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PNA molecular beacons for rapid detection of PCR amplicons

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The authors have developed a method for rapid detection of polymerase chain reaction (PCR) amplicons based on surface immobilized PNA-DNA hybrid probes ('molecular beacons') that undergo a fluorescent-linked conformational change in the presence of a complementary DNA target. Amplicons can be detected by simply adding a PCR reaction to a microtitre-well containing the previously immobilized probe, and reading the generated fluorescence. No further transfers or washing steps are involved. The authors demonstrate the specificity of the method for the detection of ribosomal DNA from *Entamoeba histolytica*. © 1998 Academic Press

KEYWORDS: PNA, nucleic-acid detection, fluorescence, microtitre, structured probes.

INTRODUCTION

Tyagi and Kramer[1] have described a homogeneous polymerase chain reaction (PCR) assay based on the properties of special structured DNA probes called 'molecular beacons'. These molecules contain a probe sequence bounded by a stem structure that holds a fluorescent moiety and a quencher moiety in close physical proximity. When the DNA probe binds its complementary target sequence in a PCR amplicon, the stem structure is disrupted, separating the quencher compound from the fluor, which now becomes detectable by fluorescence emission. By using fluors that emit energy at different wavelengths, Tyagi and Kramer have recently constructed a cocktail of molecular beacons of different colours designed for the simultaneous, real-time detection of different DNA targets.[2]

Peptide nucleic acids (PNAs) are able to form highly specific and extraordinarily-stable duplexes with complementary DNA sequences.[3,4] The authors have endeavoured to construct molecular beacons containing PNA probe portions, and to evaluate their properties and their utility in the detection of PCR amplicons. The authors chose to design PNA molecules suitable for surface immobilization because this feature will offer advantages in the future, when it will become possible to exploit molecular beacons using surface array formats. Here, the construction of surface-immobilized molecular beacons containing a PNA probe domain, and a hybrid PNA-DNA stem is reported. The authors have evaluated the performance of PNA molecular beacons in model assays for the detection of PCR amplicons from the ribosomal episome of the enteric parasite *Entamoeba histolytica*. Amplicons can be detected specifically by simply adding a PCR reaction to a microtitre well that contains immobilized PNA molecular beacons

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and reading the fluorescence generated as the amplified DNA binds to the PNA probes.

MATERIALS AND METHODS

DNA materials

All DNA oligonucleotides (obtained from Operon Technologies, Inc, Alameda, California, USA) were resuspended in 5% ammonia/water and purified by precipitation with n-butanol.[5] The primers used for amplification of target sequences from *E. histolytica* trophozoites were: RER1: 5′-CAAAA-CATTTCAATTCTTTCATC-3′, 23-mer, position 1273–TGG-3′, 21-mer, positions 700–720, 844–864, 988–1004, 1132–1147. Primers positions are given according to Bhattacharya *et al.*[6] The 10-mer modified oligo EOstem10:5′-(HS)AGACAGAAT(NH_2)A-3′-lca-Biotin, which contains a biotin at the 3′ end, an aminated thymidine at the second position and a thiolated linker at the 5′ end, was used as one of the arms of the stem. The oligo EO19: 5′-GTGTAAAA-TAGTAAAAAAT-3′, 19-mer, served as a synthetic target for the PNA-DNA probe. As negative (non-complementary) controls the following oligonucleotides were used: F5WT.34: 5′-CAGATC-CCTGGACAGGCGAGGAATACAGAGGGCA-3′, 34-mer, GM.LR74: 5′-GCGTAATACGACTCACTATAGG-ACCTGGTTGAAATTGCTGCCATTGTCTAATTAAGA-ATGCCATAGACCCATCAAT-3′, 74-mer, and a PCR amplicon from the 4RRV gene of *Rotavirus.* All PCR reactions were performed according to Estrada *et al.*[7]

PNA materials

The PNA oligomer EO24 (PerSeptive Biosystems, Inc, Framingham, Massachusetts, USA): NH_2Gly-TATTCTGTCTTTTTACTAATTTAC-Cys-$CONH_2$, 24-mer, a specific probe for detection of *E. histolytica* rDNA targets, was purified by RP-HPLC.

