Communication

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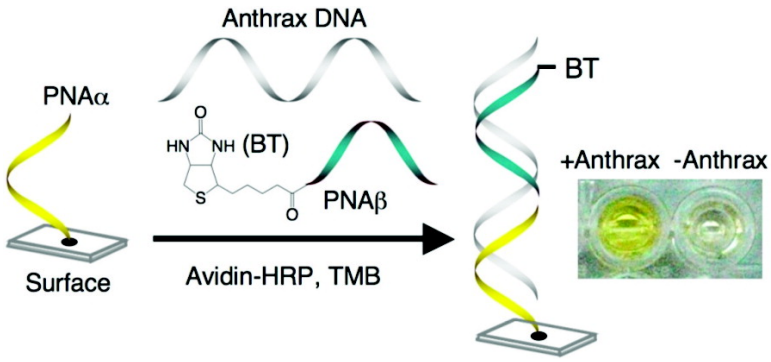
J. Am. Chem. Soc., **2007**, 129 (27), 8424-8425• DOI: 10.1021/ja072744j • Publication Date (Web): 15 June 2007



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Published on Web 06/15/2007

Colorimetric Detection of Anthrax DNA with a Peptide Nucleic Acid Sandwich-Hybridization Assay

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Technologies for genomic detection most commonly use DNA probes to hybridize to target sequences. To achieve required sensitivity, the use of PCR to amplify target sequences has remained standard practice in many labs. Direct detection methods that eliminate the requirement for a PCR step could afford faster and simpler devices that can be used outside of a laboratory. Devices based on nanotechnology have yielded impressive results,[1] yet the use of PCR is still predominant in most applications. However, replacing DNA probes with a class of synthetic nucleic acids, such as peptide nucleic acids (PNAs),[2] can significantly improve detection devices.[3] There are numerous advantages to using PNA instead of DNA probes in hybridization assays, including complete resistance to degradation by enzymes, increased sequence specificity to complementary DNA, and higher stability when bound with complementary DNA. *Due to the higher stability, it should be possible to use shorter PNA sequences compared to DNA.* Despite these advantages, the use of PNA in DNA detection systems has received sparse attention and has not replaced the use of DNA probes. We believe that one reason PNA does not dominate in this area is due to the lack of backbone modifications that can be used to adjust the properties of a probe sequence. Without the ability to improve and fine-tune the basic properties of PNA, it is likely not worth the effort and/or funds for researchers to switch from DNA to PNA probes.

We have developed a system of chemical modifications for PNAs, using cyclopentane groups, to predictably improve the melting temperature and sequence specificity of PNA−DNA duplexes.[4] Addition of one or more cyclopentane groups into a PNA sequence improves the melting temperature to complementary DNA by ∼5 °C per cyclopentane, regardless of which base is used.

To demonstrate the utility of our chemical strategy and to highlight the importance of PNA in detection, we report in this communication a simple, colorimetric sandwich-hybridization assay to detect anthrax protective antigen DNA using PNA. In this system, a key component to improving the detection limit and sequence specificity is the incorporation of a cyclopentane-modified PNA into the surface-bound probe. In the sandwich-hybridization strategy, one PNA is used as capture probe (PNAα) to recruit complementary DNA to a surface, and another PNA is used as a detection probe (PNAβ) to generate a signal (Figure 2). This assay has been developed into a convenient 96-well plate format in which PNAα is covalently attached to a DNA-Bind plate. A biotin-labeled PNAβ, in combination with commercially available avidin−horseradish peroxidase conjugate (HRP−avidin) and tetramethylbenzidine (TMB), is used to generate a signal if the target DNA is present. If a sandwich complex forms on the surface, the strong interaction between biotin and avidin will retain HRP−avidin. The HRP will then catalyze oxidation of TMB, and after stopping the reaction with sulfuric acid, a colored product that absorbs at 450 nm is generated. These items are accessible to most biomedical research facilities.

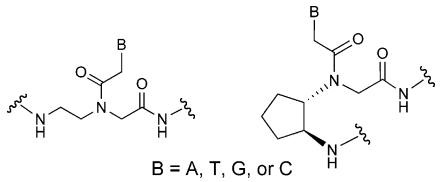

Figure 1. Structures of regular aeg PNA and cyclopentane-modified (*tcyp*) PNA.

