Quencher-free molecular beacons: a new strategy in fluorescence based nucleic acid analysis

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Molecular beacons (MBs) have been used as viable fluorescent probes in nucleic acid analysis. Many researchers around the world continue to modify the MBs to suit their needs. As a result, a number of nucleic acid probing systems with close resemblance to the MBs are being reported from time to time. Quencher-free molecular beacons (QF-MBs) are a significant modification of the conventional MB; in QF-MBs the quencher part has been eliminated. Despite the absence of the quencher, the QF-MBs can identify specific target DNA. They can also be used in SNP typing and in real-time PCR analysis for quantification of DNAs. The design, factors behind functioning and applications of different types of QF-MBs and closely related quencher-free nucleic acid probing systems (QF-NAPs) have been described in this *tutorial review*.

1. Introduction

In the past few decades, the methods for genetic analysis have grown tremendously and they continue to draw the attention of researchers world-wide. This is mainly because the genetic data thus generated is being utilized not only by biologists but also by statisticians to explore the population genomics, and by computational chemists. A recent study on global variations in the human genome revealed that around 12% of the human genome contains many forms of genetic variation such as deletions, insertions, duplications and copy of multi-site variants. They are collectively termed as copy number variations (CNV) or copy number polymorphism (CNP).[1] Thus, at present, the significant part of human genome analysis lies in identifying the CNVs. The international "HapMap" project has now been initiated to identify such genetic variations. The CNV mapping will lead to further understanding of the role of mutations in simple as well as complex diseases. Though many methods are being developed to detect genetic mutations at the single nucleotide level (widely known as SNPs),[2] it is quintessential to improve the present techniques or to develop new techniques for simultaneous detection of multiple SNPs and CNV mapping. Also, such a fresh wave of research initiatives should be aimed at devising more cost-effective, high throughput, fast and highly accurate analytical methods. It is strongly believed that in due course of time such concurrent technological advancements will lead to new diagnostic and treatment options.

In the early days, fluorescent methods of DNA analysis were highly dependent on fluorescent dyes that can complex the duplex DNA by means of non-covalent forces. Then, nucleoside building blocks containing different kinds of fluorophores (covalently linked) were used exclusively. Later on, especially in the post-genomic era, sequence specific complementary binding of short synthetic oligonucleotides containing fluorescent tags have been used in DNA analysis. Today such fluorescent oligonucleotides, called hybridization probes, play a very significant role in qualitative as well as quantitative analysis of the genome[3] and in SNP typing.[2,4]

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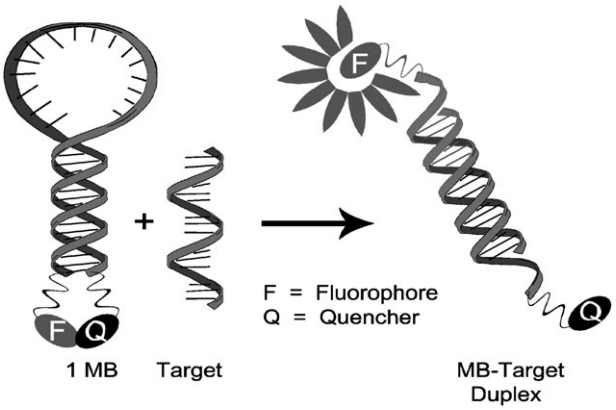

Fig. 1 Structure and function of conventional MB.

The conventional molecular beacon (MB) designed and developed by Kramer and co-workers, consists of a stable hairpin (stem-loop) oligonucleotide carrying a fluorophore at one end (usually 5′-) and a quencher attached at the other end (**1**; Fig. 1).[5] In the hairpin or closed form the fluorophore is quenched due to its close proximity to the quencher. When the fluorophore and quencher are close to each other there is efficient fluorescence resonance energy transfer (FRET) between them. In presence of the target DNA (complementary to the loop of the hairpin), the MB undergoes a conformational transformation from closed (hairpin) to open (linear) structure as shown in Fig. 1. This process is associated with an increase in fluorescence. Hence, the presence of the target is characterized by a sharp increase in fluorescence intensity.

The conventional MB introduced in the mid 90s was modified by a number of research groups to improve its function, resulting in a range of modified MBs with varied designs, fluorophores and quenchers. Some of the important changes are depicted in Fig. 2. The functions considered for improvement include eliminating false signals, increasing specificity of the MB towards the target and enhanced detection limit. It is reported that the background fluorescence intensity could be reduced significantly by using super quenchers (SQs) in place of normal quenchers (**2**; Fig. 2).[6] An array of three quencher molecules are assembled at molecular level to produce SQs with a quenching efficiency of 99.7% and the signal-to-background ratio is also enhanced significantly. It has been shown that the original design constraint of a nucleobase-paired stem is not compulsory. Such ''stemless beacons'' (**3**; Fig. 2) possess sufficient signal-to-noise (S/N) ratio for some *in vivo* applications.[7] Similar to conventional MB, the quenching of fluorophore in stemless beacon occurs through FRET. Hence, for better activity, the fluorophore and quencher should be chosen in such a way that there is maximum spectral overlap.[8]

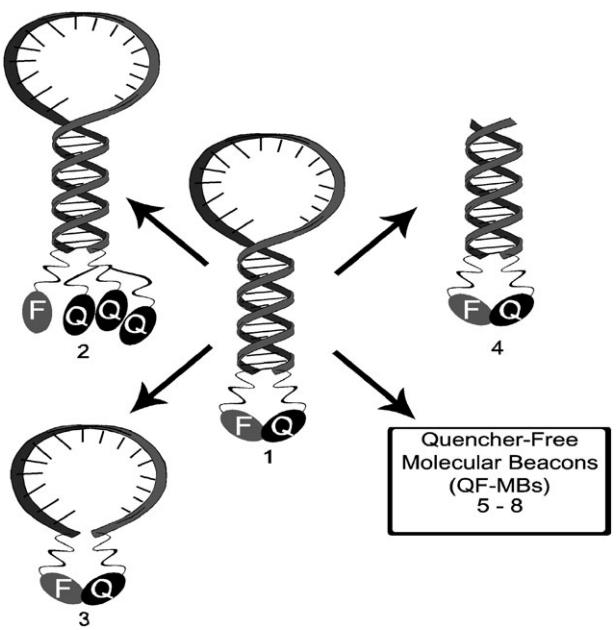

Fig. 2 Modification of conventional MB.

