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Molecular beacons attached to glass beads fluoresce upon hybridisation to target DNA

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Molecular beacons attached to glass beads have been synthesised which are non-fluorescent until exposed to a complementary target nucleic acid, whereupon they fluoresce, indicating hybridisation.

Tyagi and Kramer[1] have described a novel technology for the detection of specific nucleic acids in homogeneous solution. These probes, denoted 'molecular beacons', fluoresce upon hybridisation to their complementary DNA target. The essential feature of the probes is their stem–loop structure. The loop portion is an oligodeoxynucleotide probe for a complementary target nucleic acid. The stem is constructed of two short oligodeoxynucleotide arms, one which is terminally labelled with a fluorophore and the other with a quencher. Annealing of the arms causes intramolecular energy transfer from the fluorophore to the quencher. In this 'closed' conformation the probe is non-fluorescent. Hybridisation of the loop to its target causes the stem to open, the fluorophore and quencher are no longer in close proximity and fluorescence is emitted (Fig. 1).

Molecular beacons have been used in the detection of specific complementary sequences,[2–7] and the simultaneous detection of different pathogenic retroviruses has been reported.[8] Molecular beacons are able to distinguish between wild-type and single point mutations[3,9] and in real-time monitoring of the polymerase chain reaction (PCR) amplicon-specific probes lead to an increase in fluorescence intensity with increasing copy number of target DNA.

We have developed a modification of this technology that involves supporting the molecular beacons on solid glass particles (Fig. 2) and we have shown that glass-bound beacons exhibit similar properties to their soluble analogues. This technology could be harnessed in nucleic acid screening as hybridisation of CPG-bound beacons to target DNA or RNA generates fluorescent beads that can be isolated and analysed.

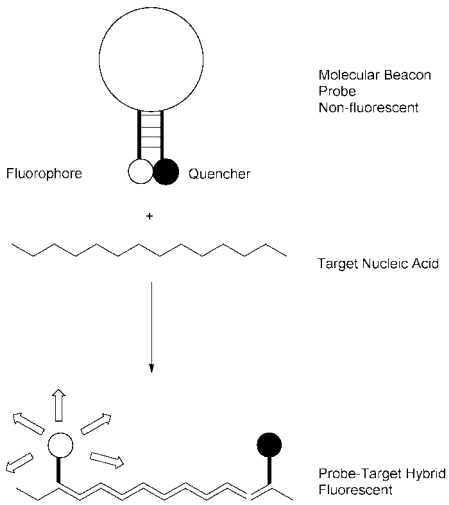


Fig. 1 Mechanism of action of molecular beacons in the presence of a complementary target nucleic acid.

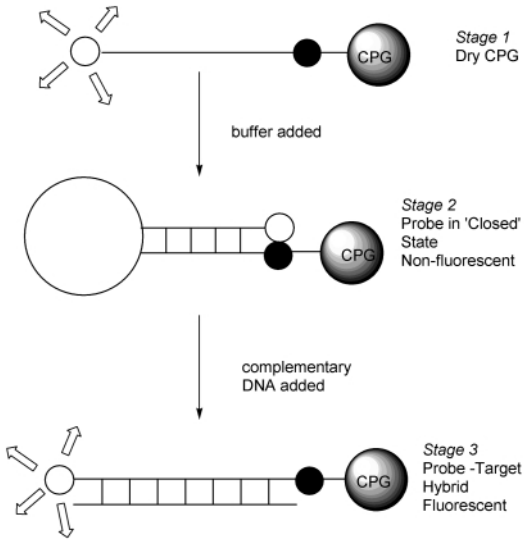
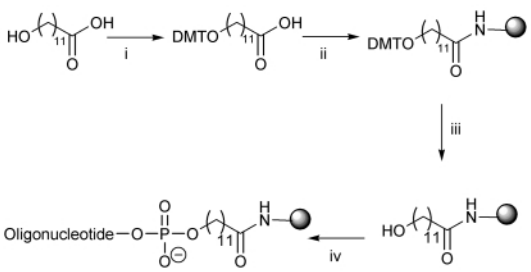


Fig. 2 Stages in assay of CPG-bound beacons.

