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Research Article

DNA ladders can be used to size polyphosphate resolved by polyacrylamide gel electrophoresis

PAGE is often used to resolve inorganic polyphosphates (polyP), but unfortunately polyP size ladders are not commercially available. Since several dyes that are commonly used to detect nucleic acids in gels also stain polyP, we examined the utility of commercially available DNA size ladders for estimating polyP polymer lengths by gel electrophoresis. Narrow size fractions of polyP were prepared and their polymer lengths were quantified using NMR. Commercially available DNA ladders and these polyP fractions were then subjected to PAGE to determine the relationship between migration of DNA vs polyP, which was found to be: $\log_{10}$(dsDNA length in bp) = 1.66 × $\log_{10}$(polyP length in phosphate units) − 1.97. This relationship between DNA and polyP size held for a variety of different polyacrylamide concentrations, indicating that DNA size ladders can readily be employed to estimate polyP polymer lengths by PAGE.

Keywords:
DNA ladder / Electrophoresis / PAGE / Polyphosphate
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Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Inorganic polyphosphates (polyP) are linear polymers of orthophosphate residues that are widespread throughout biology [1]. Although studied most intensively in unicellular organisms [1, 2], functions for polyP in higher organisms are now being revealed [3, 4]. This includes roles in blood clotting [5], angiogenesis [6], apoptosis [7], cell proliferation [8], energy metabolism [9], bone mineralization [10, 11] and tumor metastasis [12], and also as a covalent, post-translation modification for some proteins [13, 14]. Since polyP varies greatly in size depending on the source, and since the biological activities of polyP depend markedly on polymer length [15], it can be important to determine the size of polyP isolated from biological sources.

While methods other than PAGE exist for quantifying polyP polymer size, they are laborious and slow. PAGE is therefore a popular method for resolving polyP and determining the distribution of its polymer lengths [16, 17]. Although we have successfully employed narrowly size-fractionated polyP preparations for use as PAGE sizing ladders [15], polyP fractions of well-defined sizes are labor-intensive to prepare and not commercially available. We hypothesized that commercially available DNA ladders could be used as convenient size markers for estimating polyP polymer lengths using PAGE, especially since both polyP and DNA are linear, anionic polymers that can be stained using dyes such as toluidine blue or 4′,6-diamidino-2-phenylindole (DAPI) [16,18]. We therefore investigated the relationship between migration of polyP and DNA on PAGE. We now report conditions under which DNA size ladders can routinely be used to estimate polyP polymer lengths via PAGE.

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; **polyP**, inorganic polyphosphate

2 Materials and methods

2.1 Reagents

All chemicals were of analytical grade from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). The 10 bp DNA ladder was from Life Technologies (Grand Island, NY), and the 100 bp DNA ladder was from New England Biolabs (Ipswich, MA). Heterogeneous polyP preparations of varying size ranges were purchased from Sigma-Aldrich or were a kind gift from BK Giulini GmbH (Ludwigshafen, Germany).

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2.2 PolyP fractionation by preparative PAGE

Starting from heterogeneous polyP preparations, preparative PAGE was used to isolate narrowly size-fractionated polyP as previously described [15, 18]. PolyP concentrations were quantified by measuring inorganic phosphate [12] following hydrolysis in 1 M HCl at 100°C for 10 min, as described [18]. PolyP concentrations are reported here as phosphate monomer concentration.

2.3 Sizing of polyP via solution NMR

Eight narrowly size-fractionated polyP preparations were employed as size standards in this study. An aliquot of each was lyophilized and then dissolved in water containing 10% (v/v) D_2O, after which the pH was adjusted to 9. One-dimensional ^{31}P spectra of polyP samples were acquired three times at 23°C with a 3 sec recycle delay on a Varian Unity INOVA600 spectrometer with a 5 mm AutoTuneX 1H/X PFG Z probe. Chemical shifts were externally referenced with phosphoric acid at 0 ppm. Spectra were signal-averaged until the alpha phosphate peak achieved a signal-to-noise ratio of at least 5:1. All spectra were processed using MNOVA (MestreLab Research) with baseline correction and 1 Hz line broadening. An example of such a spectrum is shown in Supporting Information Fig. 1. Integration was performed to quantify the peak areas. The length of each polyP polymer was calculated from the ratios of the integration values for the alpha (terminal) phosphates and the internal phosphates, as described [15]. The polymer lengths of the polyP preparations determined using NMR are detailed in Supporting Information Table 1.