The duplex formed between two single strand linear oligonucleotides A and B containing, respectively, a quencher (say A) and a fluorophore (say B) can also be used as a fluorescent probe for the detection of a specific nucleic acid in an homogeneous solution. This duplex hybridization probe functions similarly to a molecular beacon, and its structure resembles that of a MB without the loop structure (**4**; Fig. 2).[9,10] Such duplex probes are also shown to discriminate between a perfectly matched and single nucleotide mismatched targets.[10] The (A·B) duplex probes are reported to be more specific to the target as compared to the corresponding linear single strand probes (B alone), and they can be used for simultaneous detection of multiple targets, in homogenous solution. Hybrid molecular probe (HMP) is yet another modification of the MB, where the oligonucleotides A and B are linked by means of synthetic polymers such as PEG.[11] The false positive signals during *in vivo* monitoring of the RNA in live cells are reported to be reduced in case of HMPs. Under such conditions certain hairpin oligonucleotides undergo nuclease degradation resulting in false signalling. At present, different types of MBs are used as versatile fluorescent probes in a variety of applications related to genome sequences.[3] The MBs have been used in analysis of proteins and protein–DNA interactions.[12] Significantly, the MBs are used in real-time PCR methods for quantitative DNA analysis.[13] MBs are now being used to probe the photodamage of DNA and its kinetics and as vehicles for photoinduced drug release.[14]

Recently it has been shown that fluorescent hairpin oligonucleotides can function as MB even without the attachment of additional quencher moiety and such probes can be called ''quencher-free molecular beacons (QF-MBs)''. In this tutorial review, we discuss in detail the design, function and applications of different types of QF-MBs. We emphasize with relevant examples that the mono-labelled QF-MBs, despite the absence of quencher, can also recognize the complementary DNA in a sequence specific manner. The concomitant fluorescence change is comparable to that of conventional MBs. The QF-MBs can be used successfully for detection of SNP in oligonucleotides. The structure and function of fluorescent linear single strand oligonucleotides, called quencher-free nucleic acid probing systems (QF-NAPs) that are closely related to the QF-MBs are also discussed briefly as a point of comparison.

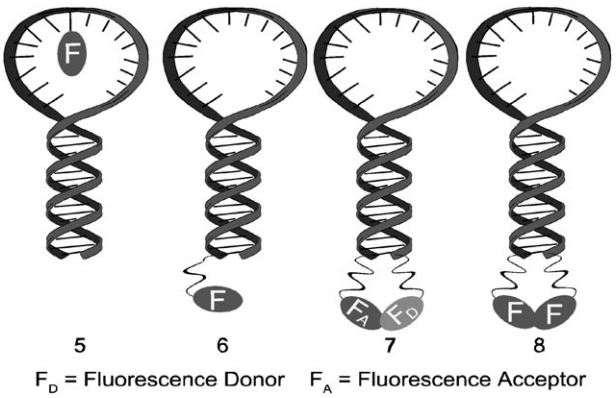

Fig. 3 Different types of QF-MBs.

2. Design of QF-MBs

The QF-MBs can be defined as hairpin shaped fluorescent oligonucleotides containing one or multiple fluorophores, attached at any part of the sequence, either directly or through a linker. The fluorophores can be modified fluorescent nucleobases or fluorescent dyes. The QF-MBs (**5–8**; Fig. 3) discussed herein are yet another modification of conventional MB. A hairpin oligonucleotide and a fluorophore are the essential building blocks required for the construction of QF-MBs. In almost all the QF-MBs the entire or part of loop is complementary to the target oligonucleotide. The essential differences between conventional MB and QF-MBs are listed in Table 1.

3. Types of QF-MBs

Based on the design, the QF-MBs reported so far can be broadly classified into two different types; (a) mono-labelled QF-MBs containing fluorophore at the middle or end of the oligonucleotide (**5** and **6**; Fig. 3), and (b) dual-labelled MBs containing two fluorophores at the stem or at the ends (for example, **7** and **8**; Fig. 3). In dual-labelled QF-MBs of the type **7** two fluorophores of different kinds are attached to a hairpin oligonucleotide. One fluorophore is attached at the 5′-end and the other fluorophore is attached at 3′-end. In case of **8** two fluorophores of same kind are attached at the ends. But none of these MBs have a quencher moiety.

3.1 Mono-labelled QF-MBs

3.1.1. QF-MBs with fluorophore at the loop or middle of the oligonucleotide. The purine and pyrimidine heterocycles found in natural nucleic acids have poor emission characteristics. Hence they are not suitable for fluorescence studies. In the recent past there have been numerous efforts to develop modified nucleosides containing fluorescent nucleobase analogues such as extended nucleobases and conjugated nucleobase analogues.[15] Kim and co-workers have designed and prepared the QF-MBs **9** (Fig. 4) containing fluorescent nucleoside analogue U^{FL} at the middle (loop) of the hairpin.[16,17] In the emissive nucleoside building block, U^{FL} the fluorophore, fluorene is anchored to the nucleobase of deoxyuridine through short conjugated ethynyl linker. Fluorene has good quantum yield (the absolute quantum yield of fluorene is 0.54 in ethanol), and it is less bulky as compared to the commonly used fluorophores such as pyrene, fluorescein, rhodamine, and coumarin derivatives. The modified nucleoside thus introduced should not affect the natural binding properties of oligodeoxyribonucleotides (ODNs). In general, a substitution at the C-5 position of the pyrimidine ring and the C-8 position of the purine ring of natural nucleobases or the C-7 position of the 7-deazapurines does not disturb their ability to form a nucleobase-pair in the resulting DNA duplex.[18] Hence, the fluorene unit was covalently attached to the C-5 position of deoxyuridine using Sonogashira coupling. The deoxyuridine building block, U^{FL} was subsequently introduced into the ODNs using the phosphoramidite method. Similar to the conventional MB, the QF-ODNs **9** are hairpin ODN. However, unlike conventional MB the fluorophores in ODNs **9** are attached at the loop, rather than at the stem end. The QF-ODNs **9** are shown to undergo DNA hybridization similar to their unmodified counterparts, so substitution of fluorene does not disturb the hybridization process.

Recently, a detailed study on photophysical properties of ODNs **9a** to **9d** containing triad nucleobases (X U^{FL} Y) of different combinations has been carried out.[17] The triad

Table 1 QF-MBs *vs.* conventional MBs

No.	Conventional MB	Quencher-free MB
1.	The fluorophore and quencher are integral parts of the design.	Quencher is not part of the design.
2.	In most cases, the fluorophore and quencher are attached at the 5′- and 3′-ends, respectively, of the hairpin oligonucleotide.	The fluorophores can be attached at any position (at the loop or at the stem ends of the hairpin oligonucleotides).
3.	[a]The ends can not be used for any further modification as they are already functionalized (with fluorophore and quencher).	In mono-labelled MBs either both the ends (as in **5**) or one end (as in **6**) is free for further modifications (for example, in surface-immobilization of the MBs).
4.	Mostly, the fluorophore and quencher are attached through a linker to the backbone of the oligonucleotide.	Nucleoside building blocks with fluorophore-modified nucleobase analogues (emissive nucleosides) can also be incorporated using phosphoramidite method).
5.	Dual-labelling (fluorophore and quencher) process is relatively complex and expensive.	Mono-labelling is relatively easy and more economical.
6.	The fluorescence quenching occurs through FRET from quencher to fluorophore.	QF-MBs have diverse quenching mechanisms. In the mono-labelled QF-MBs (**5** and **6**) the fluorophore is quenched by PET. The dual-labelled MB **7** works by FRET. In EMS MB (**8**), the fluorescence intensity changes are due to switching between monomer and excimer forms of the fluorophore.

