (D) Without ^{1}H decoupling, the alpha peak from methylated polyP expands into quartets as a result of *J* couplings from methyl protons. (E,F) 1D ^{1}H spectra of methylated polyP with (E) and without (F) applied decoupling irradiation at the alpha-phosphate frequency. The doublet peak at ~3.5 ppm (E) converts into a singlet (F) after irradiation because the proton peak is coupled to a phosphorus atom. (Other peaks in the ^{1}H spectra are from the MES buffer used in product purification and thus not affected by the decoupling.) 1D ^{13}C spectra of methylated polyP prepared with (G) natural abundance methanol or (H) 20% ^{13}C-enriched methanol. Use of ^{13}C-enriched methanol in the reaction greatly enhanced the signal at ~53.7 ppm (asterisk). (Other ^{13}C peaks are from the MES buffer.) (I) 2D HSQC analysis of the 53.7 ppm ^{13}C peak confirmed that the ^{13}C atoms are connected to the methyl protons.

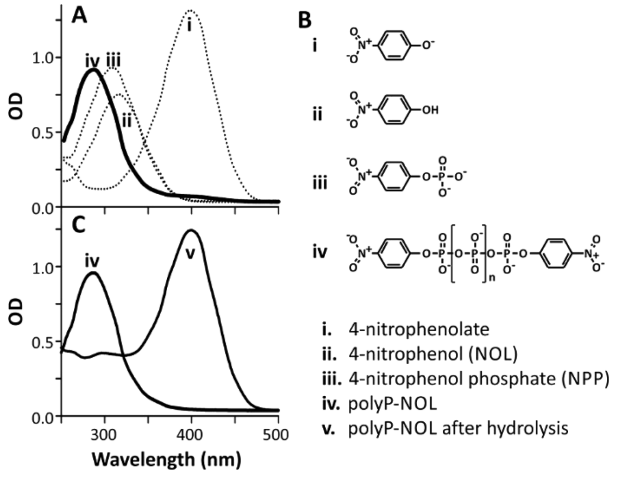

Figure 2. Product analysis and NOL substitution series using UV−vis spectroscopy. (A) Absorbance spectra of NOL substitution series with (B) the accompanying compounds' chemical structures. Each compound (except (ii)) was brought to a final concentration of 150 μM in alkaline conditions (1.5 M Tris-HCl buffer pH = 8.8, final) and scanned spectrally; NOL (ii) was scanned under acidic conditions. Absorbance maxima were the following: 398 nm (i), 317 nm (ii), 310 nm (iii), and 285 nm (iv). (C) Absorption spectra of polyP end-labeled with NOL, before (iv) and after (v) acid hydrolysis (1 M HCl for 1 h at 100 °C) followed by alkalization to pH 8.8.

released only when the last phosphate is removed from the substrate.

Endopolyphosphatase Digestion of PolyP-NOL and PolyP-MU. Covalent modification of polyP on both ends protects polyP against exopolyphosphatase (CIAP) digestion (Table 1). We therefore reasoned that two-stage assays for endopolyphosphatase activity could be devised using polyP that is completely labeled on both ends with chromophore or fluorophore. Digestion by exopolyphosphatase should be possible only after the action of endopolyphosphatase has exposed free polyP ends. In such an assay one could employ either sequential or simultaneous digestion with endo- and exopolyphosphatases. Accordingly, polyP-NOL was predigested to completion with SAP to eliminate singly labeled molecules, after which the polyP-NOL was repurified. We then used this SAP-treated polyP-NOL in a two-stage endopolyphosphatase assay in which we first digested the substrate with either Nudt3 (a hydrolase with known endopolyphosphatase activity[12]) or Nudt2 (another nudix hydrolase that cleaves dinucleotide polyphosphates, "Np$_n$Ns", but whose endopolyphosphatase activity was not known) and then monitored product release during digestion with CIAP. Figure 4 shows that few dye molecules from SAP-treated polyP-NOL were released upon incubating the substrate with either endopolyphosphatase (Nudt2 or Nudt3) or exopolyphosphatase (CIAP) alone. However, polyP-NOL was readily hydrolyzed by CIAP following pretreatment with either Nud2 or Nudt3. Additionally, this experiment demonstrated that Nudt2 has endopolyphosphatase activity (Figure 4B).

The efficiency of polyP labeling with MU was usually greater than that with NOL (Table 1), so predigestion of polyP-MU (Figure 5A) with SAP was typically not required before using this substrate to detect endopolyphosphatase activity. Figure 5B shows the reaction curves for two-stage assays of polyP-MU digestion with Nudt2 followed by CIAP. Neither Nudt2 nor CIAP alone released significant MU, while polyP-MU was efficiently digested by CIAP after incubation with Nudt2.

