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New Concepts of Fluorescent Probes for Specific Detection of DNA Sequences: Bis-Modified Oligonucleotides in Excimer and Exciplex Detection

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Abstract; The detection of single base mismatches in DNA is important for diagnostics, treatment of genetic diseases, and identification of single nucleotide polymorphisms. Highly sensitive, specific assays are needed to investigate genetic samples from patients. The use of a simple fluorescent nucleoside analogue in detection of DNA sequence and point mutations by hybridisation in solution is described in this study. The 5'-bispyrene and 3'-naphthalene oligonucleotide probes form an exciplex on hybridisation to target in water and the 5'-bispyrene oligonucleotide alone is an adequate probe to determine concentration of target present. It was also indicated that this system has a potential to identify mismatches and insertions. The aim of this work was to investigate experimental structures and conditions that permit strong exciplex emission for nucleic acid detectors, and show how such exciplexes can register the presence of mismatches as required in SNP analysis. This study revealed that the hybridisation of 5'-bispyrenyl fluorophore to a DNA target results in formation of a fluorescent probe with high signal intensity change and specificity for detecting a complementary target in a homogeneous system. Detection of SNP mutations using this split-probe system is a highly specific, simple, and accessible method to meet the rigorous requirements of pharmacogenomic studies. Thus, it is possible for the system to act as SNP detectors and it shows promise for future applications in genetic testing.

Key words: *5'-bispyrene, Trifluoroethanol (TFE), Exciplex, DNA detection, Fluorescence, Stokes shift.*

INTRODUCTION

DNA probes are single-stranded DNA molecules used in laboratory experiments to detect the presence of a complementary sequence among a mixture of other single-stranded DNA molecule. Fluorescent probes are now in widespread use in various formats of DNA/RNA assays, which use the high affinity and specificity of nucleic acid hybridisation and/or the possibility of target amplification [1-3]. Fluorescently labeled probes are used in a variety of applications, such as DNA sequencing, fluorescence in situ hybridisation (FISH), gene arrays, polymerase chain reaction (PCR), real-time PCR [1-3]. Fluorescent techniques are used extensively in nucleic acid research. Fluorescent labels give a direct simple signal, and the processes involving such groups can be easily monitored in real time repeatedly. It has been reported that hairpin forming oligonucleotides attached to a fluorescent dye at one end and a fluorescence quencher at the other showed an increase in fluorescence intensity on hybridisation to a specific DNA target [1-3]. This increase has facilitated real-time analysis of DNA sequences being amplified by PCR and detection of mutant DNA. The use of fluorescence energy transfer (FRET) between donor and acceptor dyes is another example of solution-based detection of DNA [4-9]. In this approach, changes in fluorescence emission wavelength as well as fluorescence intensity can be used to define a specific sequence in target DNA.

Detection of specific target nucleic acid sequences is commonly achieved by hybridisation of a fluorescently labeled oligonucleotide to its complementary target, but this usually has very large background fluorescence signals, commonly more than 65% when one compares the same fluorophore-labelled oligonucleotide in its single-stranded and duplex-bound forms. It is possible to decrease background fluorescence by using two short oligonucleotide probes that are complementary to neighbouring sites of the same DNA target and can form a pyrene excimer (A = B = pyrene in Figure 1). Excitation of one of the pyrene partners forms an excited state dimer (excimer) that emits at long wavelength. For excimer-DNA systems, A (e.g. pyrene) and B (e.g. pyrene) must be identical but for exciplex-DNA systems A (e.g. pyrene) and B (e.g. naphthalene) are different from each other. For exciplex or excimer emission, the partners A and B must be able to approach each other very closely; typically the distance is ~4 Å for the pyrene excimer. This distance is about the thickness of a base pair. Thus, the potential resolution of the exciplex or excimer systems theoretically approaches a single base pair.

Recently, methods based on the detection of changes in the emission of interacting pairs of fluorophores, identical as in excimers or not identical as in exciplexes, provide a promising way to determine the presence of a single nucleotide alteration in DNA. An example from this field is the pattern of excimers (exciplexes) from two spatially proximal planar fluorophores linked to DNA. Excimer fluorescence has been observed in aqueous solutions of pyrene-linked nucleic acids and their analogues [10,11]. Changes in the excimer/monomer fluorescence intensity ratio allowed the detection of complementary complexes (duplexes) [12,13]. Excitation of one of the pyrene partners forms an excited state dimer (excimer) that emits at long wavelength (Stokes shifts >100nm) [13-17]. Thus, the target assembles its own detector from components, which are non-fluorescent at the detection wavelength, and that greatly reduces background.
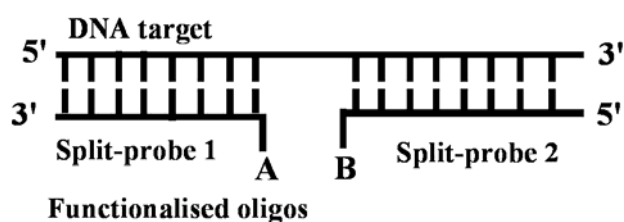
Figure 1 Split probe system