2.4 Analytical gel electrophoresis of polyP

PolyP was resolved by PAGE using commercially available, 8.6 × 6.8 cm polyacrylamide TBE gels from Bio-Rad (Hercules, CA), containing fixed 5, 10 or 15% polyphosphate, or a gradient of 4–20% polyacrylamide. Running buffer was TBE, and 5 × sample buffer contained 5 × TBE, 15% Ficoll 400, and 0.1% bromophenol blue. PolyP samples were loaded onto gels at 10 nmol phosphate monomer per lane. Electrophoresis was performed at 150 V for 20–50 min at 25° C. Gels were stained with agitation for 10 min with 0.05% toluidine blue in 25% methanol/5% glycerol, destained in three changes of 25% methanol/5% glycerol over 3 h, and imaged in white light on a GelDoc XR (Bio-Rad).

2.5 Evaluation of DNA ladder migration

PAGE migration distances for bands in commercially available DNA ladders were analyzed using ImageJ software [19]. Migration distances were determined by identifying the peak intensity of each band using the “plot lane” feature of the software. These distances were converted to R_f values by dividing the migration distance of the band by that of bromophenol blue. A standard curve was created by plotting $\log_{10}$ of the number of bp in the DNA polymer (y-axis) versus R_f (x-axis) using Sigma Plot 12.5. A cubic polynomial was fitted to the data set of identifiable DNA bands to achieve an r^2 of >0.99.

3 Results and discussion

The goal of these studies was to develop a simple, reproducible PAGE-based method for estimating polyP polymer lengths using commercially available DNA ladders.

3.1 Creation of the DNA equivalence model using 4–20% gels

Commercial DNA ladders and eight polyP preparations whose polymer lengths had been defined by NMR (ranging from 14 to 617 phosphates long) were resolved by PAGE in six separate experiments on TBE gels containing a gradient of 4–20% polyacrylamide. The R_f values for the DNA and polyP bands were measured relative to migration of the bromophenol blue dye. For the bands in the DNA ladders, the $\log_{10}$(DNA length) was plotted versus R_f, to which a cubic polynomial was fitted:

$$log_{10}\,(ds\,DNA\,length\;in\;bp) = a + b\,(R_f) + c(R_f)^2 + d(R_f)^3 \tag{1}$$

A representative example of a 4–20% gel is shown in Fig. 1A. The associated plot of DNA length versus R_f, along with the fitted polynomial, is shown in Fig. 1B.

For each individual 4–20% gel, the fitted polynomial was used to convert the R_f value of each polyP band into the equivalent DNA length predicted to migrate at that R_f value. These predicted DNA length equivalents were then used to prepare the migration equivalence model as described below. Note that the smallest polyP polymer (14 phosphates) stained poorly with toluidine blue and sometimes migrated off the gel, so this polyP band was excluded from further analysis. The two next smallest polyP polymers (44 and 50 phosphates) migrated faster than the smallest available DNA band (10 bp), so these polyP polymers were also excluded from developing the migration equivalence model.