Long chain alkyl amino-controlled pore glass (LCAA-CPG) was derivatised with a polyalkyl linker as shown in Scheme 1: first, the primary hydroxy group of 12-hydroxydodecanoic acid was protected as a 4,4′-dimethoxytrityl (DMT) ether.[10] Then, the carboxylic acid moiety was coupled to free amino groups on the CPG using DIC/HOBT (DIC = 1,3-diisopropylcarbodiimide, HOBT = 1-hydroxybenzotriazole) in 1% DIPEA–CH_2CCl_2 (DIPEA = *N*,*N*-diisopropylethylamine) to create a stable amide bond between the linker and CPG. The loading of the linker on the CPG was determined by acid-catalysed detritylation followed by quantitation at 495 nm using a UV–VIS spectrometer. The appropriate amount of CPG was used for 0.2 mmol scale oligonucleotide synthesis on a ABI 394 DNA synthesiser. Coupling efficiencies of > 98.5% were achieved by extending the coupling time from 45 s to 10 min.

In order to establish suitable conditions for the hybridisation of CPG-bound oligonucleotides to solution nucleic acids, an oligonucleotide, CGAGATACGGTTTTCACAGC (OG1), was assembled† on the LCAA-CPG. The CPG was heated (60 °C) in



Scheme 1 Derivitisation of LCAA-CPG with a polyalkyl linker and oligonucleotide synthesis. *Reagents and conditions*: i, DMTCl, pyridine, room temp.; ii, LCAA-CPG, DIC, HOBT, DIPEA, CH_2Cl_2; iii, 5% CCl_3CO_2H, CH_2Cl_2; iv standard automated DNA synthesis.

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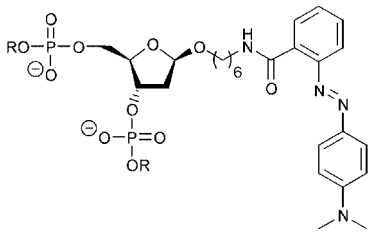

Fig. 3 Methyl Red phosphoramidite, the quencher used at the 3′ ends of CPG-bound probes.

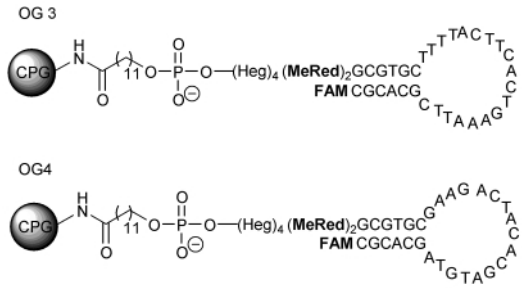

Fig. 4 Chemical structure of the CPG-bound molecular beacon probes.

conc. aqueous ammonia–ethanol (4 : 1 v/v) for 4 h and washed thoroughly with anhydrous CH_2Cl_2, to remove the cleaved protecting groups. An oligonucleotide GCTGTGAAAACCG-TATCTCG (OG2) of complementary sequence to the CPG-bound oligonucleotide (OG1) was synthesised and purified using standard methods. OG2 (0.22 OD) was mixed with the CPG-oligonucleotide (OG1) in 30 μL of an aqueous buffer of sodium hydrogenphosphate (10 mmol), EDTA (1 mmol) and NaCl (1 M) at pH 7.0, such that the CPG-bound oligonucleotide was in fourfold excess. The heterogeneous mixture was allowed to stand at room temperature for 3 h after which the absorbance (260 nm) of the CPG solution gave a reading of 0.035. This corresponds to a reduction of 84% of oligonucleotide in free solution. UV melting of the mixture gave a smooth transition as the oligonucleotide was released from the CPG into solution (T_m = 83.6 °C).