Although recent reports have shown that pyrene monomer emission is useful for fluorescent detection of a given base at the target site in DNA [18], pyrene-oligonucleotide probes employing the monomer emission usually have the disadvantage of fluorescence quenching. In oligonucleotides covalently attached through a flexible tether to pyrene at the internal phosphate [19], the nucleotide bases [20], and the terminus [21], fluorescence quenching of the pyrene strongly occurs both in the single-stranded and double-stranded forms via migration of an electron between the excited pyrene and the nucleotide bases [22-24]. To avoid these disadvantages, several efforts using the pyrene-excimer emission in place of the monomer emission have been made in the development of hybridisation probes [18,25-27]. Because pyrene-excimer emission is less sensitive to quenching by nucleobases than monomer emission, the pyrene-excimer-forming probes have been successfully used for DNA and RNA hybridisation assays [27-29].

There is current interest in the design and synthesis of fluorescence-labelled oligonucleotides with a strongly improved signal on hybridisation. Oligonucleotides labelled by fluorophores can be synthesised by different approaches [30-33]. It was reported by Paris et al (1998) that specific DNA sequences can be detected by utilising oligonucleotides containing pyrene-modified C-nucleoside [17]. In addition, Lewis et al (1997) have indicated that oligonucleotide probes labelled by a bis-pyrenyl group at the 5'-terminus can be used to observe hybridisation with a target DNA [34].

This study describes experimental structures and conditions that permit strong exciplex emission for nucleic acid detectors (conceptualised in Figure 2), and shows how such exciplexes can register the presence of mismatches as required in SNP analysis [35, 36].
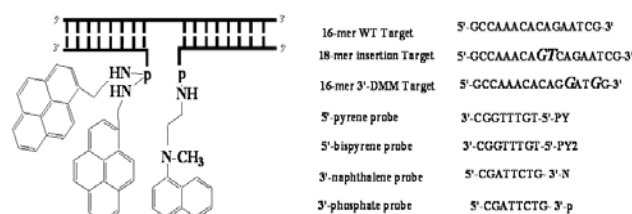

Figure 2 Target strands and nomenclature used for exciplex formation with mismatched targets. Mismatches and inserts are shown in italic. The Exci-probes used were: CGGTTTGT-5'-bispyrene and naphthalene-3'-GTCTTAGC.

Materials and methods
Materials and nature of the probes and targets
The exciplex constructs used standard DNA base/sugar structures in both target and probes. The target was a

part of the Leishmania major chromosome 1 sequence (Genbank reference [AE001274 nucleotides 90014-90029]: cccttccctc cctcgattct gtgtttggcg aggacacccg cctcgacgac atcacacacg; the bold bases provided the complement to the target used). The ExciProbe probes had the sequences 3'pGTCTTAGC-5' and 3' CGGTTTGT-5'-p. The probes were supplied with a free 5'-phosphate group (p). DNA probes and DNA targets were obtained from Proligo, Paris (Sigma- Proligo) and deuterium oxide was from Cambridge Isotope Laboratories (Goss-Scientific Instruments Ltd, UK). Distilled water was purified by ion exchange and charcoal using a MilliQ system (Millipore Ltd, UK). Tris buffer was prepared from analytical reagent grade materials.

Determination of ph
The pH values were measured using a Hanna-instruments (Portugal) HI 9321 microprocessor pH meter, calibrated with standard buffers (Sigma-Aldrich) at 20 C.
Reversed-phase HPLC
HPLC was performed on an Agilent 1100 Series system, with both diode-array and fluorescence detection for on-line acquisition of spectra. Columns were Zorbax Eclipse X DB-C8 column (l25 x 4.6 mm, 5 μm), or Luna C18 (2) (25 cm x 4.6 mm, 5 μm) using an acetonitrile-water gradient from 0% to 50%.

UV-visible spectrophotometry
UV–visible absorption spectra were measured at 20 C on a Peltier-thermostatted Cary-Varian 1E UV–visible spectrophotometer. Quantification of oligonucleotide components used millimolar extinction coefficients (260) of 79.9 for ExciProbe-3'-phosphate, 70.2 for ExciProbe-5'-phosphate and 169.4 for 16-mer target. Extinction coefficients were calculated by the nearest neighbour method [45]; the contribution of the exci-partners was small and could be neglected. Tm values (first derivative method) based on A260 were determined in Peltier-thermostatted quartz cuvettes using a Cary 4000 UV-visible spectrophotometer.

Melting temperature studies
Optical melting curves of the complexes were obtained using a Varian Cary 1E UV-visible spectrophotometer using a 1-ml optical cell with a 1.0-cm path length. Tm measurements using first derivative method, detected at 260 nm and accurate to 0.1°C, were performed at 2.5-μM component concentrations of the 1:1:1 complex in 80% TFE/Tris buffer (10 mM Tris, pH 8.4, 100 mM NaCl). Melting data were also obtained from fluorescence emission spectra: the sample was heated to 60°C at a rate of 0.25°C per minute. We used the wavelengths of λex 340nm and λem 376nm for the pyrene monomer, and λex 350nm and λem 480nm for the exciplex. Recordings were made at 0.5°C increments. The sample was cooled at a rate of 0.13°C per minute and the emission spectra were recorded once again. The melting curve obtained during cooling was used determine Tm values.

