Peptide Nucleic Acid Probes for Sequence-Specific DNA Biosensors

Joseph Wang,*,† Emil Palecek,‡ Peter E. Nielsen,§ Gustavo Rivas,† Xiaohua Cai,† Haruki Shiraishi,† Narasaiah Dontha,† Denbai Luo,† and Percio A. M. Farias†

Contribution from the Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, New Mexico 88003, Institute of Biophysics, Academy of Sciences of the Czech Republic, 61265 Brno, Czech Republic, and Center for Biomolecular Recognition, IMBG, Department of Biochemistry B, The Panum Institute, Blegdamsvej 3c, DK 2200 Copenhagen, Denmark

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Abstract: Surface-attached peptide nucleic acids (PNA) are shown to retain their unique and efficient hybridization properties, reported in solution studies. PNA recognition layers thus offer significant advantages for sequence-specific DNA biosensors, compared to their DNA counterparts. These advantages include significantly higher sensitivity and specificity (including greater discrimination against single-base mismatches), faster hybridization at room and elevated temperatures, minimal dependence on ionic strength, and use of shorter (10−15-mer) probes. Such unique properties and advantages are illustrated in connection with electrochemical detection of the hybridization event using the $Co(phen)_3^{3+}$ redox indicator and a carbon paste electrode transducer. The new capabilities and opportunities afforded by the use of PNA surface probes are discussed.

Nucleic acid hybridization forms the basis for the diagnosis of inherited or infectious diseases. DNA biosensor technologies are thus currently under intense investigation owing to their great promise for rapid and low-cost detection of specific DNA sequences. These technologies commonly rely on the immobilization of a single-stranded (ss) DNA probe onto optical, electrochemical, or mass-sensitive transducers[1−7] to recognize the complementary (target) DNA strand in a sample solution. The transducer thus converts the hybridization event into a useful electrical signal. The overall performance of these new devices is strongly dependent upon experimental variables influencing the hybridization efficiency, such as temperature, ionic strength, or probe length. Despite the stringently controlled hybridization conditions, most of these biosensors are not capable of selectively discriminating against single-base mismatches, as desired for the detection of disease-related point mutation. Higher sequence selectivity is clearly desired.

We report here on the first use of peptide nucleic acids (PNA) as the recognition layer in DNA biosensors. PNA is a structural DNA analogue containing an uncharged *N*-(2-aminoethyl)-glycine-based pseudopeptide backbone which has been shown to mimic DNA in forming Watson−Crick complementary duplexes with normal DNA.[8−11] Compared to DNA duplexes, PNA hybrids have higher thermal stability and can be formed at low ionic strengths. PNA also shows a higher specificity in the recognition of the DNA sequences and permits the use of shorter probes. In the following sections we demonstrate that the unique properties displayed by solution-phase PNA oligomers can be extrapolated onto transducer surfaces in connection with the design of DNA biosensors. The resulting biosensors offer great promise for mismatch-sensitive hybridization detection, and can operate over a wide range of hybridization conditions. The dramatic improvements accrued from the use of PNA recognition layers are illustrated in connection with electrochemical detection of the hybridization event.

Experimental Section

Apparatus. All chronopotentiometric experiments were performed with a TraceLab potentiometric stripping unit (PSU 20, Radiometer, Denmark) connected with an IBM PS/2 55SX. According to the TraceLab protocol, the potentials were sampled at a frequency of 30 kHz and the derivative signal (d*t*/d*E*) was recorded against the potential. The peak area following baseline fitting was used as the analytical signal. The three-electrode system consisted of a carbon-paste electrode (CPE) or glassy-carbon electrode (GCE), reference electrode (Ag/AgCl, Model RE-1, BAS, IN), and platinum wire auxiliary electrode. The electrodes joined the cell through holes in the Teflon cover. The body of the working electrode was a Teflon sleeve (3.5 mm i.d.) tightly packed with the carbon paste. The electrical contact was made with a stainless steel screw. The carbon paste was prepared in the usual way by hand-mixing graphite powder (Acheson 38, Fisher Scientific, PA) and mineral oil (Catalog No. M5904, free of DNase, RNase, and protease, Sigma, St. Louis, MO). The ratio of graphite powder to mineral oil was 70:30. The surface was polished to a smooth finish before use. The electrochemical cell was a 2-mL polypropylene vial. All glassware, glass-containers, pipet tips, and the cell (with the exception of the electrodes) were sterilized by autoclaving for 30 min. The electrode was rinsed with sterilized water prior to use.

