A Homogeneous Solution Method for the Detection of Point Mutations in DNA

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A simple, direct method is described for the detection of point mutations in single-strand DNA, under homogeneous conditions, that uses time-resolved fluorescence spectroscopy involving Eu^{III} ions.

In a recent communication, we described a new method for the homogeneous identification of specific sequences of DNA,[1] which utilises a cooperative sensitisation of Eu^{III} that capitalises on the unique coordination and luminescence properties of Eu^{III} ions in water.[2] Here, we report the use of this method for the specific detection of point mutations in single-strand DNA.

In our assay, two probe molecules are used for the detection of the analyte. The first is a probe bearing a complementary sequence of bases that can hybridise with the target DNA and to which is attached, at one end, a short linker molecule bearing a chelated europium ion. The main chelator we have worked with is the monoamide of EDTA, which, although it binds very strongly to the metal ion, does not completely fill its coordination sphere. The second component is a synergistic sensitiser that works by further encapsulation around the metal ion, to which is attached, by a short link, an intercalating group.† In our earlier work we utilised the sensitiser/intercalator **1** but we have found that this is rather unstable when stored for periods of days in aqueous buffers. As an alternative to **1** we have prepared the analogue **2**,‡ which has the advantage of a higher extinction coefficient, works at wavelengths >300 nm and forms solutions stable in buffers for periods of weeks.

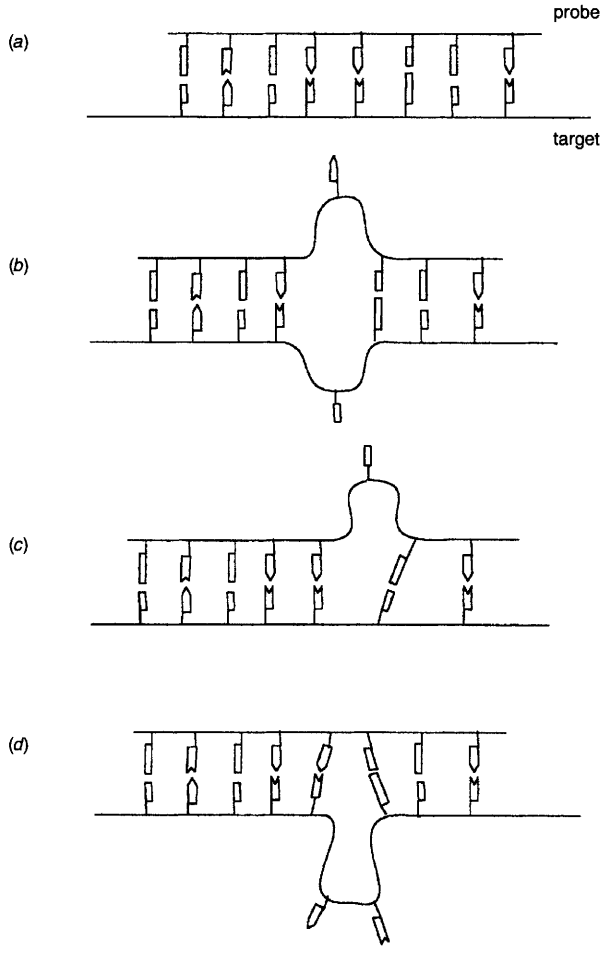


Fig. 1 Types of mismatching events ('bubbles') encountered in DNA targets: (*a*) matching probe and target; (*b*) single point mutation; (*c*) deletion; (*d*) insertion

The demands on any assay seeking the detection of a single base-pair mismatch in particular sequence of bases is high since the probe has to be able to discriminate between this and the normal sequence in which no mismatches occur [Fig. 1(*a*)]. Such mismatches, however, cause dislocations in the degree of base pairing observed ('bubbles') in the vicinity of the mismatch; three type of mutations can be identified (Fig. 1): single (or multiple) point mismatches, deletions, or insertions.

The situation is even more critical when seeking to distinguish genetic mutations in chromosomal DNA samples from cells, since, in the simplest case of a single point mutation, three situations will be realised. Material from homozygotes will be either all normal or all mutated, whilst material from heterozygotes (carriers) will be 50% normal and 50% mutated. Most assays seeking to distinguish between such material involve heterogeneous systems, *viz.* ones in which the excess of reagents are washed away from the target which is initially bound to a surface, either by itself or by the probe.[3]

Our approach capitalises on the 'bubbles' (dislocations) caused in the vicinity of a mismatch. Thus, since cooperation depends on the ability of the intercalator/sensitizer **2** to bind to the DNA as well as to the metal ion [Fig. 2(*a*)], reduced organisation of the duplex results in the reduced binding of the intercalator in the mismatch sequence as against the binding in a matching sequence [Fig. 2(*b*)]. As a consequence, one can expect less of the sensitised complex to form in the mismatch case as compared to the matching situation, provided that the probe is selected so that the preferred site(s) of intercalation coincide with occurrence of the 'bubble'.