Spectrophotofluorometry
Fluorescence emission and excitation spectra were recorded in four-sided quartz thermostatted cuvettes using a Peltier-controlled-temperature Cary-Eclipse, spectrofluorophotometer. All experiments were carried out at 5°C. 5'-bispyrene was added first, followed by target DNA and then 3'-naphthalene. Control experiments were

conducted using 5'-bispyrene first, followed by target DNA and then 3'-free oligonucleotides. Most spectra were buffer and 3'-naphthalene corrected. The mole ratio of oligonucleotide target DNA, 5'-probe and 3'-probe was 1:1:1 and the concentration of each component was 2.5 µM. Tris buffer was added also at a concentration of 2.5 µM either with or without 80% TFE, and the volume was adjusted to 100 µl with water. Excitation wavelengths of 340 nm (for the pyrene monomer) and 350 nm (for the full split probe system) were used at a slit width of 5 nm and recorded in the range of 350-650 nm. Emission spectra were recorded after each sequential addition of each component to record the change in emission due to each addition. A baseline spectrum of buffer, buffer plus water, and water plus 80% TFE was always carried out first. On each addition the solution was left to equilibrate for approximately five minutes in the fluorescence spectrophotometer and emission spectra were recorded until no change in the spectra was seen to ensure that equilibrium had been reached.

Titration experiments

Titration experiments were carried out using a stock solution of wild type target DNA of 0.159 mM concentration and diluted concentrations of 0.4, 0.8, 1.5, 1.6, 2.0 and 2.5 µM. 5'-bispyrene was kept at a constant concentration 1.7 µM, Tris buffer was used at a concentration of 2.5 µM. Total volume was 100 µl. Excitation wavelengths of 340 nm (for the pyrene monomer) and 350 nm (for the full split probe system) were used at a slit width of 5 nm and recorded in the range of 350-650 nm. A baseline spectrum of buffer and water was carried out first and then emission spectra on each dilution of target were recorded.

Synthesis and oligonucleotide modification

Attachments of N-Methyl-N-naphthalen-1-yl-ethane-1,2-diamine dihydrochloride exci-partner and 1-pyrenemethylamine to oligonucleotide probes and attachments of 5'-bis-pyrene probe were as previously described [36,37,38].

Results

Attachment of 1-pyrenemethylamine to 5'-probe to give a 5'-bispyrene probe. A typical purification chromatogram showed the elution of free 5'-probe oligonucleotide at early retention times (13.5 min), followed by 5'-pyrene oligonucleotide- conjugates (15.5 min), 5'-bis-pyrene oligonucleotide- conjugates (28 min), and finally free, unconjugated 1-pyrenemethylamine (33 min) with yields typically about 80%. Absorbance spectra of an unmodified 5'-probe oligonucleotide and 5'-bis-pyrene oligonucleotide probe indicated the attachments. In addition, the absorbance band at 348 nm is due to the presence of pyrene, which also alters the band at 260 nm. The ratio between the absorbance at 260 nm (A= 0.07) and 345 nm (A = 0.037) was 1.9, and attachments of 1-pyrenemethylamine to the 8-mer probe showed a ratio (A260/A345) of 3.0. The fluorescence emission spectra of the 5'-bispyrene probes have a maximum emission at 380 nm and 480 nm that correspond to pyrene monomer and excimer fluorescence.

Attachment of N'-methyl-N'-naphthalen-1-yl-ethane-1, 2-diamine to give a 3'-naphthalene probe. A characteristic

purification chromatogram demonstrated the elution of free 8-mer oligonucleotide at early retention times (16 min), followed by 3'-naphthalene oligonucleotide-conjugates (18 min), and finally the free unconjugated N'-methyl-N'-naphthalen-1-yl-ethane-1, 2-diamine (21 min); usual yields were about 75-85%. The shoulder at 310 nm on the 260-nm absorption band substantiates the presence of attached naphthalene.

Split-probe system using 5'-bispyrene in 80%TFE and aqueous solution. Experiments were conducted to identify if formation of exciplex occurred following the use of 5'-bispyrene instead of 5'-monopyrene in the same system as previously described [35]. Experiments were conducted in 80% TFE because previous studies using the 5'-monoyrene and 3'-naphthalene system found this to be the best co-solvent [35].

