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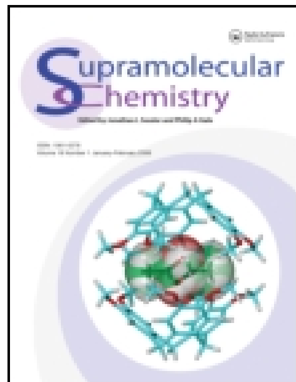

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Anthracene-modified oligonucleotides as fluorescent DNA mismatch sensors: discrimination between various base-pair mismatches

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A fluorescent anthracene-tagged DNA probe has been shown to respond to various DNA sequences by changes to its emission signal upon duplex formation. The fluorescence response for duplexes containing a single mismatch near the anthracene site has been found to be very sensitive to its composition, with the emission signal increasing for a CA mismatch and decreasing for CT and CC mismatches.

Keywords: DNA; sensor; SNP; fluorescence; anthracene

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The field of sequence-selective DNA detection is one that has greatly expanded in response to the demand for rapid and selective oligonucleotide recognition. In particular, the detection of single nucleotide polymorphisms (SNPs) has attracted much attention. An SNP, which is essentially a specific position along a sequence of DNA where the base identity (i.e. A, T, G or C) is known to vary among different individuals, is the most common form of genetic variation; it occurs with great frequency throughout the human genome and can provide an indication of a predisposition to diseases that have a genetic component (*1*).

A great deal of effort has been put into the development of fluorescent DNA sensors that are highly sequence specific (*2*). One way to achieve this is to incorporate into DNA nucleosides that are modified using fluorescent tags that can detect specific DNA bases (*3*). A wide variety of different moieties have been tagged to modified nucleosides with the aim of generating fluorescent sensors without the need for an additional quencher group (*4*, *5*). Such base-tagged systems include pyrene (*6–8*), fluorene (*9*), phenanthroline (*10*) and anthracene (*11*, *12*). Alternatively, fluorophores may be directly attached to the ribose moiety (*13–15*). A synthetically simpler approach is to incorporate the sensor unit into the oligonucleotide backbone (*15*, *16*). This has been successfully achieved with pyrene (*17*) for the purpose of sensing using excimer formation, azobenzenes for photoswitching (*18*, *19*), and acridine for site-selective RNA activation (*20*) and electron transfer studies (*21*). However, the many modified oligonucleotides that have been reported for fluorescence sensing purposes generally are limited in their ability to differentiate between all of the possible base-pairing partners. A modified nucleobase that can discriminate between the four DNA bases has recently been reported (*22*), where the sensing proceeded via different decreases in emission, with the single strand form being more emissive than any of the duplexes.

Previously, we synthesised an anthracene-based fluorescence sensor, attached to a non-nucleosidic serinol linker (Figure 1(a)), that was capable of differentiating between fully matched and singly mismatched oligonucleotides through a decrease and increase, respectively, in fluorescence emission, caused by the DNA microenvironment (*23*). However, by using prochiral serinol during the synthesis, a mixture of diastereomeric oligonucleotides was produced. Although these isomers could be separated, the linker stereochemistry was difficult to assign with complete certainty. We therefore turned our attention towards a threoninol linker (Figure 1(b)), which has previously been successfully used in other studies on functionalised oligonucleotides (*6*, *21*, *24–26*) and, as an enantiopure starting material, removes the problem of generating diastereomers. In this article, we show how a novel anthracene-containing fluorescent probe containing such a linker can discriminate between various matching and mismatching base pairs within a duplex through distinct changes in emission intensity.

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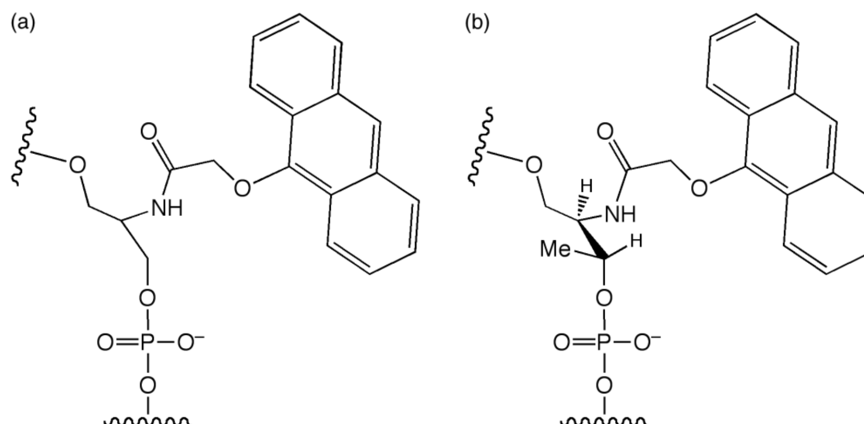

Figure 1. Structures of (a) serinol (stereochemistry not shown) and (b) L-threoninol linkers used in modified DNA probes.