[a] Special synthetic protocols should be exercised for immobilization, usually through the loop region after suitable modification.

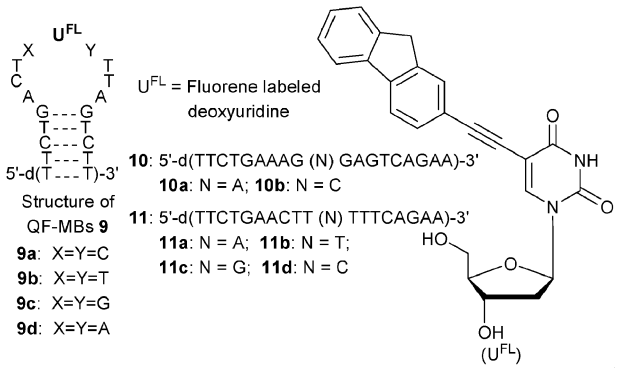

Fig. 4 Sequences and structure of QF-MBs **9**.

nucleobases are at the loop of the oligonucleotide, where the fluorescent nucleotide is surrounded by different combinations of flanking nucleobases; X and Y, for example in ODN **9a**, X and Y are cytosine (C). From a series of data it is concluded that X and Y have significant influence on the emission properties of the ODN. The fluorescence intensity of ODN **9b** was greatest (when X = Y = thymine (T)), and it was least when X = Y = guanine (G) (in ODN **9c**). Also, the triad nucleobase-pairs in the corresponding duplexes play key roles in their efficacy as SNP probes (discussed in section 5.2).

3.1.2. QF-MBs with fluorophore at the stem or strand-end. The fluorescent hairpin ODNs given in Fig. 5 are examples of the mono-labelled QF-MBs containing fluorophore at the stem or at strand end. The fluorophore can be attached either through a linker (as in **12**; Fig. 5A) or directly incorporated in the form of a modified emissive nucleoside (as in **13**; Fig. 5B) using the phorphoramidite method. The QF-MBs **12**, known as "smart probes" were designed and prepared by Sauer and co-workers.[19] In smart probes an oxazine (MR121 in **12a**) or rhodamine (R6G in **12b**) derivative is attached as fluorophore at the 5′-end of the guanosine-rich hairpin ODNs through a (C6) alkyl linker using classical *N*-hydroxysuccinimidyl ester (NHS-ester) chemistry. The full or the major part of the loop is complementary to the target DNA. On the other hand, the hairpin ODNs **13a** to **13d** contain a pyrene modified adenosine (A^{PY}) unit at the 5′-overhang position, while in **13e** the pyrene modified uridine (U^{PY}) is incorporated instead of A^{PY}.[20] In the modified nucleoside analogues; A^{PY} and U^{PY} the pyrene is covalently linked to the nucleobase through a rigid ethynyl linker using Sonogashira coupling. It may be noted that in all the ODNs **13**, the

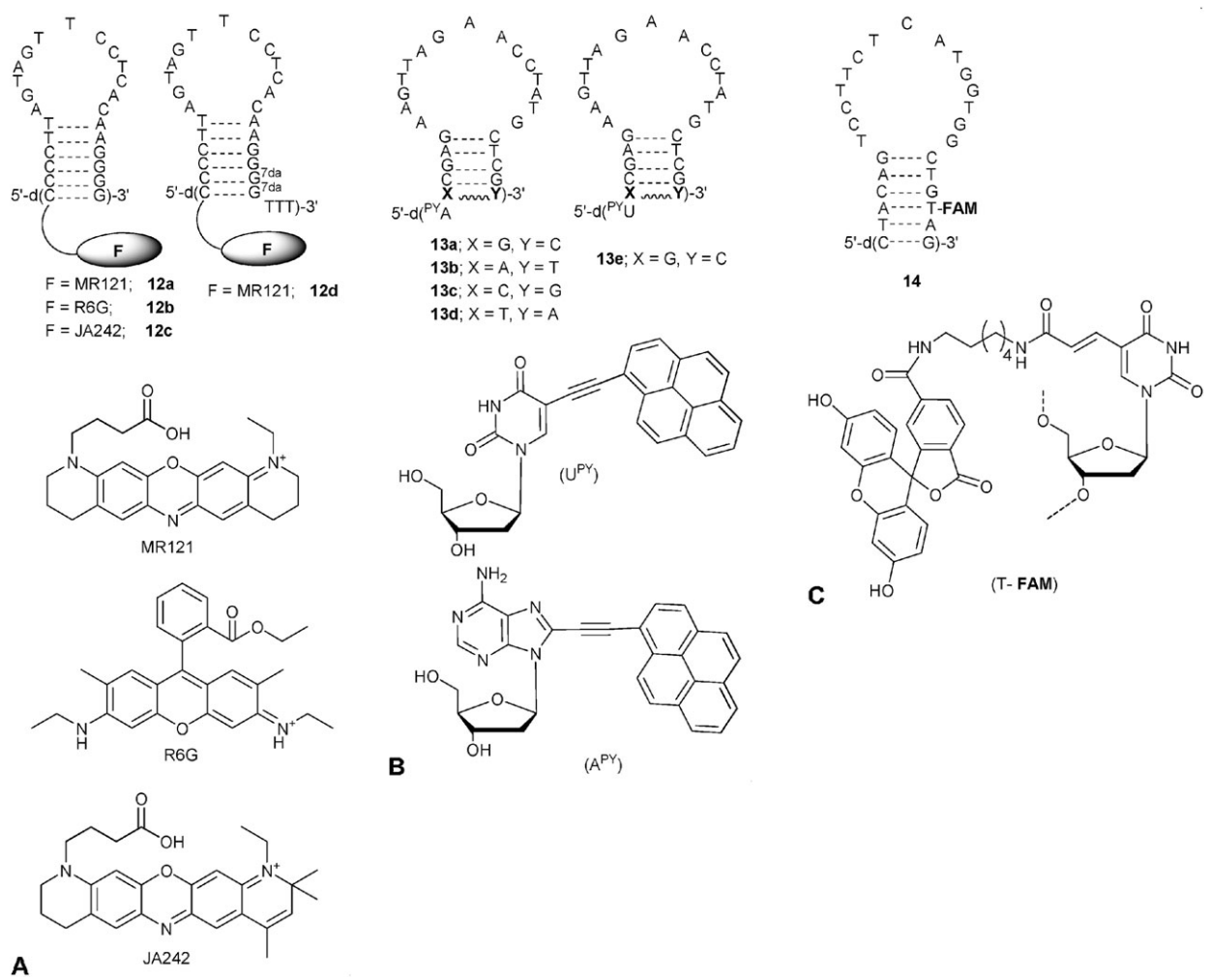

Fig. 5 QF-MBs with fluorophore at the stem end.

fluorophore-containing nucleosides lack the corresponding nucleosides in the opposite strand to form a nucleobase-pair.