HPLC was performed using a reverse phase Ultrasphere C18 column (Beckman Instruments, Fullerton, California, USA), at ambient temperature and a flow rate of 1 ml min^{-1} with 0·1 M triethylammonium acetate, pH 7·5 and acetonitrile. A two-step gradient of acetonitrile (7–25% in 18 min, followed by 25–40% in 5 min) was used for the purification of the products from each reaction. Whenever coumarin was part of the product to be purified, an in-line fluorescence detector (FluoroMonitor, Milton Roy, Ivyland, Pennsylvania, USA) was used to detect the dye.

Derivatization of the oligonucleotide EOstem 10 with DABCYL

A 300 µl solution containing 2 µg μl^{-1} oligodeoxyribonucleotide EOstem10 dissolved in 0·1 M sodium bicarbonate buffer, pH 9·0, was reacted with a 500 µl solution containing 10 mg ml^{-1} of DABCYL-NHS (Molecular Probes, Inc, Eugene, Oregon, USA, D-2245) in N,N-dimethylformamide, with continuous stirring at 25°C, over a 36 h period. The unreacted DABCYL was removed by precipitating the oligonucleotide with 2·5 volumes of ethanol and 0·1 volume of 3 M sodium acetate, pH 5·2. The derivatized oligonucleotide was then purified by RP-HPLC.

Derivatization of the PNA EO24 with AMCA

A 200 µl solution containing 240 ng μl^{-1} PNA EO24 in 0·1 M sodium bicarbonate buffer, pH 9·0, was reacted with a 100 µl solution containing 10 mg ml^{-1} of AMCA-NHS (Molecular Probes, Inc., A-1418) in N,N-dimethylformamide, with continuous stirring at 37°C, for 4 h. A volume of 20 µl of 10 mM dithiothreitol was then added to the reaction mixture and the incubation was continued for 8 h at 25°C. The unreacted coumarin was removed by centrifugation, followed by exclusion chromatography through a Biogel P4 (Bio-Rad) column. The derivatized PNA was purified by RP-HPLC.

Activation of the thiol in the PNA

The coumarin derivatized PNA was treated with 10 mM dithiothreitol and the resulting solution was stirred for 18 h at 37°C. The product was then collected by RP-HPLC directly into 100 µl of a 1 mg ml^{-1} 4,4′-dithiopyridine solution in 100 mM phosphate buffer, pH 5·5, containing 20% acetonitrile.[8] This mixture was allowed to react overnight at 25°C, after which the product (thiopyridylated PNA-coumarin) was purified by RP-HPLC, dried *in vacuo* and resuspended in 300 µl of 0·1 M triethylammonium acetate, pH 7·5, containing 20% acetonitrile.

Coupling of PNA and DNA via disulfide exchange reaction

Ten nanomoles of the derivative oligonucleotide EOstem10-DABCYL were treated with 10 mM dithiothreitol and the resulting solution was stirred for 4 h at 37°C. The reduced product was then collected

by RP-HPLC directly into the thiopyridyl-PNA-coumarin solution resulting from the previous step.[8] The reaction mixture was stirred overnight at room temperature. The product was then purified by RP-HPLC.

Thermal denaturation and renaturation profiles

Fluorescence measurements were performed on an LS-50 Luminescence Spectrometer (Perkin Elmer, Connecticut, USA) coupled to a system of two circulating water baths (Thermomix-Frigomix, B. Braun, Kronberg, Germany, and Lauda, Brinkmann, New York, USA), using 1 cm path length and 400 μl volume cuvettes (Uvonic Instruments, Inc., New York, USA). The fluorescent probe was excited at 354 nm and the emitted fluorescence measured at 450 nm. The fluorescence of a 300 μl solution containing 3·3 pmol of the probe in 10 mM Tris-HCl, 0·1 mM EDTA, 20 mM KCl, pH 7·5 buffer, was monitored as the temperature was increased from 25 to 70°C and then returned back to 20°C. A similar solution was treated with 10 mM dithiothreitol and stirred at 37°C overnight. After reduction, a new denaturation-renaturation profile was recorded. Melting temperatures were determined by computer fit of the first derivative of fluorescence emission with respect to l/T.