Table 1. Synthesised sequences.

Oligonucleotide name	Sequence
5′-CAC	5′-TGG-ACT-CAC-TCA-ATG-3′
Probe 5′-CLC	5′-TGG-ACT-CLC-TCA-ATG-3′
Target 3′-BG (B = G, A, C, T)	3′-ACC-TGA-BG-AGT-TAC-5′

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The linker containing a threoninol unit in the L configuration was synthesised based on previously reported methodologies (*23*) and was subsequently incorporated into a modified oligonucleotide (probe 5′-CLC, where L represents the L-threnoninol linker, Figure 1(b)) which, along with other unmodified sequences, was synthesised using standard solid-phase DNA synthesis techniques and purified by HPLC. The complete list of synthesised oligonucleotides is presented in Table 1. As a change from our other studies (*23*),[1] the systems were designed with no base opposite the tether site in the matching strand, in the expectation that this would give the anthracene sufficient room to fully insert inside each duplex.

A series of fluorescence titration experiments were performed on probe 5′-CLC (aqueous phosphate buffer pH 7, *ca.* 298 K) in order to determine and analyse the effect of DNA binding on the anthracene emission signal. The results from these titrations are presented in Figure 2, where it can be seen that the addition of aliquots of the matching target 3′-GG to a 1 μM aqueous solution of 5′-CLC results in a significant decrease in anthracene emission intensity at 440 nm, with quenching reaching 47% once the duplex

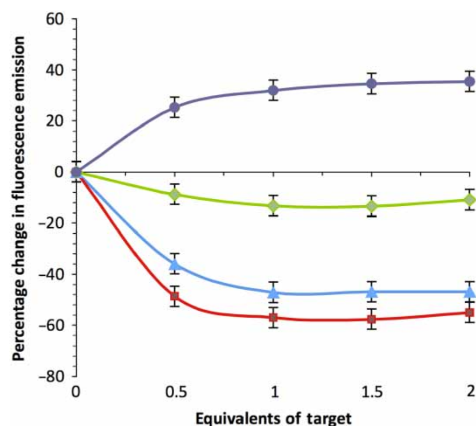

Figure 2. Fluorescence titration plots at 440 nm for probe 5′-CLC with 0.5, 1.0, 1.5 and 2.0 equivalents of fully matching targets 3′-GG (blue triangles) and mismatched targets 3′-CG (red squares) 3′-TG (green diamonds) and 3′-AG (purple circles), 10 mM (pH 7.0) phosphate buffer, 100 mM NaCl, *ca.* 298 K.

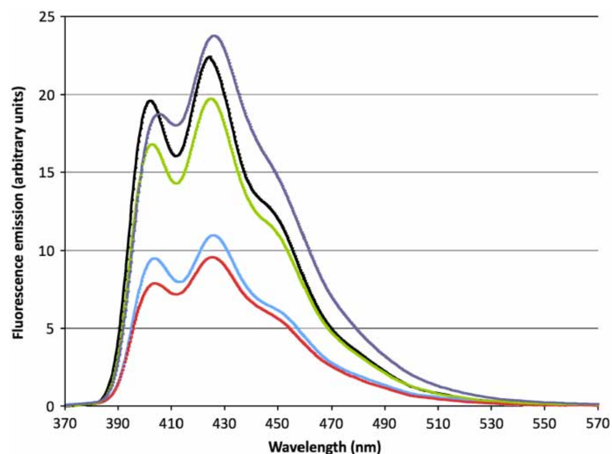

Figure 3. Fluorescence spectra for duplexes formed between probe 5′-CLC (black) and 3′-GG (blue), 3′-CG (red), 3′-TG (green) and 3′-AG (purple), 10 mM (pH 7.0) phosphate buffer, 100 mM NaCl, *ca.* 298 K.

