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Non-enzymatic quantification of polyphosphate levels in platelet lysates and releasates

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ABSTRACT

Inorganic polyphosphate has been shown to be shed upon platelet activation inducing prothrombotic stimuli on the coagulation system. Several methods have been published to detect and quantify polyphosphate in various cells and tissues, but evaluation of platelet content has only been achieved by indirect detection of orthophosphate after enzymatic digestion, thus, relying heavily on specificity of an exopolyphosphatase that is not commercially available.

We present a non-enzymatic method for quantification of platelet-derived polyphosphate featuring optimized extraction on silica spin-columns, followed by specific fluorescence detection using DAPI. This allowed us to quantify polyphosphate in platelet lysates, but also in releasates of TRAP-activated platelets for the first time.

Extraction of exogenous polyphosphate from buffer and sample matrices resulted in quantitative yields while removing matrix effects observed with direct fluorescence detection. Treatment of eluted fractions with phosphatase completely abrogated polyphosphate-specific fluorescence arguing for no additional compounds influencing the fluorescence detection. This was confirmed by no change in fluorescence intensity in samples previously treated with DNase and RNase.

Taken together, we developed a robust and easily standardizable method to quantify polyphosphate in platelet lysates and releasates that will facilitate polyphosphate related investigations of platelet physiology and coagulation.

1. Introduction

Polyphosphate (PolyP) is a linear polymer of orthophosphate residues linked by high-energy phosphoanhydride bonds [1]. This inorganic molecule is present at various chain lengths in prokaryotic and eukaryotic cells [2–4]. Microorganisms usually contain long-chain PolyP with hundreds to thousands of phosphate monomers [5], while PolyP in human cells exhibits a shorter chain length of up to 100 monomers [6].

Recently, PolyP gained interest due to its potential prothrombotic influence on the coagulation system. It has been shown that PolyP can activate factor XII being the first known physiologic activator of the intrinsic coagulation system [7]. Furthermore, PolyP accelerates thrombin generation by promoting factor V activation, inhibiting TFPI, and enhancing the feedback loop of factor XI activation by thrombin [8,9].

PolyP with an average chain length of 70–75 phosphate units has been detected in dense granules of platelets shed upon platelet activation [10]. There has been much debate over the ability of platelet-derived PolyP to start coagulation by activating factor XII. The dominant influence of these isoforms on coagulation is more likely mediated via factor V, factor XI and TFPI [11,12].

Several attempts have been made to detect and quantify PolyP in various tissues and cells. Initially, PolyP was detected by staining with toluidine blue or by chemical hydrolysis followed by staining of orthophosphate [2,13–15]. However, toluidine blue is a nonspecific dye for polyanions, and quantification can be hampered by DNA/RNA contaminants. Also methods involving chemical hydrolysis are not sufficiently specific for PolyP quantification in physiologically low concentrations. Other methods, such as NMR are only applicable at higher concentrations, and require large amounts of sample material [5].

The most commonly used method of PolyP estimation in various cells and tissues is the selective digestion of PolyP with exopolyphosphatases, and quantification of released orthophosphate with malachite green [1,3,16,17]. This indirect method is

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quite sensitive, but relies heavily on enzyme-specificity. Particularly quantification in whole cell lysates containing low amounts of PolyP next to high amounts of other potential orthophosphate sources might lead to overestimation. Furthermore, the required exopolyphosphatases are not commercially available.

A DAPI-based fluorescence assay has been proposed for the detection and quantification of PolyP [18]. The authors demonstrated that excitation at 415 nm and detection at 550 nm is highly specific for PolyP of various chain lengths. This fluorescence assay has been shown to be unaffected by DNA, RNA, ADP and ATP [18–20], but some inositol phosphates undergo a spectral shift that is similar to that of PolyP, which might impair specificity [21].

The method has been proposed for direct quantification of PolyP in lysates of microbial cells [22]. However, direct quantification from complex biological mixtures is hampered by substantial matrix effects including nonspecific autofluorescence as well as quenching of DAPI. PolyP in biological samples is usually associated with protein potentially posing a steric hindrance for DAPI. Several PolyP isolation and enrichment methods have been developed to circumvent matrix effects employing anion exchange resins [1,11], precipitation with cetyltrimethyl-ammonium bromide and barium chloride [13], and adsorption on glassmilk or silica columns [23].

Successful estimations of PolyP content in platelets have been performed, but results vary significantly, most likely due to differences in methodologies [10,24].

To our knowledge, non-enzymatic quantification of PolyP in platelet releasates has not been done yet. In this work, we present a modified silica based isolation protocol for PolyP from environmental plankton samples [20] and combined it with aforementioned fluorescence measurements using DAPI. The resulting method allows for rapid and robust quantification of PolyP from platelet lysates and platelet releasates.