On addition of target, the broad peak at 480 nm increases (Figure 3); this change in the spectrum indicates hybridisation of the probe to the target. However, on addition of 3'-naphthalene, a decrease in emission at 480 nm was seen (Figure 3). This may be due to interaction with 5'-bispyrene forming an exciplex, which may not have as strong an emission as the excimer emission because a broad structure-less peak at longer wavelengths than that of the monomer is also characteristic of exciplex formation between pyrene and naphthalene.
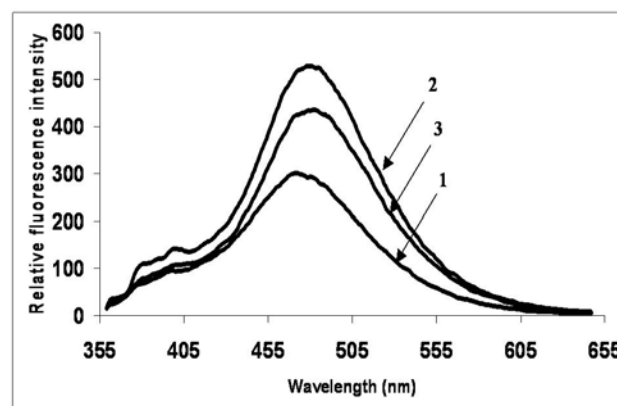

Figure 3 Fluorescence spectra of 5'-bispyrene alone (1), 5'-bispyrene and target (2) and 5'-bispyrene, target and 3'-naphthalene (3) in 80% TFE/ Tris buffer (0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5ºC. Component concentrations were 2.5 µM in a total volume of 100 µl. Excitation was at 350 nm, spectra are corrected for TFE and buffer.

The same system was studied in aqueous medium (no TFE) to see if this gives a spectrum that allows a better method of quantification. Decreased excimer emission intensity and increased monomer emission may be noted in the absence of TFE. Therefore, identification based on both monomer and excimer peaks, which will be more reliable as excimer-monomer ratio (IE/IM), could be used.

Figure 4 shows the broad structure-less peak at 480 nm and also smaller distinct peaks at 379 nm and 397 nm, which are due to the monomer that was not previously identified in 80% TFE. The intensity of excimer emission (at 480 nm) is much lower at 0% than that at 80% TFE, and the monomer peaks are much more obvious. These

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advantages make the aqueous medium preferable because identification of target depends on changes of both monomer and excimer peaks not just the excimer peak.

Addition of 3'-naphthalene to the 5'-bispyrene and target led to an increase in intensity at 480 nm. The peak lies between that of 5'-bispyrene alone and of the 5'-bispyrene when hybridised to the target and monomer peaks are quenched (Figure 4). The intensity of the excimer peak is not as strong as that of 5'-bispyrene alone. The change in spectra indicates that some interaction occurs.
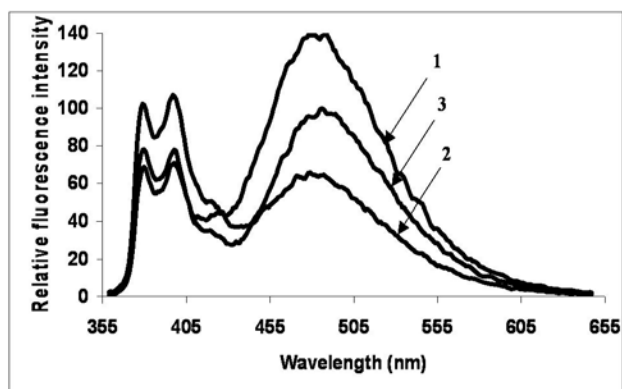

Figure 4 Fluorescence spectra of 5'-bispyrene alone (1), 5'-bispyrene and target (2), and 5'-bispyren, target and 3'-naphthalene (3) in 0% TFE/ Tris buffer (0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5°C. Component concentrations were 2.5 µM in a total volume of 100 µL. Excitation was at 350 nm, spectra are corrected for TFE and buffer.

Melting curve experiments

Using first derivative methodology, the melting temperature based on A260 of the full system in 0% v/v TFE/Tris buffer (10 mM Tris, 0.1 M NaCl, pH 8.4) was determined to be 32.0   0.8°C (Figure 5). The Tm is the same as that found for the ExciProbe system in water [35], which indicates that stabilisation of the duplex is no further enhanced or hindered. Direct comparison of Tm can be made because the target sequencing and conditions are the same. Tm of 5'-bispyrene full system is 7.0°C higher than that of the 5'-monopyrene-naphthalene Tm in 80% TFE. This difference in the Tm is related to the fact that water is known to allow intercalation and stacking interactions of the probes with DNA. These interactions also stabilise the duplex and therefore an increase in Tm would be seen. The difference in Tm between water and TFE might be due to this reason [14].

Control experiments using 3'-phosphate probe

To determine if the emission arises from exciplex formation between the wanted exci-partners (from exciplex formation between the exci-partners) and the nucleobases, control experiments were carried out. Experiments performed with a 5'-bispyrene, target and 3'-phosphate, and the 3'-probe bearing only a phosphate group at its "nick terminus" showed a decrease in the excimer peak (Figure 6). This indicates that the second exci-partner is needed for exciplex emission.