Real-time solution assays of probe-target interactions

The fluorescence of a 300 μl solution containing 3·3 pmol of the probe in 10 mM Tris-HCl, 0·1 mM EDTA, 20 mM KCl, pH 7·5 buffer, was monitored continuously in a spectrometer. After 5 min, an irrelevant target mixture was added consisting of 100 μg denatured salmon sperm DNA, 2·5 pmol of ssDNA from an asymmetric PCR reaction of *Rotavirus* amplicons, 166 pmol of the oligonucleotide F4MU.T.34 and 166 pmol of the oligonucleotide GM.LR.74 in 60 μl of the same buffer. After 12 min, 10 μl of a specific DNA target was added, consisting of 16·5 pmol of the oligonucleotide EO19 in the same buffer. The fluorescence emission was recorded continuously for another 25 min.

Probe immobilization

Probes were immobilized on streptavidin coated microtitre plates (Reacti-Bind, Pierce, Illinois, USA) using the following protocol: 50 μl of a solution containing 0·5 pmol μl^{-1} of the chimeric probe, 100 ng μl^{-1} of denatured salmon sperm DNA, 0·1% of gelatin and 1 × SSC, was incubated for 1 h at 50°C with gentle shaking (150 rpm) inside each well. The plate was then cooled to room temperature and rinsed twice with 250 μl of washing buffer (Na_2HPO_4/NaH_2PO_4 0·1 M, 0·05% Tween 20, pH 7·5).

Interaction of immobilized probes with an artificial DNA target

The microtitre plate with the immobilized probe was rinsed once with 50 mM Tris-HCl, 0·5 mM EDTA, 10 mM NaCl, pH 7·5 buffer. A 0·66 pmol μl^{-1} solution of the oligonucleotide EO19 in TE buffer (10 mM Tris-HCl, 0·1 mM EDTA, pH 7·5) was delivered into the wells of the plate, with a coefficient of decrement for the target concentration of 4/5. As controls the authors used wells containing: (1) buffer without probe or target; (2) buffer and probe but no target; and (3) buffer, probe and a cocktail of heterologous DNAs (1·5 pmol of the product from the asymmetric PCR from *Rotavirus* amplicons, 16·6 pmol of the oligonucleotide F5MU.T.34 and 16·6 pmol of the oligonucleotide GM.LR.74). Hybridization was performed 1 h at 37°C with gentle shaking (150 rpm). Fluorescence was then read in a CytoFluor® 2300 system (Millipore) using the filter set A/A (excitation 360 ± 20 nm; emission 460 ± 20 nm), at a sensitivity setting of 4. The relative fluorescence was calculated by subtracting the measured fluorescence from a control which contained the probe, but not the target. The plate was protected from light at all steps.

Detection of PCR products from *Entamoeba histolytica*

Polymerase chain reaction amplification of *E. histolytica* rDNA was performed as described.[7] The microtitre plate with the immobilized probe was rinsed once with 10 mM Tris-HCl, 0·1 mM EDTA, 12·5 mM KCl, and pH 8·3 buffer. The PCR product was denatured for 3 min at 98°C, and then delivered into the plate in aliquots containing estimated amounts of complementary target sites, starting at 5×10^{12}, with a coefficient of decrement of 5/6 from each well to the next. The negative controls were the same as for the synthetic target. As positive controls were used wells with 1×10^{12}, 5×10^{11} and $2{\cdot}5 \times 10^{11}$ molecules of the complementary oligonucleotide EO19. The rest of the procedure was as described above for the synthetic target.

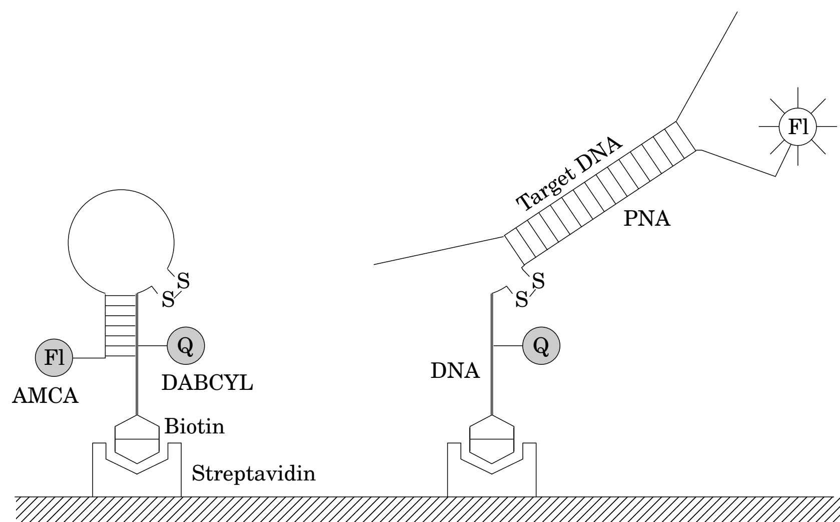

Fig. 1. The chimeric PNA-DNA probe in the 'closed' (quenched) state and, after the interaction with the target, in the 'open' (fluorescent) state.