Rashtchian and co-workers have reported a series of mono-labelled hairpin ODNs containing different types of dyes.[21,22] In a few of these ODNs, the dye is directly attached through corresponding dye-phosphoramidite derivatives, at the 3′- or 5′-end. In few other ODNs a nucleoside analogue tagged with the dye (for example, C5-fluorescein-dT) is incorporated through the phosphoramidite method, at any position of the blunt-ended stem (as in **14**; Fig. 5C) or as an overhang unit. The dye-labelling of the ODNs (to the nucleobase) could also be done post-synthetically. In blunt-ended hairpin ODN **14**, also known as self-quenched probe, the fluorescein modified thymidine (T-FAM) has been incorporated at the 3′-end as penultimate nucleoside unit. In T-FAM, thymine is tagged with a fluorophore at its C5-position through a C6 spacer as shown in Fig. 5C.

3.2 QF-MBs containing multiple fluorophores

As mentioned earlier, residual fluorescence (incomplete quenching) and poor sensitivity are some of the major drawbacks of conventional MB (**1**). In an effort to eliminate these drawbacks, hairpin oligonucleotides containing two or more fluorophores, but with no quencher moiety have been designed. The FRET MBs, excimer–monomer switching MBs (EMS MBs), dimer–monomer switching MBs (DMS MBs) are notable among them. In FRET MBs two different fluorophores–a fluorescence acceptor (F_A) and fluorescence donor (F_D)–are attached on both the ends.[23] The F_A and F_D should have overlapping emission spectra for effective FRET from F_D to the F_A. The representative FRET MB **15** shown in Fig. 6, consists of two fluorescent dyes: 6-carboxyfluorescein (FAM) is attached at the 5′-end and coumarin is attached at the 3′-end. FRET MBs containing more than two distinct fluorophores have also been designed and used as fluorescent probes.[24] For instance a hairpin ODN containing three fluorophores, namely, FAM, *N,N,N′,N′*-tetramethyl-6-carboxyrhodamine (TMR) and cyanine-5 (Cy5), has been designed in such a way that FAM is located on one end of ODN while the other two fluorophores (TMR and Cy5) are located on the other end of the ODN.

In EMS MB, two fluorophores of the same kind are attached at the 5′- and 3′-ends of hairpin ODNs. For example, hairpin oligonucleotides containing two pyrene units (one on each end) are well known EMS probes. Some of the EMS probes are given in Fig. 6. The ODN **16** containing pyrene fluorophores was reported by Fujimoto and Inouye.[25] Kim and co-workers have designed and synthesized dual-labelled quencher-free hairpin ODNs containing pyrene appended adenosine (A^{PY}) and pyrene appended uridine (U^{PY}) units in three different combinations, namely, $U^{PY}A^{PY}$ (**17a**), $U^{PY}U^{PY}$ (**17b**) and $A^{PY}A^{PY}$ (**17c**).[26] In these dual-labelled ODNs **17**, emissive nucleoside units are incorporated in the complementary positions, but, within the stem so that the ends are free for further modifications. The practical utility of the free ends in EMS MBs of the type **17** has been demonstrated by attaching a cholesterol moiety at the 5′-end of the ODN. The cellular permeability of the cholesterol-appended ODN was enhanced significantly as compared to that of free ODN.[27]

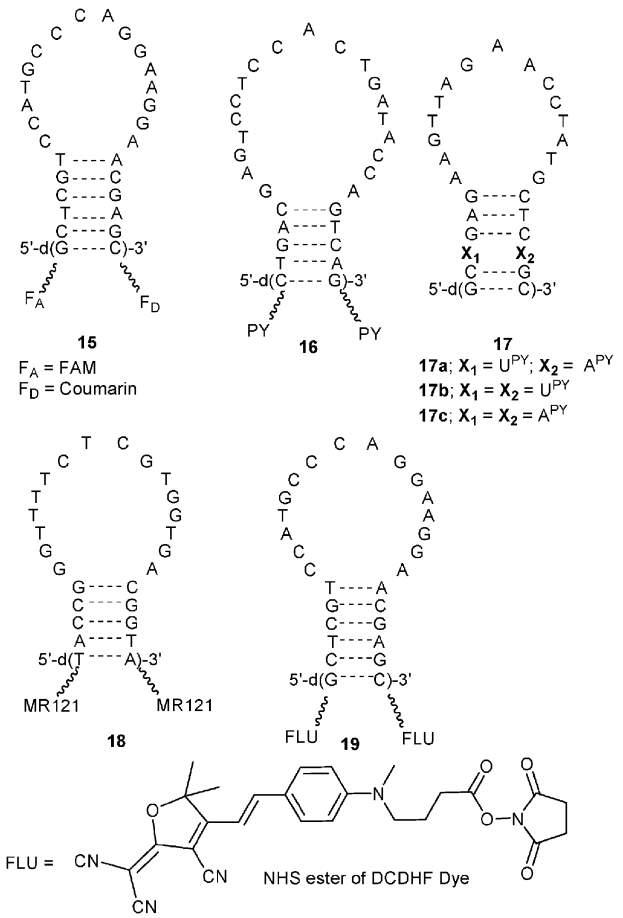

Fig. 6 Representative examples of FRET MB (**15**), EMS MBs (**16** and **17**) and DMS MBs (**18** and **19**).

Some fluorophores form non-radiative dimers when they are in close proximity. Such fluorophores have also been used in the design of QF-MBs. The ODN **18** containing two MR121 fluorophores attached at the 5′- and 3′-ends is self-quenched.[28] Similarly in ODN **19**, two dicyanomethylenedihydrofuran (DCDHF) dyes have been attached at the 5′- and 3′-ends using an NHS-ester derivative of the dye (Fig. 6).[29] The function and mechanism of dual-labelled MBs **15–19** are described in section 4.2.

4. Function and mechanism of QF-MBs

4.1 Function and mechanism of mono-lablled QF-MBs

The primary factors that are directly or indirectly responsible for the successful functioning of the mono-labelled QF-MBs are given below.

(a) Nucleobase quenching (mostly guanine quenching)

The fluorescence quenching of fluorophore by nucleobases, which is also called ''nucleobase quenching or base quenching'', is already known to occur in fluorescent oligonucleotides.[30] It is shown from a number of studies that in nucleobase quenching there is an effective photoelectron transfer (PET) between the nucleobase and the fluorophore.[19,31] Among the four different natural nucleobases, the guanine has lowest oxidation potential (1.49 V *vs.* NHE), this

is followed by adenine (1.96 V *vs*. NHE). The pyrimidine nucleobases, thymine and cytosine have the highest oxidation potential (2.1 V *vs*. NHE). These values indicate that the guanine can be oxidized very easily. In other words the PET from guanine (guanosine units) to the fluorophore is highly favoured as compared to other nucleobases. Guanosine units are reported to quench the emission of fluorescent dyes efficiently due to its good electron donating property. Hence, controlled guanine quenching in suitably designed mono-labelled ODNs plays a significant role in their function as QF-MBs. In most cases, the fluorescence intensity of hairpin or linear single strand ODNs containing guanine in close proximity to the fluorophore is lower than that of isolated fluorophore.