Detection of mismatched targets using 5'-bispyrene probe in aqueous medium

The 5'-bispyrene probe in an aqueous medium system was used to determine the effect of mismatches or inserts in the target, compared to the probe sequences on exciplex fluorescence. Different mismatch positions of the 16-mer-parent target sequence were used to analyse the effects of SNP (Figure 2). Excimer fluorescence of the pair of 5'-end and 3'-end pyrene-labelled oligonucleotide, complementary to adjacent region within target DNA, were successfully applied for detection of point mutation and quantification of DNA [12-14,17]. These different targets were used in order to determine whether the reaction between the two molecules was due to FRET or exciplex, and also if the system can identify base-pair mismatches.

Using 18-mer target containing two insertions

A target with 2 base-pairs between the two oligonucleotide probes was used in order to determine if the same changes in spectra are seen when no inserts are present. The distance between the two probes would affect the proximity of the fluorescent molecules and therefore affect the interaction between the fluorophores. No change seems to be apparent following addition of 3'-naphthalene to the system (Figure 7).
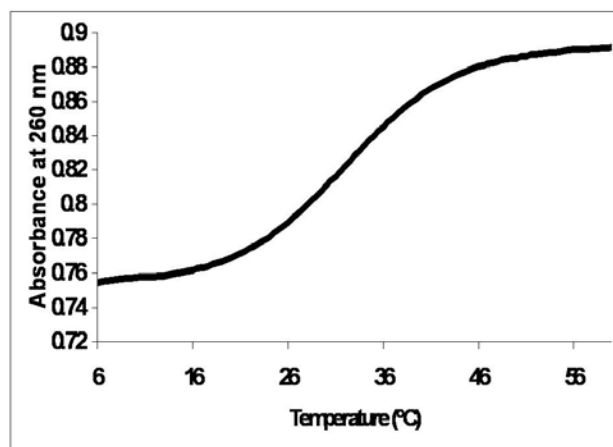

Figure 5 Melting curve of the full system in 0% v/v TFE / Tris buffer (10 mM Tris, 0.1 NaCl, pH 8.4) showing the change in absorbance at 260 nm as temperature was ramped at 0.25 °C/min.
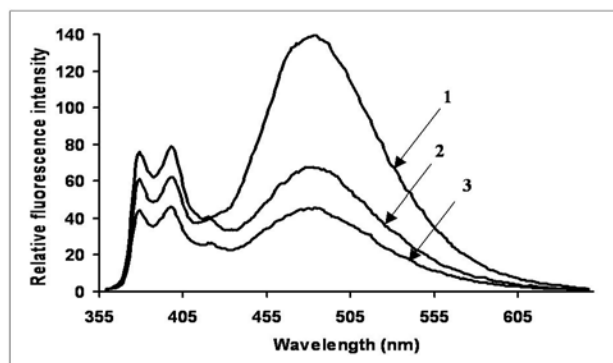

Figure 6 Fluorescence spectra of 5'-bispyrene alone (**1**), 5'-bispyrene and target (**2**) and 5'-bispyrene, target and 3'-phosphate (**3**) in 0% TFE/ Tris buffer (0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5°C. Component concentrations were 2.5 µM in a total volume of 100 µl. Excitation was at 350 nm, spectra are corrected for TFE and buffer.
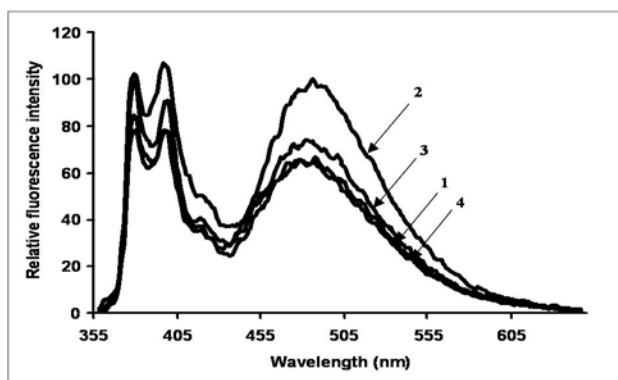
Figure 7 Fluorescence spectra of (**1**) 5'-bispyrene+WT target (-buffer), (**2**) 5'-bispyrene+WT target+ 3'-naphthalene (-3'-naphthalene and buffer), (**3**) 5'-bispyrene+3'-DMM target+ 3'-naphthalene (-3'-naphthalene and buffer) and (**4**) 5'-bispyrene+2-insertions target + 3'-naphthalene (-3'-naphthalene and buffer) in 0% TFE/ Tris buffer (0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5ºC. Component concentrations were 2.5 µM in a total volume of 100 µl. Excitation was at 350 nm, spectra are corrected (as indicated).

Using 16-mer target containing two mismatches

In order to investigate if this system could be used to identify the mismatches in the target sequences, a target containing two mismatches was used. Figure 7 shows that when the 3'-naphthalene probe is added to the 5'-bispyrene probe and the target, the exciplex intensity at 480 nm decreased to a lower extent than when WT was used. This result was unanticipated as the mismatches are on 3'-area where the 3'-naphthalene is expected to hybridise, even 5'-bispyrene probe still hybridise to target with complete complement.