RESULTS

Design and synthesis of PNA molecular beacons

The structure and mechanism of operation of the chimeric PNA-DNA molecular beacon is shown diagrammatically in Fig. 1. Figure 2 shows the base sequence and chemical structure of the PNA-DNA adduct. The DNA part of the adduct has three functional moieties: (1) a 3′-terminal biotin which serves to immobilize the entire structure by binding to streptavidin-coated mitrotitre wells; (2) a quencher moiety (DABCYL) at the 3′-terminus of the DNA stem structure; (3) a sequence which pairs with 10 bases in the PNA part of the adduct, forming the 10-base pair stem structure. The PNA part of the adduct, which is joined to the DNA part via a disulfide bond, also comprises three functional moieties: (1) a 15-base probe sequence in the unstructured loop domain; (2) 10 bases capable of pairing with the DNA part, where one base in the PNA probe performs double duty, also being part of the 10-base stem structure; (3) a fluorescent moiety (AMCA), coupled to the free terminus of the PNA.

In the absence of target, the DNA and PNA arms of the probe interact to form a stem and loop structure. This conformation places the fluorophore and the quencher in close proximity, and in this state no fluorescent emission can be detected. In the presence of the target, a rearrangement occurs, as the probe forms a larger and more stable duplex than the stem. The strands that comprise the stem are forced apart, separating the fluorophore and the quencher to a distance where no resonance can occur, thus allowing the fluorophore to emit detectable photons (Fig. 1). As the fluorophore-quencher pair the authors selected the fluorophore 7-amino-4-methylcoumarin-3-acetic

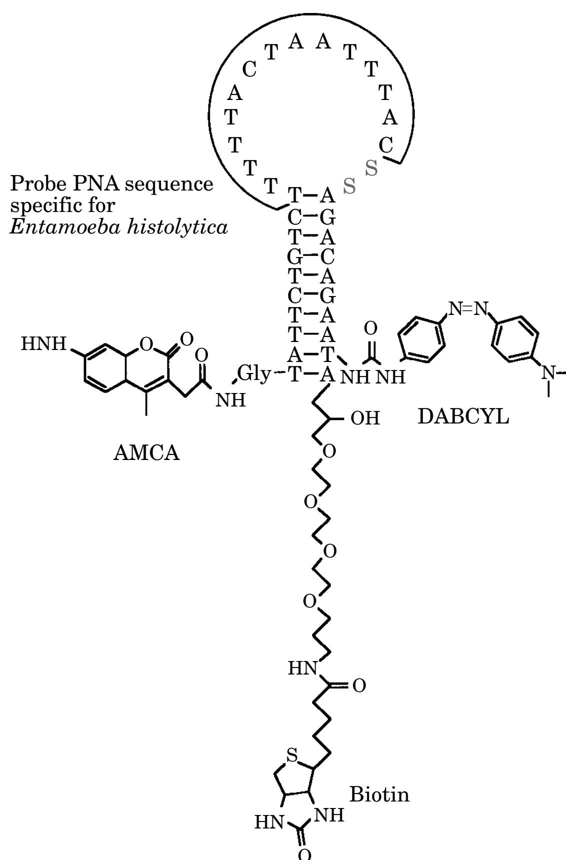

Fig. 2. Structure and sequence of the PNA-DNA molecular beacon.