To generate the DNA equivalence model, we used the polynomial derived from migration of bands in the DNA ladders to calculate the predicted equivalent DNA lengths in bp for the remaining polyP bands (81, 129, 288, 390, and 617 phosphates long) resolved on 4–20% gels. These equivalent DNA lengths are plotted vs polyP length as open circles in Fig. 2. A line was fitted to these data, yielding an equivalence model of:

$$log_{10}\,[DNA\,(bp)] = 1.66 \times log_{10}\,[poly\,P\,(phosphates)] - 1.97 \tag{2}$$

This data set therefore describes the equivalence between polyP length determined from NMR and calculated DNA

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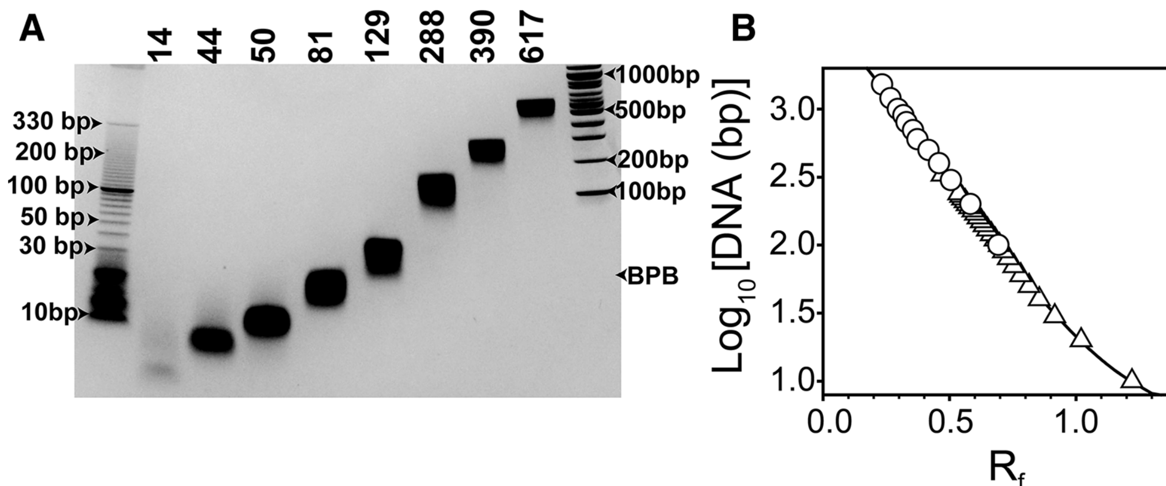

Figure 1. Representative comparison of the electrophoretic mobilities of DNA ladders and size-fractionated polyP preparations used to create the DNA equivalence model. (A) DNA ladders and polyP samples were resolved on 4–20% polyacrylamide gels. PolyP polymer lengths as determined by NMR are indicated at the top of the lanes. DNA ladders in bp (provided by the manufacturer) are indicated at left (for the 10 bp ladder) or right (for the 100 bp ladder). "BPB" indicates the migration of the bromophenol blue tracking dye. (B) Relationship between DNA length and migration on PAGE. Log_{10} of DNA lengths in bp for individual bands in the 10 bp (△) and 100 bp DNA ladders (○) were plotted against R_f. A cubic polynomial (*solid line*) was fitted to the data.