As part of our test system we have examined a sequence of DNA bases that occur in the region of the gene associated with cystic fibrosis.[4] This gene codes for the chloride carrier protein, which consists of a strand of 1480 amino acids. One of the mutations in this gene (G551D) occurs in the ATP binding exon 11, which results in a misreading of the genetic code, inserting an aspartamide group in place of glycine and, as a consequence, malfunction of the protein. The mutation is caused by substitution of a guanine-1784 by adenine.[5]

The mutated target **3** was employed, with the normal target **4** as a reference, together with the 'mutated' probe **5** and the 'normal' probe **6**.§ The probes had 5′-aminohexyl groups incorporated and the amino groups were reacted with EDTA

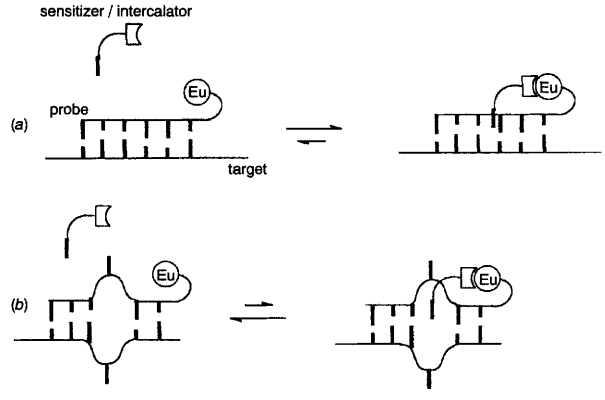
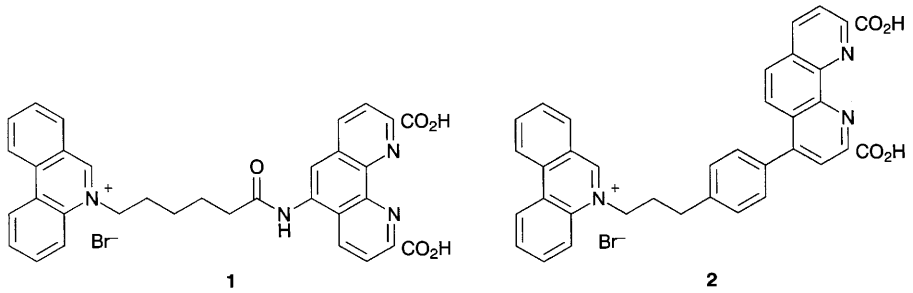
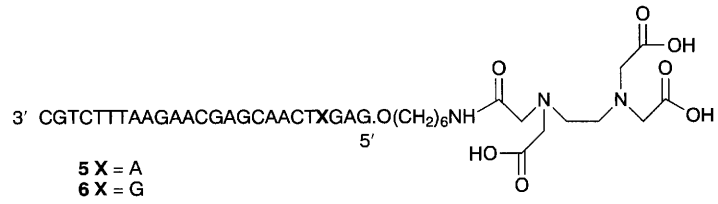


Fig. 2 Cooperation of probes with target: (*a*) with matching target; (*b*) with mismatching target. For the latter, less efficient binding of the sensitiser with the duplex moves the equilibrium to the left, resulting in a weaker signal.



5′ TGCTAAAGAAATTCTTGCTCGTTGAXCTCCACT 3′

3 X = T
4 X = C



5 X = A
6 X = G

Table 1

	Mutant target **3**			Normal target **4**		
T/°C	M-probe (blank) **5**	N-probe (blank) **6**	Ratio[b]	M-probe (blank) **5**	N-probe (blank) **6**	Ratio
22	26.5 (0.69)[a]	12.0 (0.69)	2.3:1	12.0 (0.69)	18.7 (0.69)	1:1.6
32	14.0 (0.43)	3.05 (0.43)	5.2:1	2.3 (0.43)	8.3 (0.43)	1:4.2

[a] Readings of intensity of emission at λ_{em} 615 nm by irradiation at λ_{ex} 320 nm. Results obtained for solutions of target at 1.0×10^{-8} mol dm^{-3} and probe and sensitiser concentrations of 5×10^{-8} mol dm^{-3} at pH 7.5. Sensitiser added prior to hybridisation. [b] Ratio of readings after subtraction of blank (background) reading obtained by leaving out the target DNA strands.

anhydride to form the corresponding monoamides. The probes were loaded with Eu^{III} ions and then purified by chromatography through Sephadex columns (Pharmacia, NAP 5, ×2) as described previously.[1]

Solutions of the probe and the sensitizer **2**, as a 1:1 molar mixture (200 μl) were added to the target DNA, using a hybridising solution of 25 μl Denhardt's solution and 600 μl buffer {10 mmol dm^{-3} Tween 20, [polyoxyethylene (20) sorbitan monolaurate], 1 mol dm^{-3} NaCl, 10 mmol dm^{-3} $MgCl_2$, 10 mmol dm^{-3} *N*′-(2-hydroxyethyl)piperazine-*N*-ethane sulfonic acid, pH 7.5}. Final concentration of the probe was 5×10^{-8} mol dm^{-3} and of the target was 1×10^{-8} mol dm^{-3}. The solutions were hybridised at 42 ± 1 °C for 40 min before cooling to ambient temperature (22 ± 1 °C) and measuring the luminescence. As background controls, probes were used in the absence of target DNA since no difference between these and readings in the presence of completely mismatching DNA targets was observed.[1]

At ambient temperatures (22 °C) only modest discriminations were observed (Table 1), the match to mismatch sets giving ratios of *ca.* 2:1. However, these were reproducible and gave the appropriate crossover results, *i.e.* both mutant and normal probes gave higher readings with the matching target than with the mismatched target. The discrimination increased at higher dilutions.[1] More significantly, the discrimination was very sensitive to temperature changes. Thus, for the mutant probe against the mutant target DNA, the signal intensity falls away less rapidly as one approaches the melting temperature for the duplex, compared to that observed for the mismatch pair. At a temperature of 32 ± 1 °C, for example, the discrimination (ratio of signals) is reproducibly >4:1 and this also increases with dilution.

The above results represent one of the first methods for the direct *in situ* identification of mutations in a target strand of DNA.

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Footnotes

† Groove binding is just as efficient in enhancing cooperativity as intercalation.

‡ Prepared in an eight-stage synthesis from 2-methyl-8-nitroquinoline; this synthesis will be reported elsewhere.

§ DNA probes from OSWEL DNA service, West Mains Road, Edinburgh, UK EH9 3JJ.

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