(b) Changes in the microenvironment of the fluorophore

Apart from nucleobase quenching the optical properties of a given fluorophore can be influenced by many other factors. Changes in the solvent polarity, hydrophobicity and pH of the media, intercalation of the fluorophore, and nucleobase-pair degeneracy are important factors that affect the emission characteristics of a given fluorophore. Thus in some of the QF-MBs or QF-NAPs discussed here the hybridization between fluorescent oligonucleotide probe and target DNA brings about a change in one or more of the above factors, which results in fluorescence intensity changes. We give a detailed description of the role of the above two factors (a and b) in the functioning of the individual QF-MBs and QF-NAPs in the following sections (4.1.1 and 4.1.2).

4.1.1. Function and mechanism of QF-MBs with fluorophore at the middle of the oligonucleotides. The mono-labelled QF-MBs of the type **5** (the hairpin ODNs **9**) show fluorescence enhancement upon binding to the fully matched target ODN, as depicted in Fig. 7. The nucleobase-quenching is reported to be the predominant cause of quenching of the fluorophore in the unbound ODNs **9**. Hybridization of ODN **9a** with its fully complementary ODN **10a** and its single-base mismatched ODN **10b** in solution displays, respectively, 2.2-fold enhancement and 6.7-fold decrease in emission intensities relative to that observed for **9a**. Also, the absorption maximum of the single-base mismatched duplex (**9a**·**10b**) is slightly red shifted from 334 nm to 339 nm. The ODN **9b** also behaves similarly.

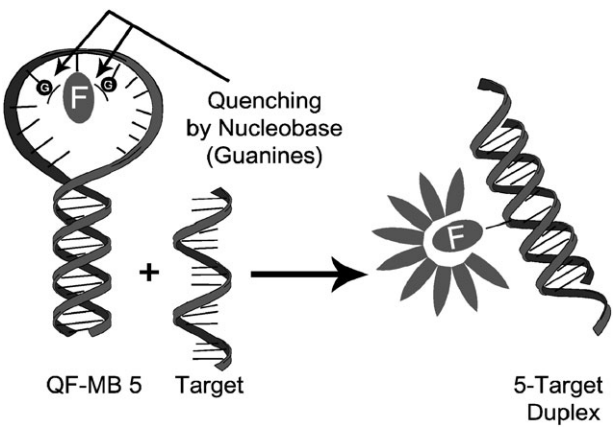

Fig. 7 Function of QF-MB containing fluorophore at the middle of the oligonucleotide.

Thus, the ODNs **9a** and **9b** display highly A-selective fluorescence emission with λ_{em} at 430 nm when excited at 340 nm.[17]

The above observations in **9** have been explained as due to interaction of the fluorene unit with the surrounding nucleobases in the probe and probe–target duplexes. The exact role of nucleobase quenching of the fluorene has been further studied using a set of ODNs (**9a** to **9d**) containing triad-nucleobase units (X U^{FL} Y) with different combinations of nucleobases (X and Y), flanking the U^{FL} unit. A detailed study of the photophysical properties of these ODNs revealed that their absorption and emission properties are strongly influenced by the flanking nucleobases. The ODN **9c** with the triad nucleobase (G U^{FL} G) showed the least fluorescence intensity (0.24) while **9b** (T U^{FL} T) showed the maximum fluorescence intensity (1.01) with reference to that of **9a** (1.00) with the triad nucleobases, (C U^{FL} C). The ODNs where U^{FL} is flanked by even one G (on either side), showed notable reduction in fluorescence intensity. Thus the guanine quenching or G-effect plays a vital role in the functioning of ODNs **9**. As anticipated, flanking abasic sites have no or negligible effect. However, the presence of the abasic site in the complementary strand (opposite to U^{FL}) has a profound effect as discussed in section 5.3. Based on these studies it is concluded that the fluorophore in ODNs 9 is quenched by neighboring guanines.

Before constructing the hairpin ODNs **9**, the photophysical properties of the linear ODN **20**, which constitutes the loop region of the ODNs **9**, were investigated. The ODN **20** exhibits an emission maximum at 425 nm.[16] Very interestingly, the fully matched duplex **20**·**21d** exhibits a 3.4-fold enhancement in fluorescence intensity (λ_{max} = 425 nm) relative to that of single stranded **20**. But the relative emission intensities of the single-base mismatched duplexes **20**·**21a**, **20**·**21b** and **20**·**21c** are, respectively 12, 12, and 24% of single stranded ODN **20**, upon excitation at 340 nm. This finding proves that even linear ODN **20** can distinguish perfectly matched and single-base mismatched oligonucleotide sequences. It should be noted that QF-MBs **9** as well as the linear ODN **20** show decrease in fluorescence intensity for the mismatched oligonucleotides.

Similarly, the linear ODN **22**, containing pyrene-labelled deoxyuridine (U^{PY}) was also observed to be a sensitive fluorescence probe to discriminate perfectly matched (**23d**) and single-base mismatched (**23a–23c**) ODN sequences by exhibiting sharp changes in its fluorescence intensity.[32] The perfectly matched duplex **22**·**23d** shows 5.6 times stronger fluorescence intensity than that of mismatched duplex **22**·**23b**. It should be noted that the **22** and **20** are structurally very similar to each other except that in **20** fluorene is used as fluorophore while in **22** the pyrene is used as fluorophore. Indeed, pyrene is shown to be more active than the fluorene.

20: 5'-d(TGGACTC $\mathbf{U^{FL}}$ CTCA ATG)-3'
21: 5'-d(CATTGAG (N) GAGTCCA)-3'
21a: N = T; **21b**: N = G; **21c**: N = C; **21d**: N = A

22: 5'-d(TGG ACAC $\mathbf{U^{PY}}$ CGGAATG)-3'
23: 5'-d(CATTCCG (N) GTGTCCA)-3'
23a: N = T; **23b**: N = G; **23c**: N = C; **23d**: N = A

The design and working hypothesis of ODNs **20** and **22** are similar to that of single strand linear probes such as HyBeacons (**24**; Fig. 8),[33] twin probes (**25**; Fig. 8)[34] and base-