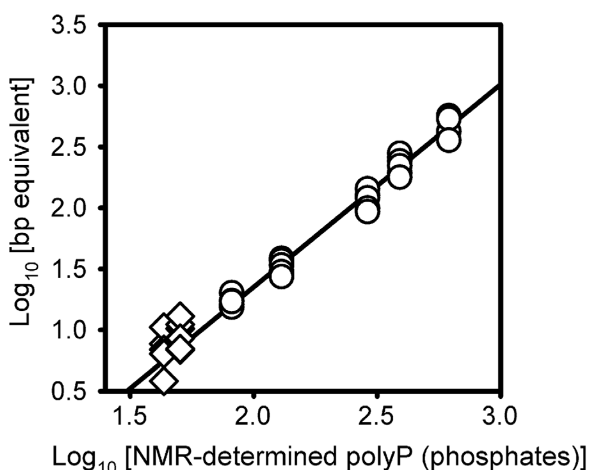

Figure 2. DNA-polyP equivalence model. Commercial DNA ladders and size-fractionated polyP preparations were resolved on six separate 4–20% polyacrylamide gels and analyzed as described in the text. The calculated equivalent DNA lengths (in bp) for the polyP bands in all six gels are plotted vs. the NMR-determined polyP lengths (in phosphates) on log_{10}-log_{10} axes. Plotted as open circles are the five polyP preparations used to create the model, which ranged in size from 81 to 617 phosphates. A line was fitted to these data ($r^2 = 0.99$), yielding the equivalence model given in the text as Equation (2). Two of the polyP preparations (44 and 50 phosphate units long, ◇) migrated faster than the smallest band in the DNA ladder, which was 10 bp. Although these two polyP sizes were not used to prepare the equivalence model, their values nevertheless lie essentially on the same line.

length based on R_f values on PAGE for 4–20% gels. Note that although the polyP polymers containing 44 or 50 phosphates were not used to create the equivalence model, their extrapolated DNA equivalence values (open diamonds in Fig. 2) fell on the same line as the equivalence data for the five larger polyP standards.

3.2 Application of the DNA equivalence model to gels of other polyacrylamide concentrations

We next evaluated whether the DNA equivalence model could be used to estimate the lengths of polyP resolved on non-gradient TBE gels containing 5, 10, or 15% polyacrylamide. Representative examples of these gels are presented in Fig. 3. As with the 4–20% gels, the $\log_{10}$(DNA length) values for the DNA ladder bands were plotted versus R_f, to which cubic polynomials were fitted. For each gel, the polynomial was used to convert R_f values of the polyP bands into equivalent predicted DNA sizes in bp, as described above for the 4–20% gels. PolyP sizes were then calculated according to the following rearrangement of Equation 2:

$$\text{polyP (phosphate units)} = 10\left[\frac{(1.97 + \log_{10}(DNA\,bp))}{1.66}\right] \quad (3)$$

The performance of this model using 5, 10, and 15% polyacrylamide gels is shown in Fig. 4A. The agreement between measured and calculated polyP lengths was excellent, with a Pearson Correlation coefficient of 0.982. A bias in performance of the model relative to the polyacrylamide concentration was not apparent from this plot. This finding indicates that the relationship described in Equations (2) and (3), although developed using 4–20% gradient gels, applies to non-gradient gels over a range of polyacrylamide concentrations.

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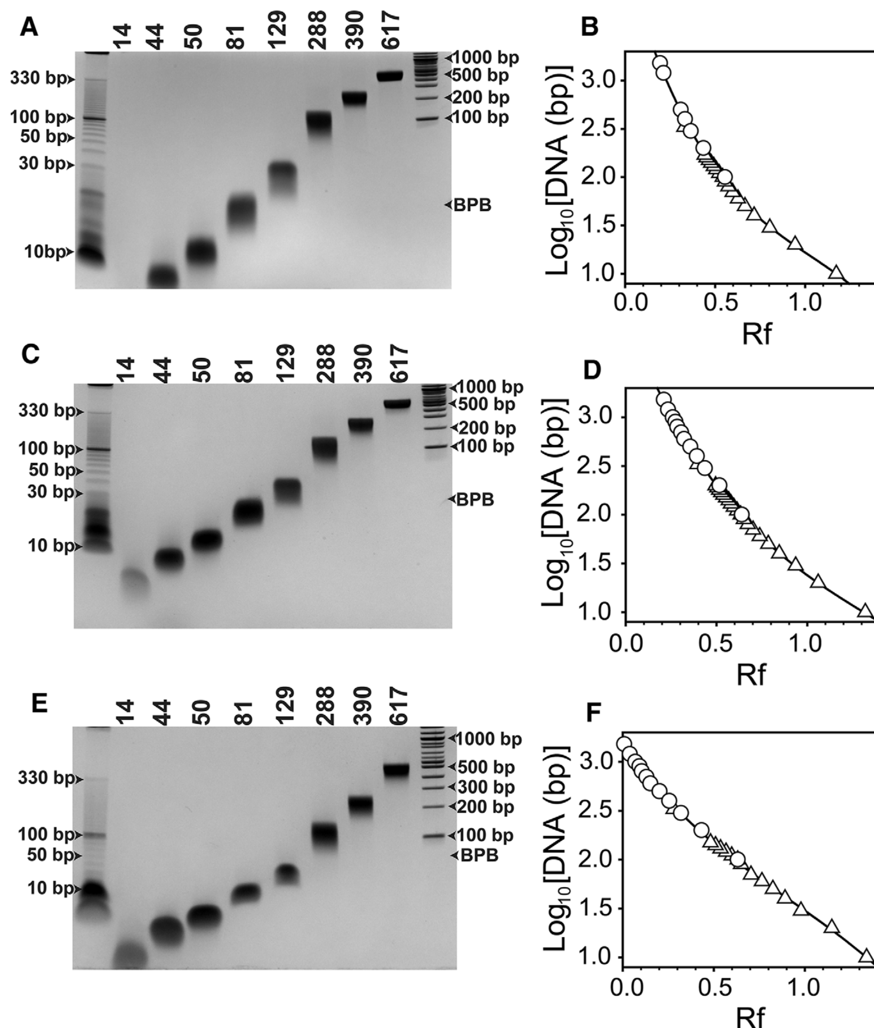

