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# Fluorescence assay of polyamide–DNA interactions

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ABSTRACT

Polyamides (PAs) are distamycin-type ligands of DNA that bind the minor groove and are capable of sequence selective recognition. This capability provides a viable route to their development as therapeutics. Presented here is a simple and convenient fluorescence assay for PA–DNA binding. PAs are titrated into a sample of a hairpin DNA featuring a TAMRA dye attached to an internal dU near the PA binding site. In a study of 6 PAs, PA binding leads to a steady reproducible decrease in fluorescence intensity that can be used to generate binding isotherms. The assay works equally well with both short (6- to 8-ring) and long (14-ring) PAs, and $K_d$ values ranging from approximately 1 nM to at least 140 nM were readily obtained using a simple monochromator or filter configuration. Competition assays provide a means to assessing possible dye interference, which can be negligible. The assay can also be used to determine PA extinction coefficients and to measure binding kinetics; thus, it is an accessible and versatile tool for the study of PA properties and PA–DNA interactions.



Polyamides (PAs)[1] are distamycin-type ligands of DNA that bind the minor groove and are capable of sequence selective recognition [1]. PAs can be thought of as higher homologs of distamycin A and the lexitropsins, and all of these DNA ligands form hydrogen bonds to functional groups in the minor groove of double-stranded DNA, providing a range of binding tendencies and selectivities [2,3]. An amino acid derivative of *N*-methylimidazole (Im), which recognizes G, and an analogous derivative of *N*-methylpyrrole (Py), which recognizes C, A, or T, form the bulk of PA building blocks. The 2-carboxy-4-amino substitution pattern of these 5-membered heterocycles confers a shape on the resulting PAs that matches the curvature of B-form duplex DNA very closely [1]. In addition, β-alanine (β) is used in PA design to confer some conformational flexibility and also can recognize C, A, and T. These are the most common building blocks found in PAs, which can be prepared in a number of topologies, from linear to circular and hairpin shapes [1]. Hairpin structures are able to form a turn composed of groups such as γ-aminobutyric acid, which binds mainly to nucleobases classified as W (either A or T). Typical C -termini of PA hairpins mimic the cationic amidine group found at the same terminus of distamycin A and are capped with cationic tails prepared from *N,N*-dimethylaminopropylamine (Dp) or 3,3′-diamino-*N*-methyldipropylamine (Ta) building blocks, both of which bind preferentially to A or T over G and C. The hairpin structure introduced by Dervan allows each strand of the PA to independently recognize one strand of duplex DNA. This arrangement makes for highly sequence selective binding [4,5]. Thus, hairpin PAs have been used effectively for biomedical programs that target specific DNA sequences, including the control of gene expression and the diminution of viral titer [6–8]. In some cases, PAs have been used successfully both in vitro and in vivo, and it is hoped that this class of designed DNA ligands will be developed as therapeutics [8–11].

Critical to the continued development of PAs as DNA ligands is a convenient assay for their DNA binding behavior. Many drug–DNA interactions have been characterized using optical techniques [12,13], and a number of them are based on the changes in intrinsic fluorescence of the ligand on binding DNA. Although PAs have limited intrinsic fluorescence, and PA–DNA binding can be characterized via observation of this weak fluorescence signal [14], high PA concentrations (>1 μM) are required—well above the range needed to measure high-affinity $K_d$ values typical for these molecules [15]. Another option for studying PA–DNA binding involves the incorporation of a fluorophore into the PA [16–18]. However, the introduction of such a dye requires additional synthetic and purification steps and can change uptake for cell-based assays, making the biological relevance of PA–dye conjugates questionable unless they are the bioactive species [19].

The most commonly performed assays of DNA binding by PAs involve calorimetry [20], surface plasmon resonance (SPR) [20], and footprinting [21]. All of these techniques require specialized

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[1] *Abbreviations used:* PA, polyamide; SPR, surface plasmon resonance; RP–HPLC, reverse-phase high-performance liquid chromatography; MeOH, methanol; TFA, trifluoroacetic acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; DMSO, dimethyl sulfoxide; MWT, molecular weight; TAMRA, carboxytetramethyl rhodamine; EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; CE, capillary electrophoresis.

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equipment and expertise that do not make them generally accessible. Fluorescence spectroscopy is a more convenient technique than any of the alternatives surveyed above, and assays such as the fluorescence of intercalator displacement (FID) have been applied to the characterization of PA–DNA binding [22]. This approach is technically easier but reliant on a second (DNA–intercalator) equilibrium that may or may not be amenable to a particular concentration regime of interest. Presented here is a simple, versatile, and convenient fluorescence assay for PA–DNA binding. Its utility is demonstrated for $K_d$ measurements, PA extinction coefficient determinations, and PA–DNA binding kinetics.

## Materials and methods

### *Chemical synthesis*

PAs **1** to **6** were prepared by automated solid-phase synthesis on Boc-β-alanine-PAM resin [23]. An ABI 433A peptide synthesizer was used. Compounds were isolated by reverse-phase high-performance liquid chromatography (RP–HPLC) with a methanol (MeOH)/$H_2O$/0.1% trifluoroacetic acid (TFA) gradient and characterized by mass spectrometry (HPLC/MS and high-resolution MS [HRMS], both using electrospray ionization $[ESI]^+$), combustion analysis (CNH), ultraviolet/visible (UV/Vis), and 500 MHz $^1$H nuclear magnetic resonance (NMR). Detailed characterizations for **1** to **5** are provided in the supplementary material, and **6** was prepared and characterized as described previously [8]. Note that, contrary to previous reports that imidazoles are not protonated in isolated PAs, elemental analysis indicated that our compounds were isolated from RP–HPLC (MeOH/$H_2O$/0.1% TFA) as tetracations with all imidazoles and the tertiary amine protonated [14,24]. Stock solutions of **6** in dimethyl sulfoxide (DMSO) were quantitated using an $\varepsilon_{305}$ of 113,300 $M^{-1}$ $cm^{-1}$, a reference value obtained by Beer Lambert plots and confirmed by NMR spectroscopy [25]. Other PA stock concentrations were obtained by dissolving a known mass (1–2 mg weighed to at least two significant figures) of lyophilized and MS-determined samples in either DMSO or $H_2O$, diluted as needed, and stored at −20 °C. If additional stocks were needed, they were quantitated from the ε at 305 nm ($\lambda_{max}$ for the peak closest to the visible region) obtained from the mass, MWT, and the absorbance of a sample in the low micromolar range. For **2**, the extinction coefficient obtained via this method was compared with the value generated by the fluorescence method as described below.