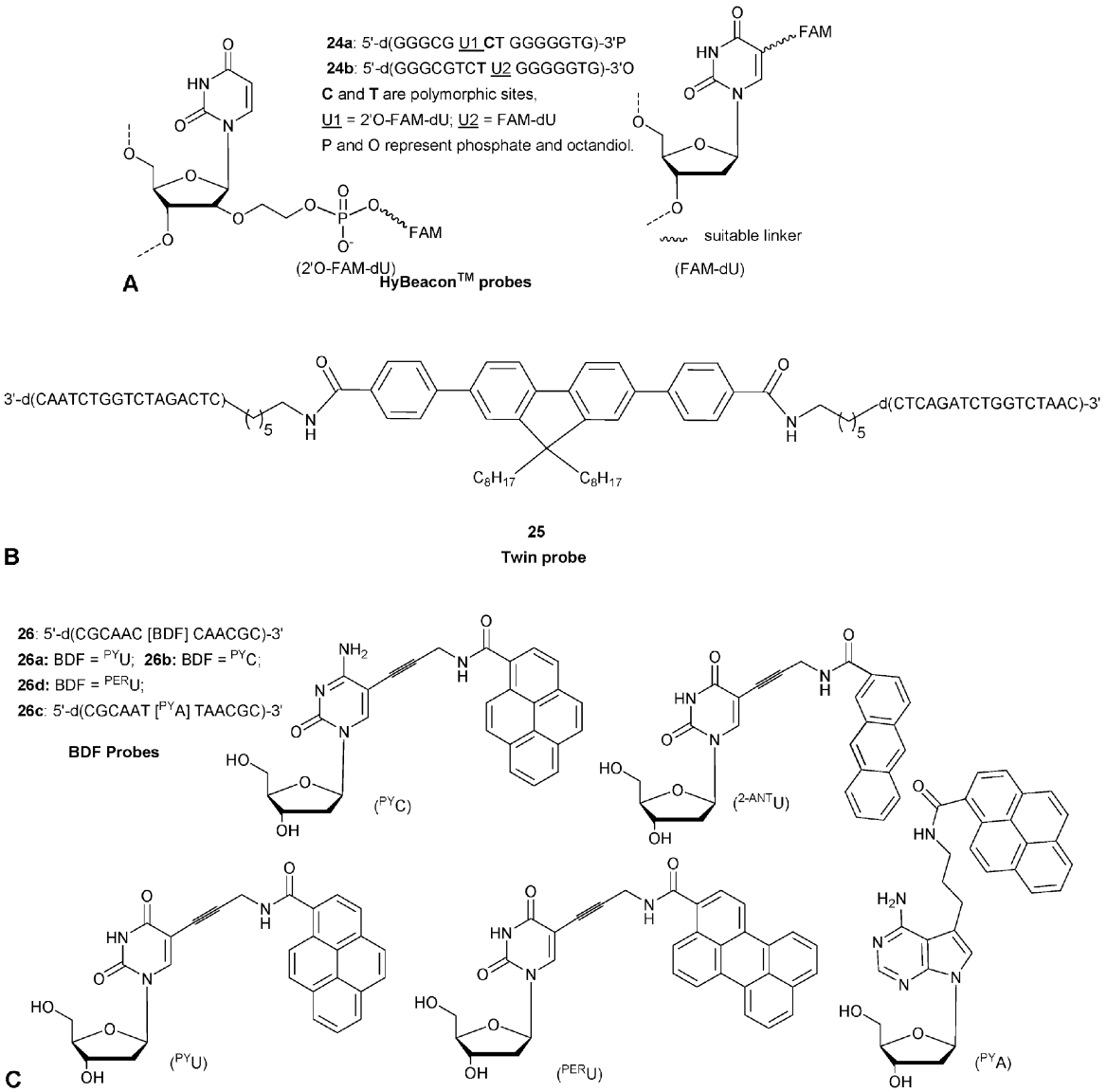

Fig. 8 Structure of HyBeacons (**24**), twin probe (**25**) and BDF probes (**26**).

discriminating fluorescent (BDF) probes (**26**; Fig. 8)[35–39] in many respects. They also undergo hybridization induced fluorescence intensity changes with the target oligonucleotides. Most importantly, these probes also distinguish the fully matched DNA sequence from mismatched sequences.

Collectively, the above results imply that in general the hairpin ODNs (QF-MBs) **9** and the linear single strand ODNs **20**, **22**, **24** and **25** are weakly fluorescent before hybridization, but they become highly fluorescent when annealed to the perfectly matched DNA sequences. Also, the fluorescence intensities of the duplexes with single-base mismatched DNAs are always lower than that of perfectly matched duplexes. For example, the unbound twin probe **25** shows low fluorescence intensity at 432 nm (when excited at 350 nm). Hybridization of the twin probe **25** with fully matched DNA sequence showed up to 10-fold increase in fluorescence intensity with an hypsochromic shift of the emission band from 432 nm to 417 nm. But hybridization of non-complementary DNA sequences showed only a marginal (1.6-fold) increase in fluorescence intensity.

Similarly the BDF probes containing different BDF nucleosides such as ^{PY}U and ^{PY}A (see Fig. 8 for their structures) report the presence of corresponding complementary nucleobases in the target DNA strands, usually by exhibiting an intense fluorescence signal. For instance, the ODNs **26a** and **26b**, containing the BDF nucleosides ^{PY}U and ^{PY}C, show fluorescence enhancement only when the A and G are present in opposite strands.[36] The solvent polarity and hydrophobicity around the fluorophore is reported to play a major role in BDF probes. Fluorophores like pyrene and anthracene show strong fluorescence when exposed to aqueous environments.[36,37] For instance, the A-selective fluorescence (a strong emission at 397 nm with quantum yield of 0.203 when excited at 327 nm) of **26a** and G-selective fluorescence emission (at 393 nm with quantum yield of 0.147 when excited 329 nm) of **26b** have been explained as due to exposure of the pyrenecarboxamide chromophore to more aqueous phase in the corresponding probe–target duplexes (A; Fig. 9).[36] The pyrene fluorophore of ^{PY}U and ^{PY}C acts as an antenna that is

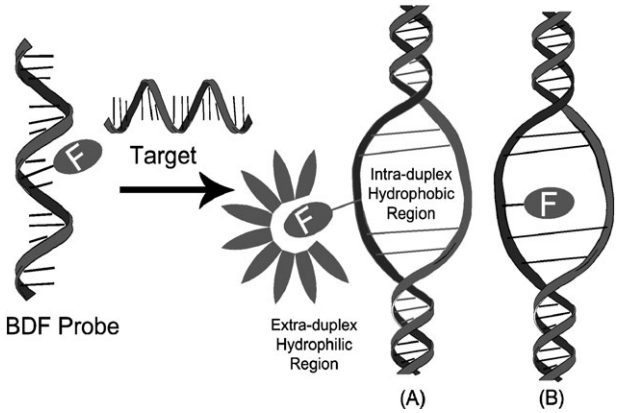

Fig. 9 Function of BDF probes in SNP typing in target DNA; there is strong fluorescence only when the base opposite to the BDF nucleoside is target base.