Hybridisation of bis-pyrenylated oligonucleotides to 16-mer complementary DNA targets (titration)

Binding of the 5'-bis-pyrene probe to the DNA 16-mer target was investigated at 5ºC in Tris buffer (0.01 M Tris NaCl, pH 8.4). A solution of 5'-bis-pyrene probe was titrated with an increasing concentration of 16-mer targets at concentrations ranging from 0.4 µM to 2.4 µM. Reaction mixtures were allowed to set under hybridisation conditions until no fluorescence changes were detected before fluorescence measurements were performed. The 5'-bis-pyrene probe exhibits a decrease of excimer fluorescence intensities on duplex formation with complementary target in a concentration dependent manner. Figure 8 illustrates that as the concentration of target increases, the excimer peak at 480 nm decreases and the monomer peaks at 379 nm and 397 nm increase. Therefore, the ratio of monomer to excimer is affected by the concentration of target. In addition, Figure 8 demonstrated a linear relationship between the fluorescence intensity at 480 nm and the concentration of the target, as well as between the fluorescence intensity at 379 nm and the concentration of the target. It is expected that these linearity changes occurred because the species of the system are interacting at a 1:1 ratio. We also showed that excimer fluorescence intensity of the 5'-bipyrene depends linearly on the concentration of target DNA and permits quantification of DNA in solution (Figure 8).

Discussion

The split-probe approach of the present study uses externally oriented DNA-mounted excimer/exciplexes from two separate exciprobes designed to produce exciplex emission by being specifically assembled in situ by hybridisation with a bio-target. In this case, a distinctive fluorescence signal can be generated only after specific self-assembly of signal-silent excimer/exciplex components with a perfectly matched target. The advantage of the excimer/exciplex-based split-probe approach is low or zero background, ultra-biospecificity, and ability to detect DNA mismatches.

Attachment of 1-pyrenemethylamine to 5'-probe showed that the fluorescence emission spectra of the 5'-bispyrene probes have maxima at 380 nm and 480 nm, corresponding to pyrene monomer and excimer fluorescence, respectively, which is consistent with the proposed structure with two pyrene residues described in the literature [37,38].

5'-bispyrene in 80% TFE and aqueous solution was used to determine if formation of exciplex occurred using 5'-bispyrene instead of 5'-monopyrene. Previous studies identified no exciplex formation in aqueous conditions because the greater stacking of the bases and increasing the hydrophobic interactions would stabilise the B-form in a medium with high water activity. In a medium with low water activity this hydrophobic effect is less important and the A-form is favoured [39]. It is therefore possible that in the 80% TFE we used to study the exciplex the tandem duplex exists in the A-form. The difference in duplex structure between the A and the B forms might affect the interaction of the exci-partners. The sugar pucker or conformation of the 5-membered ribofuranosyl ring is also different in the A-type and B-type DNA conformations. In the A-type conformation the sugar ring adopts a C3'-endo conformation, while in B-type duplexes the conformation is C2'-endo. Because the phosphate group to which the exci-partners are bound is attached directly to the sugar rings, these various conformations may change the positions of the attached exci-partners in relation to one another. This conformational change suggests that the role of TFE in enhancing exciplex signals might be due to structural effects on the duplex.
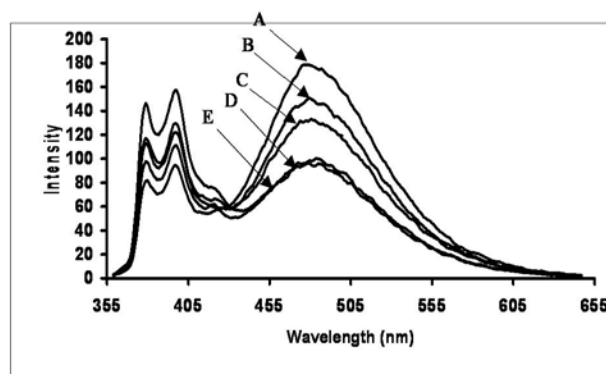

Figure 8 Fluorescence monitoring titration of 5'-bis-pyrene probe (1.7 µM) with different concentrations of complementary DNA target (**A** 0 µM, **B** 0.4 µM, **C** 0.8 µM, **D** 1.5 µM and **E** 2.0 µM) in Tris buffer (0.01 M Tris, 0.1 M NaCl, pH 8.4) at 5ºC. The total volume was 100 µl. Excitation was at 350 nm, slit width 5 nm. The inset shows the fluorescence intensity at 480 nm of 5'-bis-pyrene probe (1.7 µM) *versus* the concentration of the 16-mer target.