This experiment also demonstrates the amount of CIAP required for maximal rates of product release. Figure 5C shows

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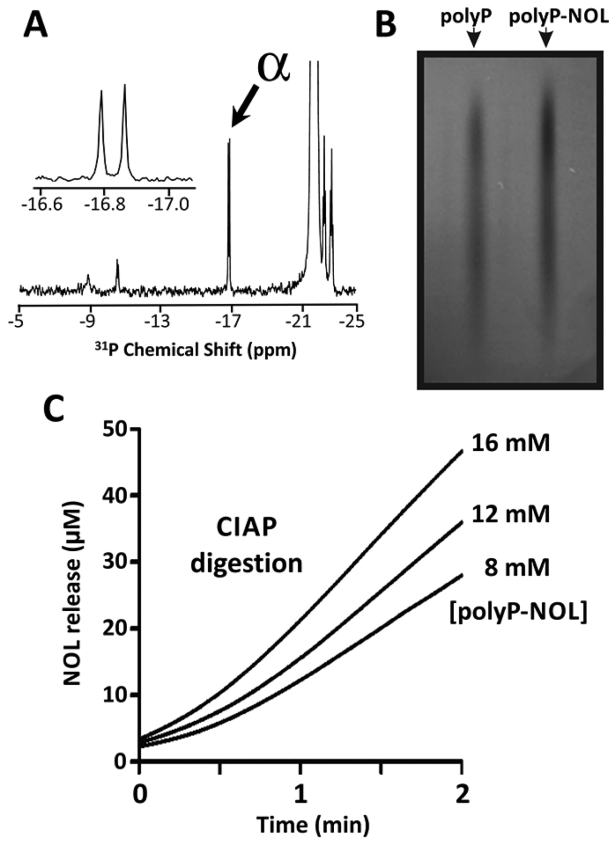

Figure 3. Analysis of polyP end-labeling with NOL. (A) 1D ^{31}P spectrum of polyP-NOL highly labeled at both ends. The alpha phosphate peak, indicated by an arrow, is resolved as a doublet (inset) upon axis expansion. (B) Comparison of polyP and polyP-NOL resolved by gel electrophoresis and detected by DAPI negative staining. (C) Enzymatic degradation of varying concentrations of incompletely end-labeled polyP-NOL (8 to 16 mM phosphate) by CIAP, with NOL release detected spectrophotometrically at 400 nm.

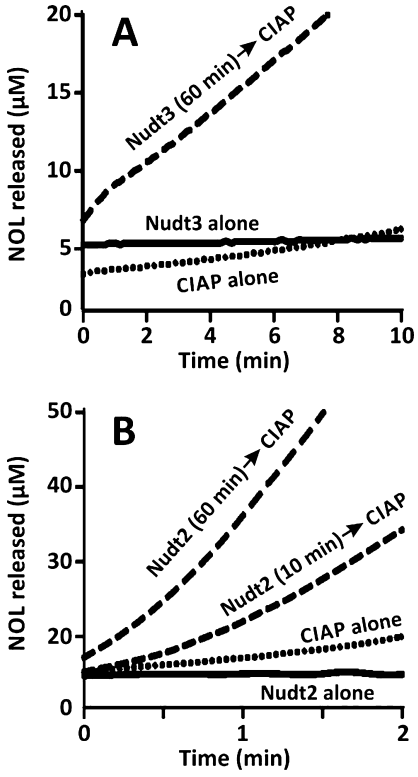

Figure 4. Sequential digestion of doubly end-labeled polyP-NOL with endopolyphosphatases and exopolyphosphatases. In both panels, doubly end-labeled polyP-NOL was first incubated with or without endopolyphosphatase (Nudt2 or Nudt3) for 10 or 60 min at 37 °C. Exopolyphosphatase (120 U/mL CIAP) was then added to the indicated samples and NOL release was quantified over time. (A) Treatment with 850 nM Nudt3. (B) Treatment with 250 nM Nudt2.

a much shorter time course of the second stage of this two-stage assay, using saturating levels of CIAP. We tested Nudt1 (a nudix hydrolase known to cleave Ap_3A but not long-chain Np_nN molecules) in a similar two-stage assay, but under the various conditions we used, the enzyme did not support MU release by CIAP (data not shown). We also examined a one-stage assay employing polyP-MU incubated simultaneously with CIAP plus varying concentrations of Nudt2. We found limited product release with CIAP alone but robust product release by the combination of CIAP and Nudt2 (Figure 5D).