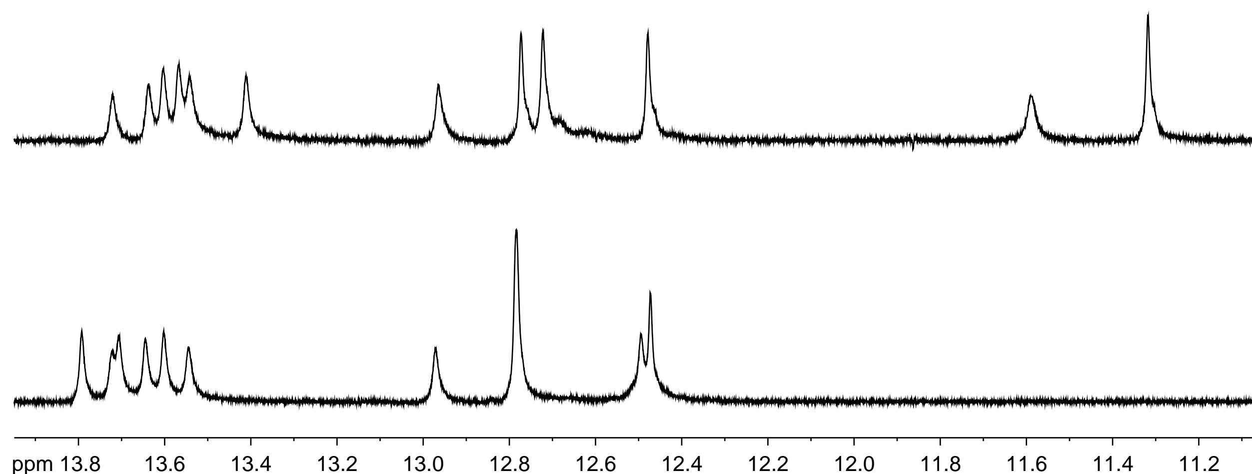

Figure 4. ^{1}H NMR spectra (900 MHz, 11–14 ppm region) of 5′-CLC/3′-GG duplex (top, 950 μM) and 5′-CAC/3′-GG duplex (bottom, 950 μM) in water (5% D_2O), 10 mM phosphate buffer, pH 7.0, 100 mM NaCl solution.

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is fully formed. No significant changes in emission intensity were observed in the presence of excess amounts of the target, which indicated the formation of a strongly bound 1:1 complex at these concentrations.

Studies with other oligonucleotides, each containing a single variation in the identity of the base in the 8th position (3′-AG, 3′-CG, 3′-TG), were then undertaken. Two titrations also showed a decrease in emission intensity (3′-CG: −55%; 3′-TG: −13%). However, 3′-AG produced an increase of +35% at 440 nm. The emission spectra for each duplex are overlaid in Figure 3. These results clearly indicate a different sensing response for each type of base pair (whether match or mismatch) with only one system (CA mismatching base pair) resulting in an increase in emission intensity. These changes in emission are supported by quantum yield measurements obtained for both the single strand and duplex forms (see Supplementary Information, available online). As described below, a series of experiments and calculations were then undertaken to examine how the position of the anthracene group with respect to the DNA helix might affect its emissive properties.

The ^{1}H NMR spectra in water of the unmodified and modified (matching base-pair) duplexes were recorded (900 MHz, for conditions see Supplementary Information, available online). In 1D NMR spectra of oligonucleotides, the most informative part of the spectrum is the downfield region where the imino protons of G(N-1) and T(N-3) bases involved in H-bonding resonate (at 12–13 and 13–14 ppm, respectively) (*27*). At 298 K, signals in both regions were observed, in the expected regions for the unmodified duplex 5′-CAC/3′-GG (Figure 4 bottom). However, for the modified matching duplex 5′-CLC/3′-GG, significant (*ca.* 1 ppm) upfield shifts in two G imino proton signals were observed, which were assigned to GC base pairs flanking the anthracene tether (Figure 4 top). Similar upfield shifts have