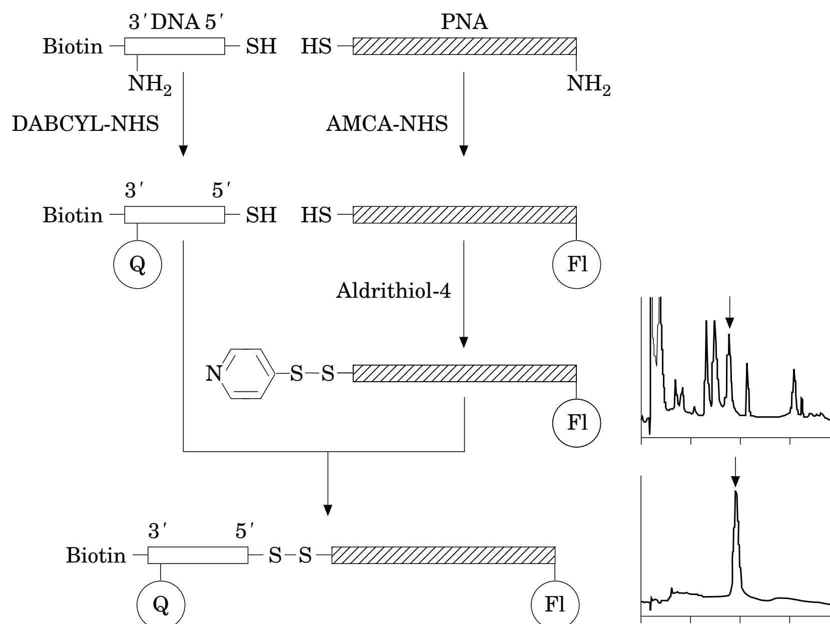

Fig. 3. Scheme of the synthesis of the chimeric PNA-DNA probe. HPLC chromatograms are also shown for the last two steps (main products indicated by arrows): thiol activation of the coumarin-derivatized PNA and purification of the final adduct (time intervals of 10 min).

acid (AMCA) and the quencher p-(dimethylaminophenylazo)benzoic acid (DABCYL). When stimulated by ultraviolet light (354 nm), AMCA emits blue fluorescence (440 nm). DABCYL is a non-fluorescent dye with an absorption spectrum that perfectly overlaps the emission of AMCA.

The sequence of the probe was designed to interact with DNA generated by PCR amplification of the ribosomal episome (rDNA) of *E. histolytica*[9] which the laboratory has used as a model system for DNA amplicon detection methods.[7] The target and probe sequences were chosen on the basis of the high stability of PNA oligomers containing at least 20–25% purine. The sequences used for the DNA-PNA stem were arbitrary, but the PNA part of the stem was also restricted to a 25% purine content.

The scheme followed to achieve the synthesis of the adduct is shown in Fig. 3. A 3′-end biotinylated deoxyribonucleotide which includes an aminated thymidine and a 5′ thiol group was coupled to the quencher DBACYL-succinimidyl ester by formation of a stable carboxamide with the aminated thymidine. A PNA molecule which has an amino-terminal glycine and a carboxyl-terminal cysteine was covalently linked to the fluorescent moiety coumarin-succinimidyl ester by the same reaction with the amino group of the glycine. Both species were then conjugated via disulfide coupling of the 5′ thiol group of the deoxyribonucleotide with the cysteine of the PNA.

Hybridization properties of the PNA-DNA molecular beacon

Thermal melting and renaturation experiments were performed using either the intact chimeric probe, or a probe in which the disulfide bond joining the DNA and PNA moieties had been cleaved by reduction with DTT. After DTT reduction, in essence, the interaction of an equimolar amount of two non-linked complementary sequences should be observed. The results (Fig. 4a) indeed show a marked difference in melting and renaturation behaviour. The profiles for the covalently-linked structured probe display a higher melting temperature (the Tm for dissociation was 52·1°C; for reassociation, 50·4°C) and a less significant hysteresis than the profiles for the separated molecules. The latter showed a considerably less stable interaction (Tm for dissociation, 46·1°C; for reassociation, 37·4°C). Remarkably, these curves were reproduced with no significant change using a buffer that contained 5 mM $MgCl_2$ (data not shown). These observations indicated that melting behaviour is relatively independent of the concentration of bivalent ions.