sensitive to the solvent polarity. When ^{PY}U and ^{PY}C form Watson–Crick nucleobase-pairs *via* hydrogen bonding with A and G, respectively, the pyrene fluorophore gets located on the outside of the duplex (a highly polar aqueous phase) and so it shows a strong fluorescence. On the other hand, when ^{PY}U and ^{PY}C do not form nucleobase-pairs with complementary nucleobases, the pyrene is folded into the hydrophobic intra-duplex region (B; Fig. 9), and so the fluorescence is quenched effectively. But the BDF probe **26c** containing the nucleoside ^{PY}A shows a sharp decrease in fluorescence intensity for the matched duplex (T in the opposite strand).[39] The pyrene in ^{PY}A is reported to be quenched by intercalation into the hydrophobic intra-duplex (B; Fig. 9). Recently it has been reported that the fluorescence behavior of 3-perylene carboxaldehyde and 3-acetyl perylene towards solvent polarity is just the opposite to that of pyrene and anthracene. That is the ODNs containing ^{PER}U unit (**26d**) shows strong fluorescence when it is within the duplex.[38]

4.1.2. Function and mechanism of QF-MBs with fluorophore near ends of the oligonucleotides. The QF-MBs **12–14** containing the fluorophores at the stem ends also function similarly to the ODNs **9** as illustrated in Fig. 10. The fluorescence intensity of the ODNs increases after binding to the fully complementary target DNAs. However, in few derivatives of

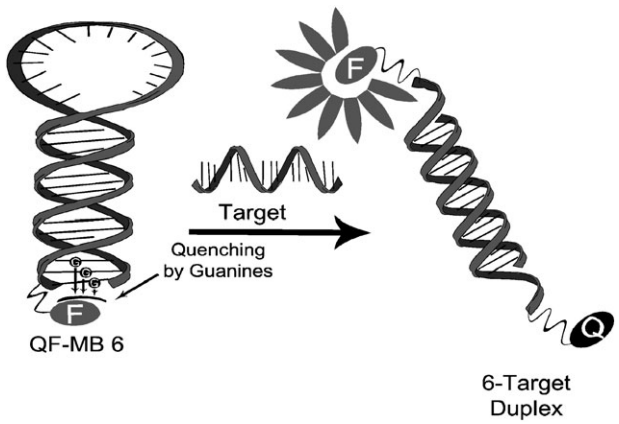

Fig. 10 Function of QF-MBs containing fluorophore at the stem end.

ODN **14** the hybridization causes fluorescence quenching (decrease in fluorescence intensity).

From a series of detailed studies it is now fully believed that in hairpin ODNs **12a** and **12b** the covalently linked fluorophores such as oxazine (**12a**) or rhodamine (**12b**) derivatives are quenched by PET from neighboring guanosine residues or G–C nucleobase pairs present at the stem.[19] The results of intermolecular quenching experiments with different dyes (rhodamine and oxazine derivatives) and DNA nucleotides indicated involvement of strong static fluorescence quenching. The fluorescence quenching depends on factors like structure of the fluorophore, the distance between the guanosine and the fluorophore, the nature of the linker and conformation of the fluorophore. The quenching efficiency increases with number of guanosine units. Stem with four guanosines is reported to be the optimum structure required for efficient quenching in ODNs **12**. Formation of ground state complexes between guanine and fluorophore is the primary prerequisite for efficient electron transfer. The end-capped conformation of MR121 in ODN **12a** is nearly coplanar with respect to the last G–C nucleobase-pair and it favors quenching. But the fluorophores R6G and JA242, respectively in ODNs **12b** and **12c** failed to adopt such conformation and so they are not quenched. Similarly, the C6-linker is also reported to be essential to provide the much needed conformational flexibility and so necessary interaction geometries for efficient quenching. The relative fluorescence quantum yield of MR121 in the ODN **12a** is reduced to 0.2 with respect to that of free dye.

The double-stranded stem of the DNA-hairpin facilitates efficient electron transfer from the guanosine residue to MR121 with shallow distance dependence. Moreover, it is shown that the overhanging single-stranded purine or pyrimidine nucleotides at the 3′-end also affect the quenching efficiency by influencing the interaction geometry of the fluorophore with guanosine. The quenching is increased if the overhanging strand stabilizes the stacking interactions. Importantly, substitution of guanosine by stronger electron-donating nucleotides, such as 7-deazaguanosine residues enhances quenching. The 7-deazaguanosine has a lower oxidation potential (0.8–1.0 V *vs.* SCE) than that of guanosine.[19] For example, the relative fluorescence quantum yield of the smart probe **12d** containing two 7-deazaguanosine units is 0.04, which is much lower than that of corresponding ODN with guanosine units.

The absorption and emission spectra of ^{PY}A-containing ODN **13a** and ^{PY}U-containing ODN **13e** were very different from each other. The ODN **13a** showed a sharp difference in fluorescence intensity (when excited at 400 nm) between the matched and mismatched duplexes. But the ODN **13e** failed to show hybridization induced fluorescence intensity change. In ODN **13e** the fluorophore (pyrene) is quenched by the covalently linked uracil both in the bound and unbound states. In unhybridized hairpin ODNs **13a–13d** the fluorescence is quenched due to an electronic interaction (PET) between pyrene and neighboring nucleobases (X). The stem is stabilized by π-stacking of pyrene with terminal nucleobase-pair (X–Y). The degree of quenching of the 5′-nucleobases (X) follows the order C > G > T > A, irrespective of the presence of

matched or mismatched nucleobase (Y) in the opposite strand. If the neighboring nucleobase is A (**13b**) no quenching occurs in the hairpin state; but, if it is G or C there is maximum quenching (**13a** or **13c**). This observation (quenching of these pyrenyl residues through electron transfer from the neighboring nucleobases) is consistent with the QF-MBs **9**. Also, formation of a stable nucleobase-pair (X–Y) is the other key factor for effective SNP discrimination *i.e.* if X and Y are mismatched nucleobases, the quenching is reduced even if the neighboring base (X) is G.[20] Among all the ODNs **13**, the ODN **13a** with neighboring G–C nucleobase pair shows the highest match/mismatch discrimination factor. But, if the pyrene is replaced by fluorene units (*i.e.*, nucleosides, A^{FL} and U^{FL}) the resulting ODNs do not show notable discrimination factors due to smaller size and poor stacking ability of fluorene as compared to pyrene units.