Data presented in this paper (Figure 3) indicates that the probe hybridised to the target and this is consistent with the reported literature [38]. It has been reported that excimer fluorescence of the 5'-bispyrene is sensitive to their interactions with the complementary strands as the 5'-bispyrene probe exhibits an increase in the excimer fluorescence intensities upon binding with corresponding targets [38]. Enhanced excimer fluorescence of the 5'-bispyrene in the duplexes could mean that hybridisation releases the nucleobase stacked pyrene residue. Possibly, the short linker between pyrene residue and oligonucleotide 5'-phosphate provides some steric constrains and avoids intercalation of the pyrene residue in rigid duplexes. Although in the flexible single-stranded probe (8-mer) the interaction of 5'-bispyrene with nucleobases can occur on duplex formation, these interactions are disordered and pyrene excimer is restored. Thus, enhancement of excimer fluorescence on hybridisation indicates that pyrene excimer is formed when conjugate binds the complementary strand. There is a possibility that bis-pyrenylated oligonucleotides possess their own structure with one or two pyrene residues involved, which affects the duplexes stability. The decrease in emission at 480 nm seen following addition of 3'-naphthalene might be due to interaction with 5'-bispyrene to form an exciplex. The resulting emission might not be strong as the excimer emission, and gives a broad structureless peak at longer wavelengths than that of the monomer. This is also characteristic of exciplex formation between pyrene and naphthalene. A previous study involving naphthalene and anthracene showed that exciplex emission was not as intense as excimer emission of the methylanthracene [40]. An alternate implication may be that there may be no interaction between the compounds but addition of naphthalene simply causes quenching of the spectrum or the addition of naphthalene may disturb the spatial arrangement of the 5'-bispyrene molecules, causing decreased excimer emission.

Using an 80% TFE system was not a convenient method to quantify the target concentration because it relies on the increase and decrease of intensity at 480 nm. Quantification, therefore, might not be reliable. This system has no advantages over normal fluorescent signals not using excimer/exciplex because these also rely on increase/decrease in fluorescence intensity and very small shifts in wavelength. The only advantage of the system we tested is the greater sensitivity of a split probe system. The same system was studied again in aqueous medium (no TFE) in order to see if this gives a spectrum, which would allow a better method of quantification. Decrease in excimer emission intensity and increase in monomer emission was noted in the absence of TFE, and so identification can then be based on both monomer and excimer peaks, which will be more reliable because of the use of the excimer-monomer ratio (IE/IM).

The spectrum shown in this paper is similar to that previously reported for bis-pyrenyl probes [29,37,38]. The changes in spectrum observed following addition of the 5'-bispyrene probe indicate hybridisation of the probe to the target. As there is a decrease in excimer peak at 480 nm and an increase in monomer peaks, this could suggest that excimer formation is being reduced on hybridisation and that there is greater emission from the monomers

[38]. This may be due to distortion of the intramolecular structure of the bispyrene, one of the pyrene residues involved in stacking interactions with adjacent nucleobases of oligonucleotide, which interferes with excimer formation. Previous NMR studies using the same DNA sequences of the probes and targets showed that the DNA duplex existed in a B-DNA structure and the pyrene existed in the minor groove. It also suggested that the pyrene stacks with its neighbouring base on hybridisation [41,42]. Studies looking at the effect of a pyrene residue 'dangling' from an oligonucleotide also suggest that it stacks onto a nitrogenous base [43]. It is possible that lack of excimer formation is due to one residue intercalating or stacking while the other one is not. Consequently, the residues will be at some distance from each other. Stacking has been reported previously in polar medium with purine residues, in particular guanine, and was shown to cause quenching [34]. There is one guanine residue relatively close to the binding site of the 5'-bispyrene probe on the target strand; however, the proximity of this guanine would depend on the configuration of the double helical structure on duplex formation. Bichenkova et al also support these interpretations, as they add TFE to prevent pyrene-DNA interactions occurring on hybridisation to target and the exciplex emission increases, whereas in water quenching is observed [35]. Previous research on the length of the linker arm between pyrene and the oligonucleotide probe in the ETPH system suggests this affects excimer emission due to the configuration of the molecules and therefore illustrates the importance of the spatial arrangement on excimer formation [14].

The spectrum shown in figure 4 is very similar to those previously reported for bis-pyrenyl probes [29,37,38]. When the target is added to the 5'-bispyrene probe, there is a large increase in monomer peaks and a large decrease in the excimer peak at 480 nm. The changes in spectrum indicate hybridisation of probe to the target. As there is a decrease in excimer peak at 480 nm and an increase in monomer peaks, this indicates that excimer formation is reduced upon hybridisation and there is greater emission from the monomers [38]. This might be due to distortion of the intramolecular structure of the bispyrene and one of the pyrene residues may involve in stacking interactions with adjacent nucleobases of oligonucleotide, which interferes with excimer formation.

The change in spectra when 3'-naphthalene is added to the 5'-bispyrene and target indicates that some interaction is taking place. The 3'-naphthalene might interact with one or both of the pyrene residues to form an exciplex. It is also possible that the 3'-naphthalene allows better excimer conformation of the pyrene residues, for example the addition of the second probe may force both pyrenes into the DNA double helix, or may force both of them out of the helix, leading to an increase in excimer emission due to their orientation. A previous study did not observe exciplex between 3'-naphthalene and 5'-monopyrene in 0% TFE [35]. Our use of 5'-bispyrene instead of 5'-monopyrene and 3'-naphthalene in the presence of 80% TFE led to a decrease in intensity at 480 nm. This result is not surprising. Different interactions occur between the probes and target in organic solvent and aqueous medium because the medium affects the structure of the DNA, and

the emission of excimer and exciplex is greatly dependent on the secondary structure of the DNA [37].