The PNA-DNA molecular beacon was highly specific for the target DNA sequence. The beacons were incubated in a cuvette inside a spectrofluorimeter and the response to the addition of different DNA targets was followed in real time. High concentrations of heterologous DNAs, comprising a mixture of genomic

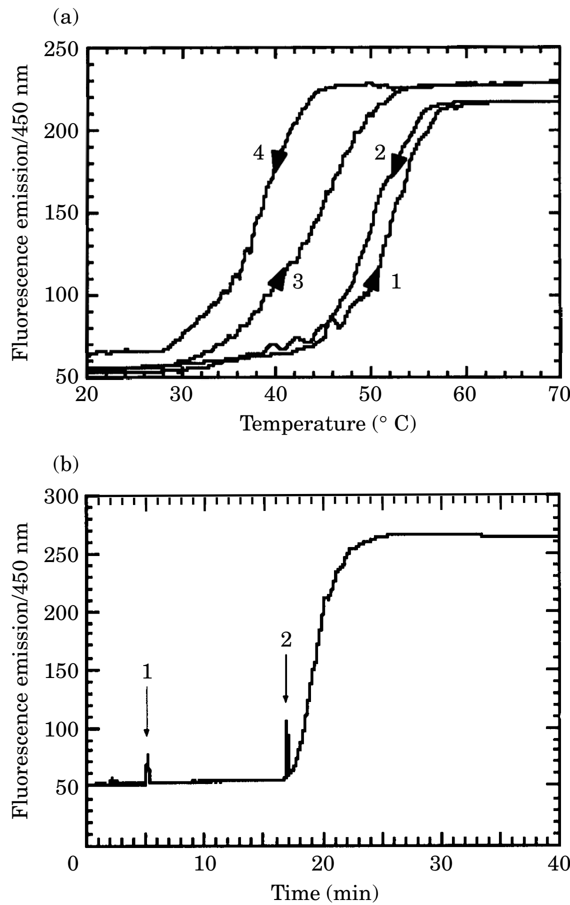

Fig. 4. (a) Melting (1) and renaturation (2) profiles of the 'native' chimeric PNA-DNA probe and for the probe after treatment with DTT (3 and 4, respectively). (b) Specificity of the probe for *Entamoeba histolytica* targets: (1) addition of a cocktail of heterologous DNAs; (2) addition of the correct oligonucleotide target.

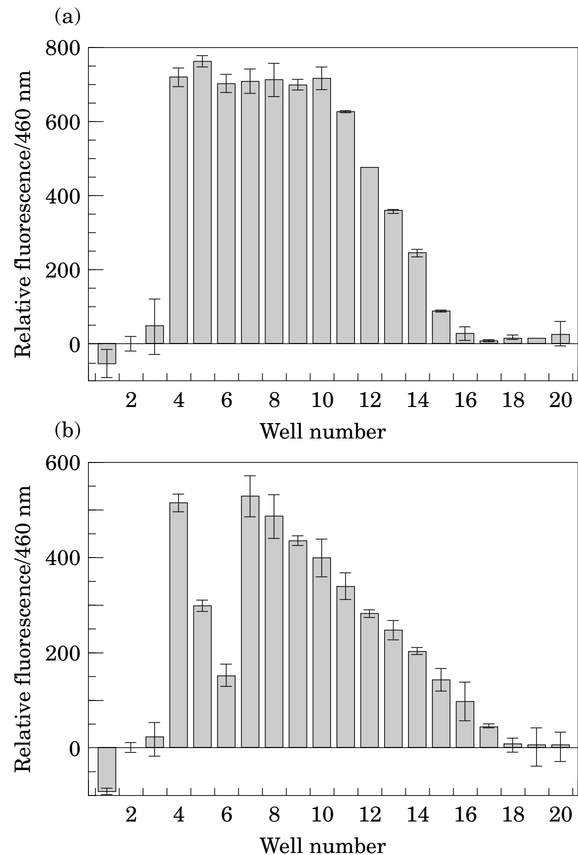

Fig. 5. (a) Detection of the complementary oligonucleotide target by the PNA-DNA probe. Wells No. 4–20; oligonucleotide EO19, from $2{\cdot}0 \times 10^{13}$ to $1{\cdot}6 \times 10^{10}$ molecules, with a decrement coefficient of 16/25. (b) Detection of the rDNA polymerase chain reaction (PCR) product from *Enthamoeba histolytica*. Wells No. 4–6, controls with $1{\cdot}0 \times 10^{12}$, $5{\cdot}0 \times 10^{11}$ and $2{\cdot}5 \times 10^{11}$ molecules of EO19, respectively; wells No. 7–20, PCR product, from $5{\cdot}0 \times 10^{12}$ to $4{\cdot}7 \times 10^{11}$ molecules, with a decrement coefficient of 5/6. For both (a) and (b): well No. 1, control without probe and target; well No. 2, control with probe but without target; well No. 3, control with probe and a cocktail of irrelevant sequences.