DISCUSSION

This study had two goals: expand the covalent coupling chemistry for polyP, allowing for facile linkage of alcohols to the terminal phosphates of polyP via phosphoester bonds; and use this chemistry to develop high-throughput methods for detecting and quantifying the enzymatic digestion of polyP. We now report that the water-soluble cross-linker, EDAC, can be used to efficiently generate phosphoester linkages between alcohols and the terminal phosphates of polyP. We utilized this coupling chemistry to generate new chromogenic and fluorogenic substrates for detecting the enzymatic hydrolysis of polyP, based on phosphoester end-labeling of polyP with NOL or MU, respectively. Additionally, with our polyP substrates, we were able to monitor and distinguish endo- and exopolyphosphatase activities in real time.

Previously, we reported that EDAC could be used efficiently to couple compounds with primary amines to the terminal phosphates of polyP via phosphoramidate linkages, and this chemistry has allowed us to link a variety of probes to polyP;[13] for example, we used this method to biotinylate polyP, which we then employed to detect and quantify interactions between polyP and blood clotting proteins.[21,22] Although phosphoramidate linkages are relatively stable under neutral and alkaline conditions, the linkages are highly acid-labile.[23] It would be advantageous, therefore, to be able to efficiently link the terminal phosphates of polyP to organic compounds via phosphoester linkages, which, unlike phosphoramidate linkages, resist acid hydrolysis at physiological temperatures.[23] A previous study used combinations of carbodiimides (other than EDAC) in conjunction with polyP and alcohols in anhydrous organic solvents to generate phosphoester linkages to polyP; however, the reactions also caused substantial polyP hydrolysis to much shorter polyP chains and the apparent formation of cyclic and branched polyP adducts (which are unstable in aqueous solution).[24] In this report we identified aqueous coupling conditions for EDAC-mediated formation of phosphoester linkages to polyP that resulted in labeling just the terminal phosphates and that did not appreciably shorten the polyP chains.

Full-length polyP capped on either end with chromogenic or fluorogenic dyes was used to detect phosphatase activity. PolyP-

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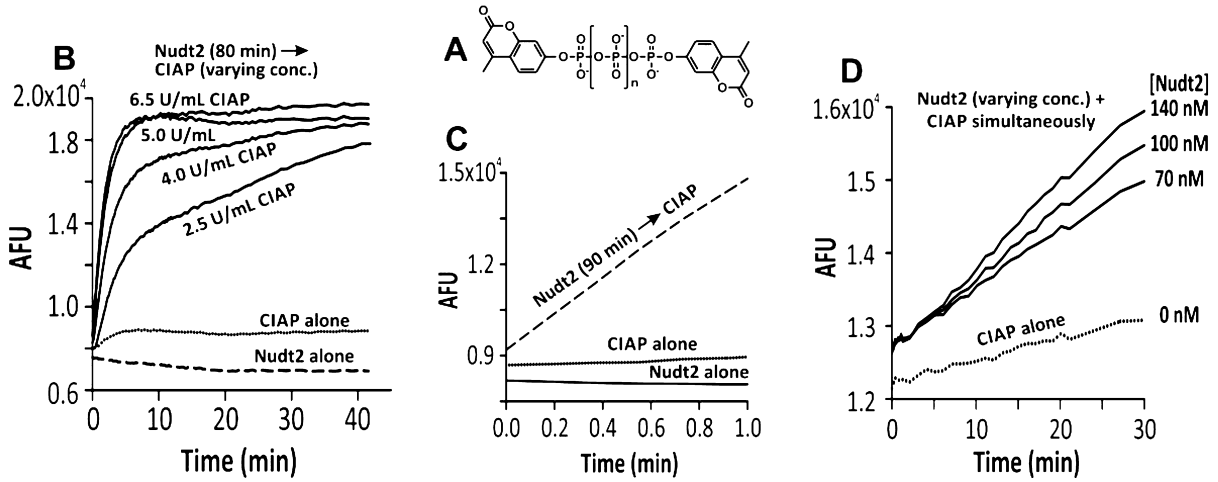

Figure 5. Digestion of polyP-MU by mixtures of endo- and exopolyphosphatases. (A) Structure of doubly end-labeled polyP-MU. (B) Sequential digestion of doubly end-labeled polyP-MU with endopolyphosphatase and exopolyphosphatase. PolyP-MU was first incubated with or without endopolyphosphatase (250 nM Nudt2) for 80 min at 37 °C, after which varying concentrations of exopolyphosphatase (CIAP) were added to the indicated samples and the increase in fluorescence (AFU) was quantified over time. (C) Sequential digestion of doubly end-labeled polyP-MU, first, with or without 250 nM Nudt2 for 90 min at 37 °C, after which 5 U/mL CIAP was added to the indicated samples and fluorescence was quantified over time. (D) Simultaneous digestion of polyP-MU with the indicated concentrations of Nudt2 and 55 U/mL CIAP, during which fluorescence was quantified over time.