Control experiments were carried out to determine if the emission arises from formation of exciplex between the wanted exci-partners, or from the interaction between the exci-partners and the nucleobases. The results support the observation that the emission detected in this work results from interaction between the exci-partners, and the interpretation that the exciplex signals detected in the full system occur from correct hybridisation of the probe oligonucleotides to the target strand and interaction of the exci-partners rather than pyrene or naphthalene interacting with the nucleotidebases.

The 5'-bispyrene probe in aqueous medium system was used to determine the effects of mismatches or inserts in the target, compared to the probe sequences on exciplex fluorescence. Different mismatch positions of the 16-mer-parent target sequence were used to analyse the effects of SNP on exciplex intensities. Excimer fluorescence of the pair of 5'-end and 3'-end pyrene-labelled oligonucleotide, complementary to the adjacent region within target DNA, was successfully used to detect point mutations and to quantify DNA [12-14,17]. These different targets were used to determine if the reaction occurring between the two molecules was due to FRET or exciplex and also if the system can identify base pair mismatches.

A target with 2 base-pairs between the two oligonucleotide probes was used in order to determine if the same changes in spectra are seen when no inserts are present. The distance between the two probes would affect the proximity of the fluorescent molecules and therefore affect the interaction between the fluorophores. Our results show that no change is apparent when 3'-naphthalene is added to the system. Exciplexes and excimers only form when the two probes are in close proximity (3-4 Å). If they are further away (10-100 Å), the change in fluorescence intensity is likely to be due to FRET rather than exciplex formation. The insertion of 2 base-pairs between the two probes would make the distance longer than 4Å, as the distance between each base pair in duplex structure is 3.4Å. The absence of a change in fluorescence intensity indicates that the increase in intensity was due to interaction of 3'-naphthalene and 5'-bispyrene probes and only occurs when they are close together. Therefore, when the two probes attach to target, the change in intensity may be due to exciplex formation. Theoretically, if the change was due to FRET, the increase in fluorescence intensity would still be seen. However, it could also be due to the 3'-naphthalene allowing better configuration of the two pyrenes, so when the two probes are far apart, interaction of naphthalene cannot affect the spatial arrangement.

Mismatches are expected to affect the proximity of the fluorophores of the 5'-bispyrene and of the 3'-naphthalene, particularly if mismatches are so close to the contact site of the two probes that it causes distortion of the configuration when WT is used [44]. Due to mismatches, the helical structures of 3'-DMM are expected to differ from that of WT, especially as there are more guanine residues on 3'-DMM than on WT. Previous studies have reported changes in fluorescence when extra Gs or Ts were in the target sequences [34]. Structural changes

would therefore affect the spatial arrangement of the molecules and some differences in the spectrum would be expected. Also, the mismatch causes the structure to be less stable, which might increase or decrease excimer formation. These results demonstrate that the system can identify the mismatches and insertions, which are common polymorphisms in DNA.

Hybridisation of bis-pyrenylated oligonucleotides to 16-mer complementary DNA targets showed that the 5'-bis-pyrene probe exhibits a decrease of excimer fluorescence intensities on formation of duplex with a complementary target in a concentration dependent manner. In addition, as the concentration of target increases, the excimer peak at 480 nm decreases and the monomer peaks at 379 and 397 nm increase, indicating that the ratio of monomer to excimer is affected by the concentration of target. However, Kostenko et al demonstrated that the changes in spectra were heavily dependant on the binding sites and secondary structure of the duplex, and demonstrated that different base pair sequencing titrations give very different spectra [38], which indicates that the 5'-bispyrene probe is very sensitive. Work carried out in this study illustrates a linear relationship between the fluorescence intensity at 480 nm and the concentration of the target, and also between the fluorescence intensity at 379 nm and the concentration of the target. The linearity changes because it is expected that the species of the system interact at a 1:1 ratio. We showed that excimer fluorescence intensity of the 5'-bipyrene depends linearly on the concentration of target DNA and enables quantification of DNA in solution.

Conclusion

We demonstrated that oligonucleotides possessing a 5'-bis-pyrene probe at the designated position can be synthesised easily by phosphoramidate chemistry. From the emission spectrum of the split-probe system in water, it appears that 5'-bispyrene alone displays excimer emission. When a target with a two base-pair insertion between the contact site of the 3'- and 5'-probes was used, no change in excimer signal on hybridisation was observed, suggesting that the interaction occurs only when the two probes are within 1-2 base pairs of each other.

This exciplex formation was observed for the first time in water between pyrene and naphthalene. The preliminary results about mismatches and insertions observed in this work indicate that it is possible for exciplex systems to act as SNP detectors. Careful design of probes for such systems could lead to the ability to detect single-base mismatches, which can be used in a variety of commercial and scientific applications, including genotyping, medical diagnostics, gene delivery, assessment of gene expression, and drug discovery.

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