Figure 3. Representative comparisons of the electrophoretic mobilities of DNA ladders and size-fractionated polyP preparations resolved on gels with fixed percentages of polyacrylamide. DNA ladders and polyP samples were resolved on (A) 15%; (C) 10%; or (E) 5% polyacrylamide gels. PolyP polymer lengths as determined by NMR are indicated at the top of the lanes. DNA ladders in bp (provided by the manufacturer) are indicated at left (10 bp ladder) or right (100 bp ladder). "BPB" indicates the migration of the bromophenol blue tracking dye. Log_{10} of DNA lengths in bp for individual bands in the 10 bp (△) and 100 bp DNA ladders (○) were plotted against R_f for: (B) 15%; (D) 10%; or (F) 5% polyacrylamide gels. Cubic polynomials (*solid lines*) were fitted to the data.

3.3 Reproducibility of polyP size values obtained using the DNA equivalence model

Using thirteen additional size-fractionated polyP preparations of unknown polymer lengths, we resolved the preparations on 5, 10, 15%, and 4–20% polyacrylamide gels and evaluated the inter-assay variability for determining their polymer lengths using the model. The sizes of these polyP samples estimated using our DNA equivalence model are presented in Fig. 4B, with a mean coefficient of variation of 0.08 ± 0.03 ($\pm$ standard deviation)

4 Concluding remarks

Advances in understanding the physiologic roles for polyP in mammalian biology [20] require more sensitive methods to detect, characterize, and quantify inorganic polyP. The method described here for estimating the polymer lengths of polyP via PAGE on TBE gels using commercially available DNA ladders is simple, easily accomplished, inexpensive, and reproducible. The method performed equivalently well on gels containing a range of different polyacrylamide concentrations, including gradient and non-gradient gels. Our model was reliable for sizing polyP longer than 62 phosphate units (the size that comigrated with the smallest DNA band), but the lack of availability of dsDNA standards shorter than 10 bp and the technical difficulty of staining shorter DNA and polyP bands using the same stain made it difficult to precisely evaluate shorter polyP polymers. Nevertheless, it seems likely that this method could be applied to the comigration of shorter polyP preparations with either ssDNA or dsDNA, although verifying this would require additional investigation.