2. Method

2.1. Materials

A Polyphosphate standard with an average length of 100 phosphate units was purchased from Kerafast (Boston, Massachusetts, USA). Prostaglandin I_2 was obtained from Cayman (Ann Arbor, USA). Thrombin receptor activating peptide-6 was purchased from Multiplate (Multiplate Services GmbH, Munich, Germany). Silica spin columns and Qiagen protease were obtained from a QIAmp DNA blood mini kit (Qiagen, Hilden, Germany). DNase and RNase cocktails were from Ambion (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All other reagents were purchased from Sigma Aldrich (Vienna, Austria), if not stated otherwise.

2.2. Sample preparation

Blood was drawn with a 21 gauge needle from the antecubital vein, without applying venostasis, into pre-citrated S-Monovette premarked tubes (3 ml) from Sarstedt, containing 0,30 ml 0,106 mol/l trisodium citrate solution. Platelet rich plasma was obtained by centrifugation (200 × *g*, 10 min). Platelet counts were obtained using a Sysmex XS-1000i.

For measurements of PolyP-content in platelets, platelets were pelleted from platelet rich plasma by centrifugation (2200 × *g*, 10 min) after addition of prostaglandin I_2 (0,5 μM). Platelets were resuspended in sample buffer devoid of divalent ions to prevent chemical hydrolysis of PolyP (5 mM Tris, 100 mM NaCl, 5 mM EDTA, pH 7,4), and subjected to repetitive ultrasonic bursts (6 × 15 s) on ice. Samples were kept on ice until further treatment.

Washed platelet suspensions were prepared from platelet rich plasma by addition of prostaglandin I_2 (0,5 μM) followed by centrifugation (2200 × *g*, 10 min). Platelets were washed twice with sample buffer. Platelet counts were obtained using a Sysmex XS-1000i. For platelet releasate studies washed platelets were activated with TRAP-6 (32 μM) at 37 °C under constant stirring for 15 min, and then removed by centrifugation (2200 × *g*, 10 min). The pellet of activated platelets was lysed as described above. Supernatants and lysates were kept on ice for further treatment. All samples were immediately subjected to protease digestion according to manufacturers' instruction at 56 °C for 10 min, and kept on ice for PolyP isolation.

2.3. Polyphosphate extraction

250 μl of each sample was mixed with 750 μl 5,5 M guanidine isothiocyanate (GITC) and 2 ml ethanol. Mixtures were immediately loaded onto the spin columns in 5 × 600 μl aliquots, respectively, and washed twice with a sample buffer-ethanol mixture (1:1, v/v). Spin columns were dried in a high speed centrifugation step (11.000 x *g*, 3 min), and PolyP was eluted into new containers with 5 × 90 μl aliquots of sample buffer, respectively.

2.4. Fluorescence measurements

550 μl of sample buffer and 50 μM of 4′,6-diamidino-2-phenylindole (DAPI) were added to the PolyP elutions. Measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer equipped with a sipper. Excitation and emission wavelengths were set for highest PolyP specificity according to Aschar-Sobbi et al. (excitation 415 nm, emision 550 nm) [18]. Fluorescence was recorded for one min, respectively, and repeated until signal remained stable (standard deviation < 2% of the mean).

2.5. Direct detection of PolyP in platelet lysates

Platelet lysates were prepared as described above. Additionally 50, 100, 150, 200, and 250 ng PolyP were added respectively, and samples were directly subjected to fluorescence measurements using 50 μM DAPI. Fluorescence intensities were compared with intensities of corresponding PolyP standards in sample buffer. Differences were evaluated using Student's *t*-test.

2.6. Evaluation of PolyP recovery rate

PolyP standards were added to sample buffer to give an overall amount of 50, 100, 150, 200, and 250 ng PolyP in 250 μl respectively. These standard mixtures were subjected to PolyP extraction as described above, followed by fluorescence measurements. Another set of standards was brought to 1 ml with sample buffer for direct fluorescence measurements. All procedures were performed in triplicates.

2.7. Evaluation of matrix effects

Platelet lysates and releasates were spiked with 50, 100, 150, 200, and 250 ng PolyP 100 respectively, before PolyP extraction. The corresponding fluorescence signals were compared with the signals of PolyP standards that have been extracted from buffer. All procedures were performed with three samples.

2.8. Evaluation of polyP-specificity

Elutions from platelet lysates and platelet releasates were subjected to PolyP digestion with alkaline phosphatase from calf mucosa (0,05 U/μg PolyP, 37 °C, 6 h). Fluorescence signals were compared with corresponding non-digested samples.