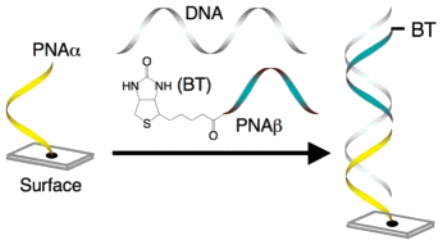

Figure 2. PNA-based sandwich-hybridization assay. PNAα is the capture probe, and PNAβ is the detection probe.

In the construction of PNA probes, all PNAs were made with a single sequence that corresponds to the protective antigen (PA) portion of the anthrax genome, which is highly conserved.[5] Next, PNAα probes were designed with extended linkers on the N-terminal for covalent attachment to the DNA-Bind plate. The PNAβ probes were outfitted with additional lysine groups so that biotins could be attached. To ensure that there was enough room for the HRP−avidin complex to bind, additional linkers were added onto the lysine side chains (Table 1).

PNAα probes were attached to the surface of each well of the DNA-Bind plate, and free sites were blocked using a buffer consisting of BSA and lysine. Detection conditions were optimized using synthetic DNA. Ultimately, a set of conditions were developed that involve incubating a solution of target DNA and PNAβ in the well of a DNA-Bind plate that contains PNAα, followed by washing, incubation with HRP−avidin, another washing, and then detection with 1-Step Turbo TMB, a commercially available TMB and peroxide solution. The enzymatic reaction was stopped by the addition of sulfuric acid, and then absorbance at 450 nm was measured. Using unmodified PNA under these conditions, only picomole quantities of DNA could be detected (Table 2, entry 4).

Several strategies were explored to boost the detection signal and lower the DNA detection limit. A *tcyp*-modified PNA residue was incorporated into PNAα (PNAα(2)), an additional biotin was attached to PNAβ (PNAβ(2)), and a commercially available polymer of HRP−avidin (poly-HRP−avidin) was used in which the ratio of HRP to avidin is approximately 40:1. Table 2 represents the absorbance values at 450 nm (over background) obtained when using different combinations of PNAα and PNAβ at several different concentrations of synthetic DNA and when using HRP−avidin (entries 1−4) and poly-HRP−avidin (entries 5−8). The

Table 1. PNA Capture Probes (α) and PNA Detection Probes (β)

entry	PNA sequence[a]	T_m (°C)[b]
α(1)	H_2N-$(egl)_5$-ATCCTTATCAATATT-$CONH_2$	50.5
α(2)	H_2N-$(egl)_5$-ATCCTTAT$_{tcyp}$CAATATT-$CONH_2$	55.6
β(1)	H_2N-TAACAATAATCC-Lys-Lys-$CONH_2$ $(egl)_2BT$	54.1
β(2)	H_2N-TAACAATAATCC-Lys-Lys-Lys-$CONH_2$ $BT(egl)_2$ $(egl)_{10}BT$	55.1

[a] *t*cyp = PNA residue derived from (*S*,*S*)-*trans*-1,2-cyclopentane diamine (Figure 1), BT = biotin, egl = 8-amino-3,6-dioxaoctanoic acid. [b] T_m represents the melting temperature for the duplex formed between the indicated PNA and antiparallel DNA. Conditions for T_m measurement: 150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.0, 0.1 mM EDTA, UV measured at 260 nm from 90 to 25 °C, in 1 °C increments. All values are averages from two or more experiments.

Table 2. Absorbance at 450 nm for DNA Detection with Different PNA Probes and Using HRP−Avidin (entries 1−4) versus Poly-HRP−avidin (entries 5−8)

		Femtomoles of Anthrax DNA			
entry	PNAs[a]	10^3	10^1	10^{-1}	10^{-6}
1	α(2)+β(2)	0.23	0.02	0.01	0.01[b]
2	α(2)+β(1)	0.16	0.01	–	–
3	α(1)+β(2)	0.19	0.01	–	–
4	α(1)+β(1)	0.12	–	–	–
5	α(2)+β(2)	0.99	0.15	0.15	0.09[c]
6	α(2)+β(1)	1.05	0.03	0.01	0.01[c]
7	α(1)+β(2)	0.44	0.01	–	–
8	α(1)+β(1)	0.20	–	–	–

[a] See Table 1 for structures of PNAs. Standard deviation values for absorbances range from 0.01 to 0.03. All experiments in Table 2 were performed with synthetic anthrax DNA sequences. [b] Data for 50 zmol DNA. [c] Data for 10 zmol DNA.