There are many automated systems available for analyzing PAGE data using dsDNA size ladders resolved on the same gel to convert the migration data of unknown bands into DNA lengths in bp. Such automated gel analysis systems should be readily usable in the method described in this paper. Thus, once the migration data of polyP bands have been converted into "bp" from the automated analysis, these bp values can be further converted into polyP sizes (in phosphate units) using Equation (3). Alternatively, if the

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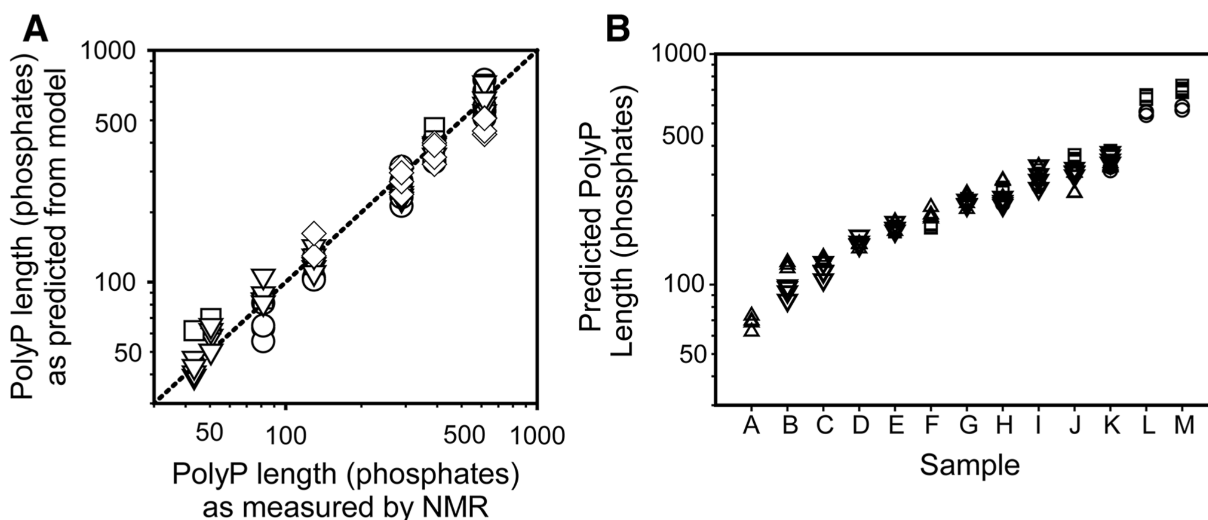

Figure 4. Performance of the DNA equivalence model. (A) Application of the equivalence model to estimate polyP lengths using various gels. PolyP standards of known lengths (determined by NMR) and bands in 10 bp or 100 bp DNA ladders were resolved on 5% (○), 10% (□), or 15% (▽) polyacrylamide gels (four each) as visualized in Fig. 3. Predicted polymer length of each polyP band was calculated by reference to migration of DNA ladders resolved on the same gel, as described in the text. These predicted polyP lengths are plotted here vs. their lengths determined by NMR, with the dotted line representing equivalence between predicted and measured lengths. Concurrence between measured and predicted polyP polymer sizes had a Pearson correlation coefficient of 0.982. (B) Reproducibility of the polyP sizing method. Thirteen narrowly size-fractionated polyP samples ("A" through "M" on the *x*-axis), but of undetermined length, were resolved together with commercial DNA ladders on 5% (○), 10% (□), 15% (▽) or 4–20% (△) polyacrylamide gels (four gels each). Polymer lengths of the polyP bands were predicted from the polynomial fit of the migration of the DNA ladder and the equivalence model as defined in Fig. 3. The resulting (calculated) polyP polymer lengths using this method are plotted on the *y*-axis.

Table 1. PolyP lengths represented by common DNA ladder bands

DNA (bp)	Equivalent polyP length (phosphates)
10	62
25	107
50	162
75	207
100	246
200	374
300	478
400	568
500	650
1000	986

equivalent polyP length for each DNA band is input into gel evaluation software, then the sizes calculated by the software for unknown polyP bands will be output directly in phosphate units. For the reader's convenience, the polyP length equivalents for several common DNA ladder bands are presented in Table 1.

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