### *Fluorescence spectroscopy*

Quantitation of DNA binding was achieved by observing the change in fluorescence intensity of TAMRA (carboxytetramethyl rhodamine)-labeled oligonucleotide as a function of PA concentration. 5′-CCT GGA GAG GAA GCC AAG TGT TTT CAC TTG GCT TCC TCT CCA GG-3′ (**HP1**) and 5′-GCT AGA TAT ATA GCT TTT TAG CTA TAT ATC TAG C-3′ (**HP2**) were purchased with HPLC purification from Integrated DNA Technologies (IDT, Coralville, IA, USA) either unlabeled or labeled with dU-TAMRA (at C5 via *N*-hydroxysuccinimide [NHS] ester) at either T34 or T37 for **HP1** and at T3 for **HP2**, as shown in Fig. 1. The DNAs were rinsed twice with Milli-Q water using a Centricon unit, annealed from boiling water, quantitated using the vendor's extinction coefficient, and either used directly or aliquoted, lyophilized and stored at −20 °C until use.

The experiments were performed in acid-prewashed quartz cuvettes in buffer (10 mM Hepes, 50 mM NaCl, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.4) on a T-formatted Fluorolog-3 (Spex) spectrofluorimeter. The temperature was

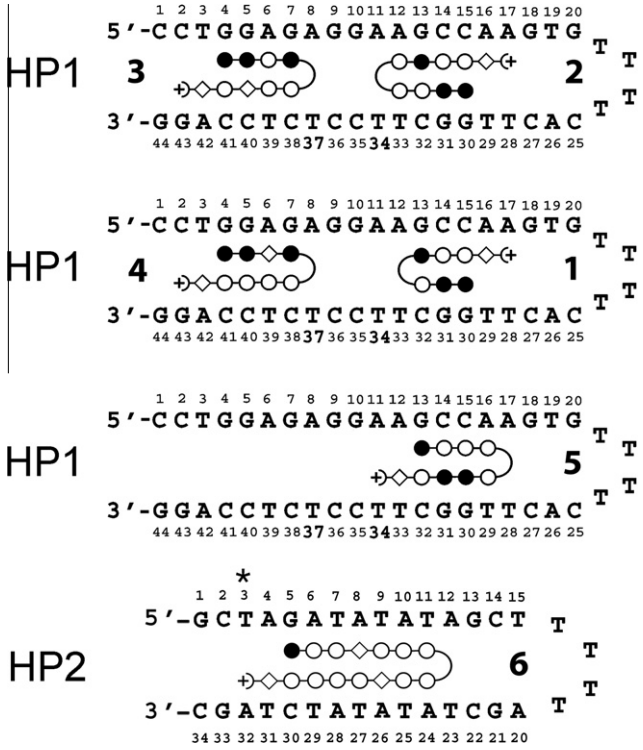


**Fig.1.** Summary of hairpin design with dye placement. For **HP1**, TAMRA dye was placed at either T34 or T37 as indicated. Illustrated PAs are numbered and correspond to Table 1 entries. PA positions are based on expected binding location as dictated by conventional recognition rules. For **HP2**, TAMRA dye was placed at T3 (∗). Closed circles: imidazole; open circles: pyrrole; open diamonds: β-alanine.

maintained at 25 °C with a thermostatted cell holder equipped with a magnetic stirrer. TAMRA-labeled oligonucleotides were excited at 559 nm, and the resulting emission was observed through a monochromator set at 580 nm or passed through a 592-nm bandpass filter (Edmund Optics, Barrington, NJ, USA). In most cases, the intensity at the emission maximum decreased as PA was added. Intensity values were obtained in triplicate and averaged.

Intensities were normalized to indicate the fraction of DNA bound and then plotted versus PA concentration and the data fit to Eq. (1),

$$\Theta = \frac{K_a[L]}{1 + K_a[L]} \quad (1)$$

where Θ is the fraction of duplex bound, [L] is the total PA concentration, and $K_a$ is the association constant. Each reported $K_d$ value represents an average of at least three separate experiments.

### *Competition fluorescence binding assay*

Increasing amounts of unlabeled DNA hairpin PA were added to a fluorescent DNA–PA complex preformed at a concentration 10-fold above the $K_d$, and the resultant intensities were measured and averaged. The resulting data were fit to a system of equations describing both the labeled DNA–PA equilibrium (known $K_d$) and the unlabeled DNA–PA equilibrium (unknown $K_d$) using Scientist software (MicroMath, Salt Lake City, UT, USA) as described previously [26–28].

### *Dissociation kinetics*

To observe $k_{off}$ for **2**, 75 nM unlabeled DNA hairpin was added to a 30-nM 1:1 complex of **2** and TAMRA-labeled DNA hairpin (denoted ∗DNA) in 10 mM Hepes, 50 mM NaCl, and 1 mM EDTA