Two molecular beacons were assembled by solid phase DNA synthesis on the polyalkylamide LCAA-CPG previously described (Scheme 1). Both oligonucleotides were labelled at the 5′ end with fluorescein (FAM);

CGCACGCTTAAAGTCACTTCATTTTCGTGCG (OG3)

CGCACGATGTAGCACATCAGAAGCGTGCG (OG4).

Four hexaethylene glycol (Heg) spacers were incorporated between the CPG and the methyl red quencher (MeRed) to minimise steric interference from the solid support (Figs. 3, 4). Two complementary solution oligonucleotides were synthesised;

AAAATGAAGTGACTTTAAG (OG5), CTTCTGATGTGC-TACAT (OG6).

The molecular beacon-functionalised CPGs were heated (60 °C) in conc. aqueous ammonia–ethanol (4 : 1 v/v) for 4 h, to deprotect the oligonucleotides. Both solutions became fluorescent during this period implying loss of oligonucleotide from the CPG (*ca.* 70% loss of oligonucleotide from the resin). However subsequent thorough washing of the CPG afforded glass beads that remained fluorescent, indicating that some of the oligonucleotide was still present on the beads. Cleavage of a proportion of the silicon–oxygen bonds within the glass is inevitable under these strongly basic conditions. Alternative deprotection conditions or CPG material may be beneficial for future applications.

The heterogeneous beacon assay was carried out in two stages. First, the CPGs (OG3 and OG4, 2 mg) were placed in an aqueous buffer of sodium hydrogenphosphate (10 mmol), EDTA (1 mmol) and NaCl (1 M) at pH 7.0 to facilitate annealing. Then, the complementary oligonucleotides (OG5 and OG6) were added and after 2 h at room temperature any increase in fluorescence due to hybridisation (stem dissociation) was noted. Fluorescence was monitored under a fluorescence microscope (LEITZ DM IL) and photographed (ASHAI PENTAX K1000).

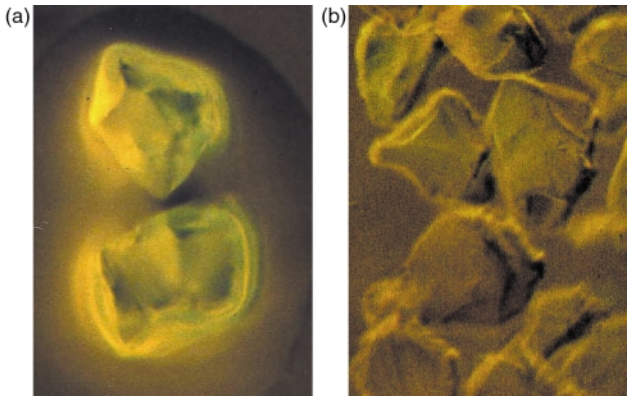

Fig. 5 (a) CPG-beacon in buffer and (b) CPG-beacon in buffer and complementary target nucleic acid.

Addition of a suitable annealing buffer causes the beads to become non-fluorescent [Fig. 5(a)], indicating the presence of a hairpin loop. Introduction of a complementary oligonucleotide in solution causes a significant increase in the fluorescence of the beads, due to the loop portion of the probe annealing to its target, allowing the solid-supported molecular beacons to exist in the fluorescent 'open' state [Fig. 5(b)]. Exposure of the CPG-beacons to a non-complementary oligonucleotide in the same buffer did not increase the level of fluorescence on the beads.

In summary, we have synthesised CPG-bound molecular beacon probes. These single-stranded probes have been shown to detect the presence of their target nucleic acids. At present, we are investigating the properties of alternative resins that have more uniform and spherical structures and we are applying the CPG-bound probes to the differentiation of nucleic acid sequences by variation of the fluorophore.

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Notes and references

† All oligonucleotides were prepared in the Oswel Research Products Laboratory, Boldrewood, University of Southampton, Southampton, UK SO16 7PX.

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