DNA, an unrelated PCR product and oligonucleotides with different lengths and sequences, had no effect on the fluorescence emitted by the system (Fig. 4b, target addition No. 1). On the other hand, the addition of a complementary oligonucleotide target led to a rapid increase in fluorescence (Fig. 4b, target addition No. 2).

Detection of artificial DNA targets in microtitre plates

The results for the interaction of the immobilized PNA molecular beacons with a synthetic oligonucleotide target are shown in Fig. 4a. The immobilized probe is capable of detecting targets that differ in amount by one order of magnitude, from $1{\cdot}2 \times 10^{11}$ to $1{\cdot}1 \times 10^{12}$ molecules. The interaction is highly specific, since the negative controls, which contained more than 2×10^{13} irrelevant molecules, did not generate a significant signal.

Specific detection of rDNA amplicons in microtitre plates

Figure 5b shows the results of an assay for the detection of PCR amplicons containing repeated sequences of the rDNA of *E. histolytica*. The number of target sites was calculated by estimating the number of molecules of rDNA amplicons in a polyacrylamide gel and then correcting for the number of sequence repeats in each amplicon. The assay signal was highly

specific for *E. histolytica* rDNA amplicons and the fluorescence intensity was proportional to the number of estimated target sites within the range of 5×10^{12}–8×10^{11} molecules. However, the signal generated by the PCR products was approximately four times lower than the signal generated by an equivalent number of artificial DNA targets. This observation suggests that the renaturation of PCR products competed with the amplicon/immobilized PNA interaction, possibly due to DNA helix stabilization by the presence of residual monovalent and divalent ions derived from the PCR reaction buffer. This problem would be less acute if the molecular beacons were in solution, because the rate constant for PNA/DNA reassociation would then be much higher.

DISCUSSION

The authors have documented a simple method for detecting PCR amplicons using surface-bound fluorescent probes. The principle of exploiting resonance energy transfer for homogeneous DNA detection assays was first described by Morrison in 1987.[10] In 1996 the authors described 'molecular beacons', a novel probe design[11] combining increased specificity and a very efficient fluorescence quenching system. Tyagi and Kramer demonstrated the usefulness of molecular beacons for the detection of PCR products in real time, as well as a remarkable capability for discrimination of single-base mismatches. Recently, they showed that DABCYL, when used in a molecular beacon format, can serve as a universal quencher for a variety of dyes.[2] This observation had greatly expanded the number of fluorescent dyes that may be used in molecular beacon format, making possible multiplex assays in solution. The recent work of Tyagi and Kramer also suggests that the excellent allele discriminating properties of molecular beacons may be due to the hairpin structure of the probe. The work presented here extends the applications of molecular beacons to include the use of PNA probes, which the authors have employed in a novel surface-immobilized format.

The authors experimental protocol for the detection of DNA amplicons post-PCR is remarkably simple. After a standard PCR is performed, an aliquot of the product is transferred to a microtitre plate and, after a short incubation period, the data is recorded using an automated fluorescence plate reader. The use of a PNA probe permitted the use of relatively short (15-base) sequence of high binding affinity. The improved hybridization stability of PNA over DNA made possible the direct detection of double-stranded PCR products using a simple heat denaturation step, because the PNA probes are able to compete to a reasonable extent with the reassociating DNA strands.[4] The relative insensitivity of the DNA-PNA beacon's melting behaviour to the concentration of magnesium ions suggests that bivalent ion concentration should be less critical when using PNA molecular beacons as opposed to DNA molecular beacons.