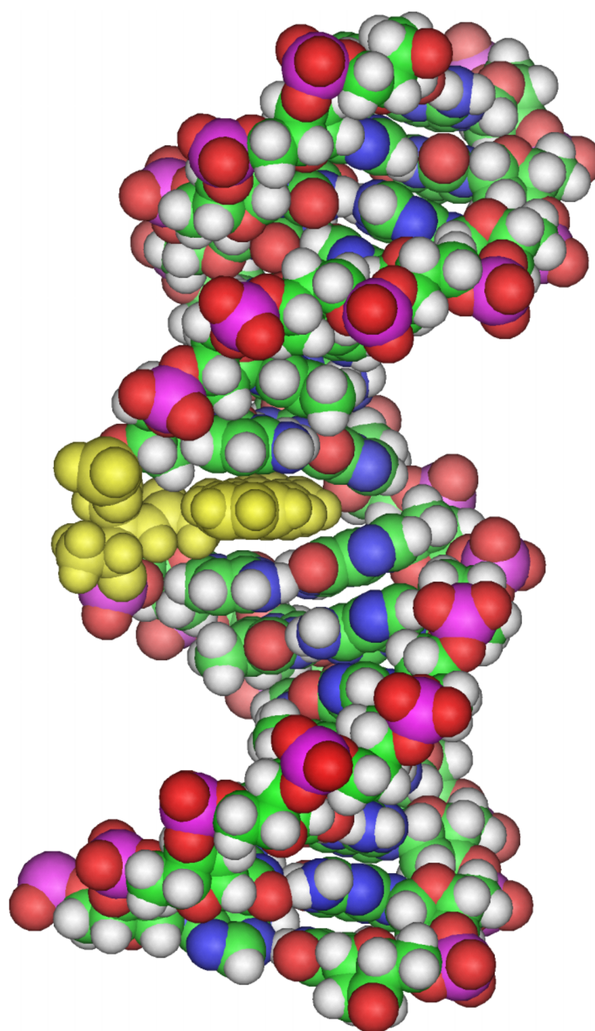

Figure 5. AMBER model of the duplex 5′-CLC/3′-GG.

Table 2. Melting points of DNA duplexes in °C.

Target 3′-BG	Unmodified probe 5′-CAC	Anthracene probe 5′-CLC
3′-GG	49.5	61
3′-CG	35.5	45.5
3′-TG	34.5	46
3′-AG	38	46.5

Note: Conditions: Each oligonucleotide 5 μM with 10 mM phosphate buffer, 100 mM NaCl, pH 7.0.

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been observed previously in the duplexes that interact with intercalating acridine (*28*) and azobenzene (*29*) moieties.

A computational model of the duplex 5′-CLC/3′-GG also indicated a significant interaction of the anthracene with the base pairs of the helix through intramolecular intercalation, with the moiety again flanking the central GC base pairs within the major groove of the duplex (Figure 5), consistent with the NMR findings.

DNA melting studies confirmed that duplexes between probe 5′-CLC and each of the targets form at the temperature used for the fluorescence studies. The T_m values of these duplexes are presented in Table 2 and show that the modified duplexes are more stable than the equivalent unmodified duplexes (where an adenine nucleoside replaces the anthracene non-nucleosidic linker).

The melting temperature for the duplex 5′-CLC/3′-GG is significantly higher (+11.5°C) than that of the unmodified 5′-CAC/3′-GG system, which is a further evidence for a strong association of the anthracene unit with the matching duplex, and is consistent with previous findings (*21*), including those from studies with conventional intermolecular anthracene intercalators (*30*). The high T_m value is also consistent with an even greater interaction of the anthracene with the duplex than in related probe systems containing a base site opposite the tether site (*22*).[1] Furthermore, the fact that all the anthracene-modified duplexes give uniformly higher T_m values than their unmodified counterparts, whether the duplex contains a matching base pair or not, indicates that the anthracene group takes part in effective and broadly similar stabilising interactions with the duplex in each case (*vide infra*).

As for explaining the fluorescence response of the anthracene tether to duplex formation, quenching via photoinduced electron transfer, as suggested previously (*31*, *32*), could be expected to arise from a stacking contact with at least one of the adjacent GC pairs, but the different emission intensities observed for various mismatching base pairs are more difficult to rationalise. However, the similar T_m data and induced CD spectra (see Supplementary Information, available online) for the three mismatched duplexes clearly imply that their structures resemble one another and indicate that the anthracene environments, with respect to proximate base pairs in the duplex, are approximately the same in each case. Therefore, factors of electronic rather than steric/positional origin appear to be largely responsible for a change in base identity within a base pair bringing about such a dramatic change in anthracene emission intensity. The increase in fluorescence intensity for the system 5′-CLC/3′-AG can be rationalised by a combination of the hydrophobic environment of the duplex preventing quenching by water and the relatively weak quenching of the proximate adenine, a phenomenon that has been previously observed when anthracene intercalates in AT vs. GC-rich regions of DNA (*30*). This quenching effect has, in fact, been quantified in the case of pyrene intercalators by consideration of the free energy of electron transfer between bases as a result of their different redox potentials (*31*). Such an analysis also predicts cytosine to have the greatest thermodynamic driving force for quenching by electron transfer and adenine to have the weakest, which is consistent with what is observed in this study.

In conclusion, we have shown how an anthracene-tagged DNA probe can demonstrate a different fluorescent response to various base-pair combinations within a duplex, which is of relevance to the development of novel SNP sensors. It appears that the unusual selectivity of the sensing signal (i.e. the emission being highly sensitive to the identity of the base pair in the duplex) stems from an optimal interaction of the anthracene with the duplex, which is associated with the absence of a free base opposite the tether site. A range of studies with different base sequences using a range of anthracene tethers are now planned[1] in order to rationalise these findings further.

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Note

1. Monomer synthesis and detailed spectroscopic studies on these and related probe systems, including comparative studies on strands with D-threoninol linkers, will be reported elsewhere in a full paper.

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