Table 3. Absorbance at 450 nm for Detection of Mismatches Located Directly Across from the *t*cyp in PNAα(2) and Comparison with Unmodified PNAα(1)[a]

	Mismatch Comparison at 10^3 fmol Anthrax DNA			
PNAs[a]	none	TG	TC	TT
α(2)+β(2)	0.31	0.05	0.04	0.05
α(1)+β(2)	0.12	0.04	0.01	0.04

[a] See Supporting Information for DNA sequences used in detection. Standard deviation values for absorbances range from 0.01 to 0.05. All experiments in Table 3 were performed with synthetic anthrax.

results of Table 2 demonstrate that all these strategies help improve the signal associated with DNA detection and lower the overall DNA detection limit. In the best combination (entry 5), 10 zmol of DNA can be detected (see Supporting Information for more details).

Compared to unmodified PNA, incorporation of *t*cyp into PNA can improve the discrimination of single base mismatches in DNA.[4] In the context of the detection assay, we examined the changes in absorbance at 450 nm associated with single base mismatches for *t*cyp PNA versus regular PNA. The results in Table 3 show that, under the same conditions, the *t*cyp PNA (PNAα(2)) shows much clearer differences in signal between matched and mismatched DNA sequences than the regular PNA (PNAα(1))

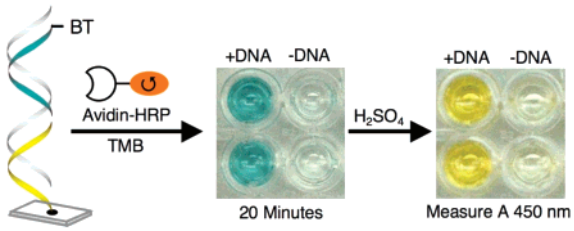

Figure 3. Signal amplification from PNA-based sandwich-hybridization using PNAα(2) and PNA*β*(2) with 10^3 fmol DNA and HRP−avidin. Four wells of a 96-well plate are shown, and each column represents identical conditions. The blue color results from initial oxidation of 1-Step Turbo TMB, and the yellow color is produced once the enzymatic reaction is stopped.

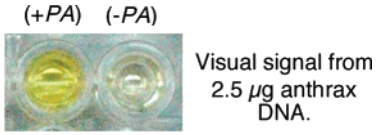

Figure 4. Colorimetric detection of protective antigen DNA (PA) from *Bacillus anthracis* Ames 35 strain (+*PA*) and Ames 33 strain (−*PA*). Signal obtained from PNA-based sandwich-hybridization using PNAα(2) and PNA*β*(2) with poly-HRP−avidin.

Using the most sensitive detection system from our research, we examined the ability to detect protective antigen (PA) DNA obtained from a whole cell extract of *B. anthracis*. In this study, DNA was extracted from two cell lines of anthrax, one that has PA-DNA (Ames 35)[6] and one that lacks PA-DNA (Ames 33).[7] Our detection system was clearly able to distinguish the two cell lines, giving a colored signal visible to the naked eye (Figure 4).

The high thermal stability of PNA−DNA duplexes allows shorter PNA probes to be used compared to DNA. A DNA-based system with equivalent thermal stability of the capture and probe sequences would require DNAs ~30−45 bases long, which could reduce sequence specificity. The ability to introduce chemical modifications with predictable effects into the PNA allows us to equalize the T_m's of the two PNA probes for their DNA targets, which likely promotes uniform hybridization of all probes.

Acknowledgment. This work was supported by the intramural research program at NIDDK, and the Biodefense Program of NIAID, NIH. The authors would like to thank Dr. John Lloyd for collection of mass spectra, and Dr. Stephen L. Leppla and Dr. Haijing Hu (NIAID, NIH) for samples of anthrax.

Supporting Information Available: PNA characterization and detailed procedures for performing the DNA detection assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA072744J