* To whom correspondence should be addressed.
† New Mexico State University.
‡ Academy of Sciences of the Czech Republic.
§ The Panum Institute.
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Chemicals. The 10-mer PNA oligomer was synthesized at Nielsen's laboratory (University of Copenhagen, Denmark), while the 15-mer PNA oligomer was received from PerSeptive Biosystems Inc. (Framingham, MA). DNA oligomers (10-mer and 15-mer, as their ammonium salts) were obtained from Life Technologies (Grand Island, NY). The base sequences of these PNA and DNA oligomers are as below:

10-mer PNA	H-AGTGATCTAC-NH_2 (P-sequence)
15-mer PNA	H-TGTACGTCACAACTA-NH_2 (P-sequence)
10-mer DNA	5′AGTGATCTAC3′ (P-sequence)
15-mer DNA	5′TGTACGTCACAACTA3′ (P-sequence)
10-mer DNA	5′GTAGATCACT3′ (T-sequence)
15-mer DNA	5′TAGTTGTGACGTACA3′ (T-sequence)

Note that the 10-mer and 15-mer DNAs (P-sequences) have the same base sequences as the 10-mer and 15-mer PNAs, respectively. The 10-mer and 15-mer DNAs (T-sequences) are complementary to the 10-mer and 15-mer DNAs and PNAs (P-sequences), respectively. Tris(1,10-phenanthroline)cobalt(III) perchlorate was synthesized at NMSU using the method described by Dollimore and Gillard.[12] Trifluoroacetic acid (TFA, Catalog No. T6508) was purchased from Sigma (St. Louis, MO).

The PNA stock solutions (100 mg/L) were prepared with 0.1% aqueous TFA and divided into aliquots. The aliquots were frozen when not in use. The DNA solutions were made with the TE buffer (10 mM Tris−HCl, 1 mM EDTA, pH 8.0). All aqueous media used were sterile distilled water. Unless otherwise stated, all data were obtained at room temperature (22.0 ± 0.5 °C).

Procedure. The chronopotentiometric measurements of the DNA and PNA at the CPE and GCE were performed in 0.2 M acetate buffer (pH 5.0). The anodic signal at around +1.0 V, corresponding to the oxidation of guanine moiety, was used as the analytical signal.

The sequence detection with DNA or PNA probe consisted of four steps: probe immobilization, hybridization, indicator binding, and chronopotentiometric transduction. During the electrode transfer to the next solution, its surface was rinsed with a specific buffer solution (see below). **(a) Probe Immobilization.** A freshly smoothed carbon paste electrode was immersed in a stirred acetate buffer solution (0.2 M, pH 5.0) containing a DNA or PNA probe and was held at a potential of +0.50 V for 2 min. **(b) Hybridization.** The electrode was rinsed with 5 mM phosphate buffer (pH 7.0) for a short time (5 s) and was immersed into the stirred phosphate buffer hybridization solution containing a target for a desired time, while holding its potential at +0.5V. **(c) Indicator Binding to the Hybrid.** The electrode was rinsed with 0.02 M Tris−HCl buffer (pH 7.0) for 10 s. The $Co(phen)_3^{3+}$ was accumulated onto the surface hybrid by placing the electrode into the stirred Tris−HCl buffer solution containing 0.2 mM $Co(phen)_3^{3+}$ for 1 min while holding the potential at +0.5 V. **(d) Chronopotentiometric Transduction.** The accumulated $Co(phen)_3^{3+}$ was measured by using an initial potential of +0.5 V and a constant current of −6.0 μA.

Repetitive measurements were carried out by renewing the surface and repeating the above assay format. The reported chronopotentiometric response represents the difference in indicator peak areas at the hybrid- and probe-coated electrodes.

Results and Discussion

Electrochemical detection of hybridization events[2] was employed for assessing whether the unique properties of solution-phase PNA are retained upon immobilizing these oligomers onto transducer surfaces. Such development of biosensors based on PNA-modified electrodes requires knowledge of the interfacial and redox behaviors of these DNA analogues. While such properties of DNA were reviewed recently,[13] similar studies on PNA have not been reported. Computerized chronopotentiometry was employed for this purpose (and for all other experiments), owing to its effective discrimination against background contributions at carbon-electrode transducers.[14,15] Such operation involves passing a constant current through the working electrode and monitoring the variation of its potential as a function of time. (In the differential display used in this study, the quantitative signal is the peak area.) Figure 1 compares the response of the 15-mer PNA (A) and its corresponding DNA oligonucleotide (B) at the carbon-paste (a) and glassy-carbon (b) electrodes. Both PNA and DNA display well-defined anodic peaks, at similar potentials, as expected from the presence of the electroxidizable guanine moiety. The PNA response, particularly at the CPE, is greatly enhanced following the 2-min accumulation (solid vs dotted lines), suggesting a very strong adsorption.