NOL preparations that were incompletely labeled on both polyP ends were useful substrates for detecting exopolyphosphatase activity, which released free NOL upon the complete hydrolysis of the singly end-labeled polyP. On the other hand, polyP-NOL and polyP-MU preparations that were fully labeled on both polyP ends were highly resistant to exopolyphosphatase digestion, and this property was used as the basis of a two-stage assay in detecting endopolyphosphatase activity. In such assays, endopolyphosphatase digestion creates free polyP ends which are then substrates for exopolyphosphatase (CIAP) digestion, which in turn releases free dye from polyP-NOL or polyP-MU.

Not surprisingly, assays using the fluorogenic substrate, polyP-MU, could be conducted using lower substrate concentrations than those using the chromogenic substrate, polyP-NOL, owing to the greater sensitivity of fluorescence-based detection methods; however, assays using fluorogenic substrates require more specialized equipment and sample handling than do simple chromogenic assays, prompting us to develop both types of substrates in this study.

We constructed a two-stage assay to demonstrate the activity of a known endopolyphosphatase (Nudt3), and to demonstrate that another nudix hydrolase (Nudt2) also exhibits endopolyphosphatase activity. Nudt3, sometimes called DIPP1, or simply DIPP, is a nudix-type enzyme with multiple known in vitro substrates: capped mRNA, oxo-8-dGTPase, inositol pyrophosphates, dinucleotide polyphosphates, and inorganic polyphosphate (reviewed by McLennan[25]). Many nudix-type phosphatases are clinically important enzymes and their overexpression can be markers of disease. Nudt2 (Apah1), for example, is an Ap_4A hydrolase that, when overexpressed in breast cancer, correlates with poor prognosis.[26] In addition to processing Ap_4A, Nudt2 can hydrolyze long-chain Np_nNs such as Ap_6A. We hypothesized that this nudix enzyme, though previously not described as having endopolyphosphatase activity on inorganic polyP, might be able to cleave polyP and that we might detect this cleavage using our substrates in conjunction with CIAP. This was confirmed with our novel substrates. Like Nudt2, the human short-chain exopolyphosphatase, h-prune, is also implicated in tumor survival,[7] again providing a connection between alterations in polyP degradation and human health.

It should also be noted that the substrates only release a signal (free dye) when the last phosphate of the polyP chain is removed by an exopolyphosphatase. While this reaction was efficiently catalyzed by CIAP, other exopolyphosphatases might digest polyP to very short chains but not completely to monophosphate and therefore would not be expected to release the dye from these substrates. Such enzymes could be studied using these substrates in sequential assays employing CIAP.

We anticipate that the utilization of chromogenic and fluorogenic polyP substrates will aid further inquiry into how polyphosphates are turned over enzymatically. For example, an additional class of enzymes that could conceivably be studied using these substrates are kinases that utilize polyP as a substrate/phosphate donor. Also important is the development of a method for making stable ester linkages to the ends of polyP. Applications for such phosphoester linkages could include attaching polyP to surfaces, fabricating polyP-containing nanoparticles, and attaching fluorescent probes to polyP, in addition to creating the chromogenic and fluorogenic substrates for polyP-degrading enzymes reported here.

■ CONCLUSIONS

Using carbodiimide-mediated chemistry, we selectively esterified the terminal phosphates of inorganic polyP polymers with various alcohols. In a proof-of-principle experiment, we used methanol in esterification reactions and confirmed the product through 1D and 2D ^{31}P, ^{1}H, and ^{13}C NMR analyses. We also showed that polyP could be similarly end-labeled with chromogenic or fluorogenic alcohols to form adducts. These adducts were shown to be useful substrates for polyP-degrading enzymes, allowing us to monitor enzyme activity spectrophotometrically in real time; furthermore, we used these substrates to identify a new function for the clinically significant enzyme,

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Nudt2. The chemistry and substrates developed in this work are likely to be useful for synthetic and clinical applications.

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Notes

The authors declare no competing financial interest.

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