A limitation of the PNA-DNA molecular beacon assay described here is the narrow dynamic range for DNA detection in microtitre format. This, however, is not an inherent limitation of the molecular beacon system, being due instead to factors that can be improved considerably. An important detrimental factor was the relatively high background fluorescence of the instrument (Millipore Cytofluor® 2300) and microtitre dishes (Pierce, Reacti-Bind) that were used. A second limiting factor was the coumarin-based fluorescent moiety, which gives a relatively poor fluoresce quantum yield and is subject to rapid photobleaching. The initial choice of this fluor was based on the excellent fluorescent overlap with the absorbance spectrum of DABCYL. However, as mentioned above, a variety of high quantum yield fluorescent compounds, including fluorescein and other dyes, can be effectively quenched by DABCYL when employed in the molecular beacon format.[2] By employing fluorescent dyes with higher quantum yields, as well as optimized surfaces and detection instrumentation, it should be possible to realize a significant increase in the sensitivity and dynamic range of surface-immobilized PNA molecular beacons.

The use of a DNA oligonucleotide as part of the PNA-DNA molecular beacon served to facilitate the authors' original prototyping studies for the choice of an optimal length for the stem portion of the molecule, allowing inexpensive modifications of this critical parameter. However, in the future it will be preferable to synthesize molecular beacons where the entire molecule is made of PNA. Molecular beacons made entirely of PNA could be constructed with relatively small stem structure (5–6 bases), for a total PNA length of 25–27 bases. Weiler *et al.* have recently described the solid-state synthesis of arrays containing hundreds of different PNA oligomers.[12] It should of course be possible to adapt this chemistry to the synthesis of PNA molecular beacons on surface arrays. By combining PNA array technology with fiber-optic, site-addressed detection instrumentation, it should become possible to perform multiplex fluorescence detection in arrays of different PNA molecular beacons bearing unique probe sequences. Such array formats, which can be adapted for use within a

thermocycler, would permit the simultaneous detection of a very large number of DNA targets. Most importantly, the same, highly optimized fluor/quencher pair could be used in all the molecular beacons because on a surface array format the signals are spatially resolved. Another exciting prospect, which remains to be explored, is the possibility that PNA molecular beacons may offer improved stringency for the discrimination of point mutations, as is the case for DNA molecular beacons.[2,4]

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REFERENCES

1. Tyagi, S. & Kramer, F. R. (1996). Molecular beacons: probes that fluoresce upon hybridization. *Nature Biotechnology* **14**, 303–8.
2. Tyagi, S., Bratu, D. & Kramer, F. R. (1998). Multicolor molecular beacons for allele discrimination. *Nature Biotechnology* **16**, 49–53.
3. Nielsen, P. E., Egholm, M., Berg, R. H. & Buchardt, O. (1991). Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. *Science* **254**, 1497–500.
4. Egholm, M., Buchardt, O., Christensen, L. *et al.* (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. *Nature* **365**, 566–8.
5. Sawadogo, M. & Van, D. M. (1991). A rapid method for the purification of deprotected oligodeoxynucleotides. *Nucleic Acids Research* **19**, 674.
6. Mittal, V., Sehgal, D., Bhattgcharya, A. & Bhattacharya, S. (1992). A second short repeat sequence detected downstream of rRNA genes in the Entamoeba histolytica rDNA episome. *Molecular and Biochemical Parasitology* **54**, 97–100.
7. Estrada, G., Colin, L., Gaytan, P., Alagón, A. & Lizardi, P. M. (1996). Sequence-specific detection of PCR-amplified DNA by restiction enzyme release of hybrids. *Molecular and Cellular Probes* **10**, 179–85.
8. Zuckermann, R., Corey, D. & Schulz, P. (1987). Efficient methods for attachment of thiol specific probes to the 3′- ends of synthetic oligodeoxyribonucleotides. *Nucleic Acids Research* **15**, 5305–21.
9. Mittal, V., Bhattacharya, A. & Bhattacharya, S. (1992). Organization of repeated sequences in the region downstream to rRNA genes in the rDNA episome of Entamoeba histolytica. *Archives of Medical Research* **23**, 17–8.
10. Morrison, L. E., Halder, T. C. & Stols, L. M. (1989). Solution-phase detection of polynucleotides using interacting fluorescent labels and competitive hybridization. *Analitical Biochemistry* **183**, 231–44.
11. Lizardi, P. M., Tyagi, S. & Kramer, F. R. (1996). Detectably labeled duel conformation oligonucleotide probes, assays and kits. European Patent Application EPO 745 690 A2.
12. Weiler, J., Gausepohl, H., Hauser, N., Jensen, O. N. & Hoheisel, J. D. (1997). Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. *Nucleic Acids Research* **25**, 2792–9.