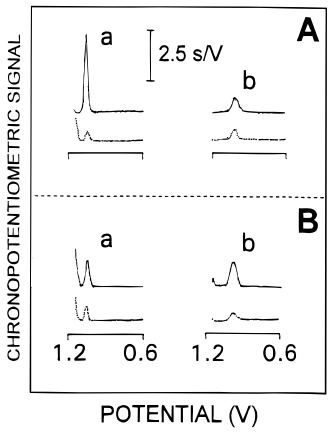

Figure 1. Chronopotentiograms for 1 mg/L 15-mer PNA (A) and 15-mer DNA (P-sequences) (B) at a CPE (a) and a GCE (b) following a 2-min accumulation at +0.2 V. Dotted traces denote the corresponding responses without accumulation. Electrolyte solution: 0.2 M acetate buffer (pH 5.0). Constant current: + 6 μA. Initial potential: +0.2 V.

The strong adsorptive accumulation of the PNA oligomers was exploited for preparing PNA-modified surfaces. The adsorptive immobilization process on the CPE transducer was monitored via the intrinsic anodic response of PNA (i.e., the guanine oxidation signal). Conditions for attaining a full surface coverage were assessed by measuring the dependence of the chronopotentiometric guanine peak area upon the PNA solution concentration or the adsorption time. Using the 1 mg/L solution of the 15-mer PNA oligomer, surface saturation was observed for adsorption periods longer than 2 min. The stability of the immobilized PNA layer was examined by monitoring its signal dependence upon the immersion time in a stirred blank solution (20 mM phosphate buffer, pH 7.0; operating potential, +0.5 V). No diminution of the guanine response was observed over a 30-min period, indicating a stable probe layer.

The adsorptive immobilization of short PNA oligomers leads to very effective hybridization biosensors. The attractive performance of such devices, over a wide range of hybridization conditions, is illustrated in Figure 2. An electroactive indicator, $Co(phen)_3^{3+}$, commonly used for electrochemical detection of DNA hybridization,[2] was employed for monitoring the PNA−DNA surface duplex formation. (While this metal complex is known to associate with the DNA double helix through both intercalative and minor-groove binding,[16] it appears based on solution studies[17] that the latter dominates its interaction to the PNA−DNA duplex.) The increased peak area of the $Co(phen)_3^{3+}$ marker, upon its association with the surface hybrid,

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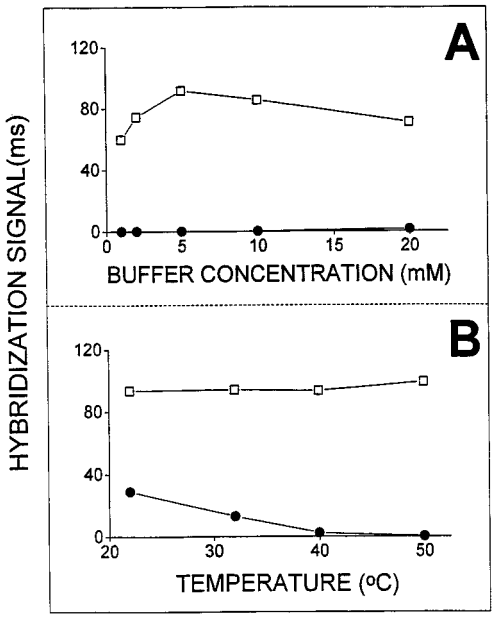

Figure 2. (A) Effect of the buffer concentration on the PNA−DNA (□) and DNA−DNA (●) hybridization at the CPEs. The PNA or DNA probe was immobilized onto the CPE from a stirred acetate buffer solution (0.2 M, pH 5.0) containing 1 mg/L of the 15-mer PNA or DNA oligomers (P-sequence). The immobilization proceeded for 2 min using a potential of +0.5 V. The coated electrode was immersed in the stirred 1 mL-hybridization solution containing the target (1-mg/L 15-mer DNA T-sequence) and different concentrations of phosphate buffer (pH 7.0). The hybridization proceeded for 5 min, while holding the electrode at +0.5 V. Then the electrode was placed in the stirred indicator solution (0.2 mM $Co(phen)_3^{3+}$ in 20 mM Tris−HCl buffer, pH 7.0) for 1 min while holding the potential at +0.5 V. The associated marker was measured by applying a constant current of −6.0 μA and an initial potential of +0.5 V. (B) Effect of the temperature on the hybridization. Hybridization solution: 0.02 M phosphate buffer (pH 7.0) plus 0.3 M NaCl used for the DNA probe (●), or 5 mM phosphate buffer (pH 7.0) used for the PNA probe (□); the hybridization was performed at elevated temperatures; all other conditions as part A.

thus serves as the hybridization signal. Figure 2A displays the influence of the buffer concentration upon the hybridization response. While the DNA-based biosensor (●) is not responding to its 15-base complementary strand using low buffer concentrations, the PNA device (□) offers convenient detection of this target over the entire ionic-strength range examined. The response of the PNA sensor increases gradually between 1 and 5 mM buffer concentrations, and decreases slightly up to 20 mM. Minimal ionic strength is apparently essential for providing the counterions for the formation of the PNA−DNA surface duplex. In contrast, and as expected for short strands, even the addition of an accelerator (1% poly(ethylene glycol)) did not result in a detectable signal at the DNA-coated transducer. Only an excess salt (0.3 M NaCl) increased the response of the DNA biosensor (not shown).

The effect of the hybridization temperature is examined in Figure 2B. At ambient temperature, both the PNA- and DNA-based devices yield a defined hybridization response, except that the PNA-biosensor signal is more than 3-fold higher. (Note the greatly different ionic strengths of the hybridization solutions.) Raising the temperature leads to a gradual decrease of the DNA-biosensor response, and its disappearance above 40 °C. In contrast, the hybridization response of the PNA biosensor remains unchanged up to 50 °C. The T_m of 15-base PNA−DNA and DNA−DNA complexes are 69 and 53 °C, respectively.[10] Overall, the profiles of Figure 2 (A and B) indicate that the use of surface-bound PNA probes provides a much

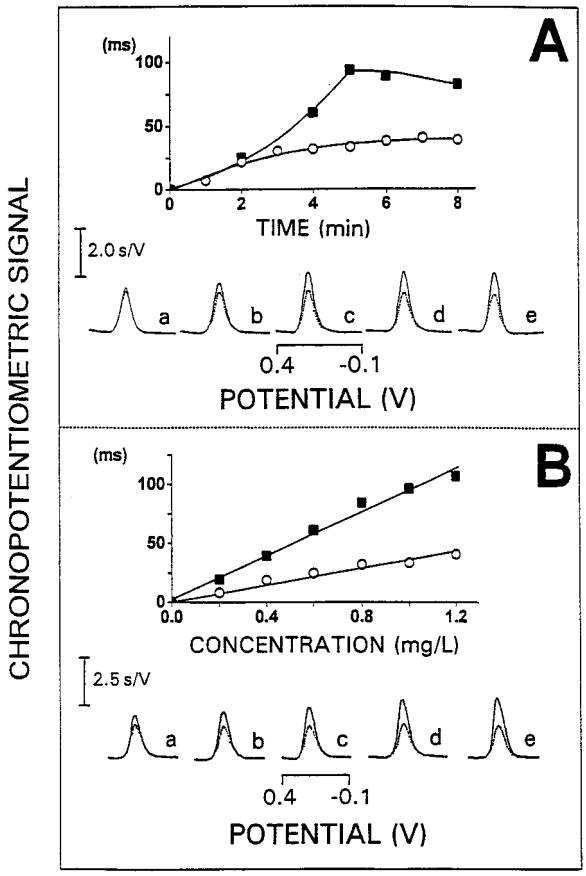

Figure 3. Effect of the hybridization time (A) and target concentration (B) upon the indicator response of the PNA-based biosensor. (A) Chronopotentiometric response to 1 mg/L of the 15-mer DNA (T-sequence) following different hybridization periods: 2 (a), 4 (b), 5 (c), 6 (d), and 8 (e) min using the 15-mer PNA probe (P-sequence), along with the resulting peak area vs time plot (■). Also shown is the analogous time-dependent plot using the 10-mer PNA probe (P-sequence) and its complementary DNA target (T-sequence) (○). (B) Chronopotentiograms for increasing level of the target 15-mer DNA (T-sequence) in 0.2 mg/L steps (a−e) using the 15-mer PNA probe (P-sequence). Also shown is the resulting calibration plot (■), along with calibration plot for a similar experiment using the 10-mer PNA probe and its complementary DNA (○). Dotted lines denote the corresponding response for the blank solution (i.e., absence of a target). Hybridization for 5 min (B) in a 5 mM phosphate buffer solution. Immobilization and measurement conditions as in Figure 2A.

greater latitude in the selection of the hybridization conditions in connection with the operation of DNA biosensors.

Figure 3A shows chronopotentiograms for the indicator at the 15-mer PNA probe after different hybridization periods. The response to the 15-mer target increases rapidly with the time up to 5 min, after which it decreases slightly. No peak enhancement is observed in the absence of the target (dotted traces). A slower increase of the response with the hybridization time and a lower sensitivity is indicated (from the inset, ○) for the detection of the 10-mer DNA target at the 10-mer PNA coated electrode. Figure 3B displays the response of the 15-mer PNA biosensor for increasing levels of the 15-mer target DNA (in 200 ng/mL steps, a−e), using 5 min of hybridization; linearity prevails up to 1000 ng/mL. Linearity up to 1200 ng/mL is indicated (from the inset, ○) for a similar experiment using the 10-mer PNA probe and its complementary DNA target. A detection limit of 50 ng (10 pmol) of the 15-mer target was estimated from the response following 10 min of hybridization in the 5mM phosphate buffer solution.

The high specificity of the PNA biosensor is illustrated in

Table 1. Effect of Various Nucleic Acids on the Hybridization Response[a]

		A (nucleic acid alone) relative signal (%)[c]		B (mixture) signal change (%)[d]	
nucleic acid[b]	concn (mg/L)	PNA probe	DNA probe	PNA probe	DNA probe
ssDNA	1	+6.6	+57.2	+7.2	+36.7
dsDNA	1	−12.2	+43.8	+5.1	+31.8
tRNA	1	−14.6	+34.8	−1.2	+4.3
42-mer DNA	1	−10.5	+71.0	−6.8	+66.4
	2	−18.3	+86.0	−15.3	+79.1
36-mer DNA	2	−3.7	+70.2	−1.0	+50.2
21-mer DNA	1	−4.0	+92.0	−1.9	+95.0
15-mer DNA*	1	+21.0	+97.0	+19.0	+90.0

[a] The electrode with the immobilized 15-mer PNA or DNA probe (P-sequence) was immersed (A) into the solution of the non-complementary nucleic acid (interferent) alone, or (B) into the mixture of the complementary 15-mer DNA (T-sequence, 1 mg/L) with the interferent given in the table. All measurements were performed at room temperature; other conditions as in Figure 2B. [b] The sequences of the DNA oligomers are as below: 42-mer DNA, 5′-ACT-GCT-AGA-GAT-TTT-CCA-CAC-TGA-CTA-AAA-GGG-TCT-GAG-GGA-3′; 36-mer DNA, 5′-CCA-CAT-GGC-CTG-TAC-TTT-AAA-AGC-TTC-CGG-ATG-ACC-3′; 21-mer DNA, 5′-ACT-GCT-AGA-GAT-TTT-CCA-CAT-3′; 15-mer DNA*, 5′-TAG-TTG-T**T**A-CGT-ACA-3′ (one-base mismatch of the 15-mer DNA T sequence, as indicated in bold). [c] Signal obtained in the presence of interferents alone relative to the target response (taken as 100%). [d] Change in the 1 mg/L target response (taken as 100%) after addition of the non-complementary nucleic acid.

Table 1. The sensor was challenged with various non-complementary oligomers and chromosomal DNA, including a 15-mer oligonucleotide containing a single-base mismatch. The substantial response of these oligomers at the DNA-coated electrodes is minimized at the PNA biosensor (columns A). Similarly, in mixtures with the complementary strand, these non-complementary oligonucleotides have a profound effect upon the target signal at the DNA biosensor and a negligible effect upon that observed at the PNA-coated electrode (columns B). Note, in particular, that the single-base mismatch oligonucleotide leads to a 90% interference using the DNA recognition, but only to a 19% change for the PNA probe. Such single mismatch discrimination is of great diagnostic significance and agrees with the solution-phase studies of Nielsen and co-workers.[10] Note also that such high sequence selectivity is achieved without a stringent control of the hybridization conditions, and using a 15-base-long oligomer. Elevated temperatures[6] or changes in the solvent accessibility[7] were used previously for achieving mismatch discrimination in connection to electrochemical detection.

Conclusions

We have demonstrated for the first time that PNA probes offer an efficient surface hybridization in combination with the high specificity of DNA binding. We have also shown that the unique properties displayed by solution-phase PNA oligomers can be extrapolated onto transducer surfaces in connection with the design of DNA biosensors. While the advantages of PNA surface probes have been presented in connection to electrochemical detection of hybridization events, they should benefit other (e.g., optical, piezoelectric) transduction modes. Such use of DNA mimics opens up exciting opportunities for DNA diagnostics, in general, and for the rapid screening for nucleic-acid sequences, in particular.

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