A detailed study on fluorescence intensity, polarization and lifetime of linear (not shown) and hairpin ODNs of the type **14** containing a fluorophore (T-FAM) near the 3′-end (Fig. 5) were carried out to understand the effect of neighboring nucleobase or nucleobase-pair and secondary structures (linear and hairpin) on optical properties of the ODNs. These studies led to the conclusion that in the case of linear ODNs the presence of a 3′-terminal C causes 87% quenching, but the quenching is eliminated when it forms duplexes, where there is terminal A–T nucleobase-pair. As anticipated the quenching is restored if the terminal nucleobase pair is C–G. Presence of purine nucleobases or nucleobase-pairs reduced the fluorescence significantly. In few linear ODNs where the dye is attached close to the 3′-end, and the 3′-terminal nucleobase is either G or C there is up to 10-fold quenching of the fluorescence upon hybridization with complementary ODNs. If the dye is positioned within the same sequence context and close to the 5′-end, no quenching upon hybridization is observed. Most importantly, the quenching is very efficient in hairpin ODNs with a blunt end G–C or C–G nucleobase-pair as compared to the corresponding linear ODNs. Also, the hairpin ODNs exhibited the largest change in fluorescence intensity and polarization upon duplex formation. The G–C nucleobase-pair does not affect the fluorescence of the nearby dye in the duplex structure if the nucleobase-pair is located internally in the double strand.[22]

4.2 Function and mechanism of dual-lablled QF-MBs

The function of dual-labelled FRET MB is depicted in Fig. 11. In the absence of the target oligonucleotide the MB is in its stem-closed form and shows efficient energy transfer (FRET) from the donor (F_D) to the acceptor (F_A), generating mostly fluorescence from F_A. In the presence of the target oligonucleotide the MB opens efficiently and hybridizes with the target DNA. The energy transfer is reduced significantly in the stem-open form and the fluorescence signal generated by the duplex is clearly distinguishable from that of the probe (in stem-closed form). For example, in the case of ODN 15 in the absence of the target DNA, there in an efficient energy transfer (FRET) from the coumarin (donor) to the FAM (acceptor), generating mostly fluorescence from FAM. In the presence of the complementary

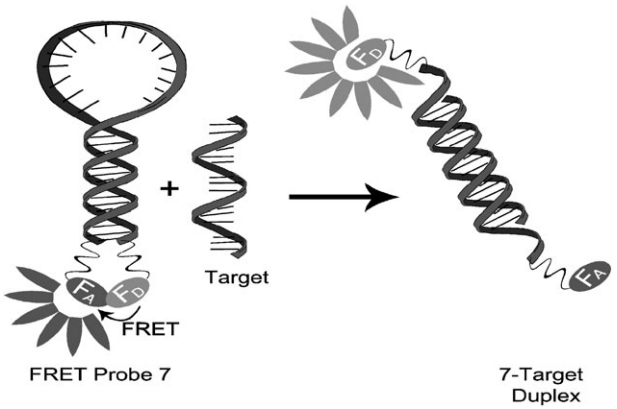

Fig. 11 Function of FRET MBs labelled with two fluorophores (F_D and F_A).

target DNA the energy transfer is almost blocked. A ratiometric analysis of the fluorescence signal from the donor and acceptor, $(I_{FA})/(I_{FD})$ is measured before and after binding the target. The ratio increases sharply upon binding to the target ODN. The coumarin/FAM MB (15) could be used to detect the target accurately in a wide concentration range (1 nM to 1 μM). The conventional coumarin/DABCYL MB failed to yield a linear response in concentrations above 10 nM. The basic criterion for a pair of fluorophores to be used in a FRET probe is that there has to be a spectral overlap between the emission spectrum of the donor and absorption spectrum of the acceptor.

The FRET MBs containing more than two fluorophores also work through a similar mechanism, where one fluorophore acts as primary energy donor while the other two fluorophores act as energy acceptors. For instance, in FRET MB containing FAM/TMR/Cy5 system, FAM acts as a primary light absorber (energy donor). The TMR acts as the primary energy acceptor (secondary energy donor), while Cy5 acts as secondary acceptor.[24] In the absence of target oligonucleotide, the MB exists in the stem-closed form and excitation of FAM initiates an energy transfer cascade from FAM to TMR and further to Cy5 generating a unique fluorescence signal defined as the ratio of the emission from each of the three fluorophores. In the presence of the complementary target DNA, the MB opens efficiently and hybridizes with the target separating FAM and TMR by a large distance, so that energy transfer from FAM to TMR is blocked in the stem-open form. Thus opening of the MB generates a distinct fluorescence signal, which is different from that of the stem-closed form of the MB. FRET MBs containing metal complexes as acceptor have also been used in identification of target ODNs. For example in one of the recent reports it has been shown that ODNs containing a quinolinone derivatives as donor and a (bathophenanthroline)ruthenium(II) complex as acceptor can be used as a sensitive probe for identification of fully matched complementary ODNs.[40] The long decay times of the fluorescence of the Ru-complex is reported to be advantageous for highly sensitive time-gated measurements. Indeed, so far, FRET is the most commonly used technique in MB design and applications of different FRET-based MBs have already been reviewed.[41]

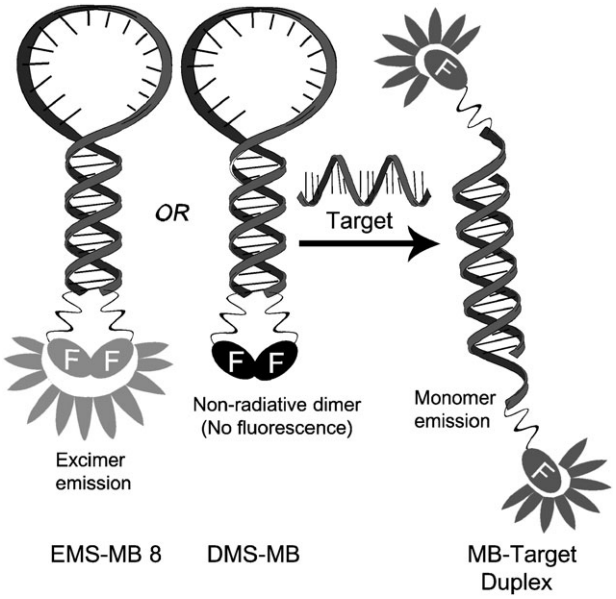

Fig. 12 Schematic representation of excimer–monomer switching molecular beacons (EMS MBs) and DMS MBs. The EMS MB depicted above is dually end-labelled (with pyrene at 3′- and 5′-ends) single-strand hairpin oligonucleotide. In the absence of target DNAs, the stem-closed EMS MB emits the excimer fluorescence predominantly. Upon hybridization with target DNA, it undergoes a dynamic conformational change and emits the monomer fluorescence.

The design of the EMS MBs is quite similar to that of FRET MBs. However, the fluorescence signal is generated by a different mechanism. A schematic representation of the function of EMS probe containing pyrene fluorophore is depicted in Fig. 12. In the absence of the target oligonucleotide the EMS MB exists in hairpin form in which the fluorophores are close enough to form an excimer and there is maximum excimer-emission. But after binding to the target the probe assumes linear form. As a result, the excimer-emission is decreased while that of monomer is increased. Thus the monomer to excimer intensity ratio is nearly 0 before binding, and a many-fold increase occurs upon binding to the target. For instance, the probe **16**, shows a pyrene excimer emission (I_{exim}) at 498 nm and monomer emission (I_{mono}) at 382 nm.[25] The ratio of (I_{mono})/(I_{exim}) was estimated to be 0.2, but after addition of target the value went up to 20. The sensitivity of **16** is reported to be much higher than the corresponding FRET MBs containing 5′-FAM and 3′-DABCYL dyes. Also, the output pattern of the EMS probes with [0,1′,1] offers unambiguous detection of target as compared to that of conventional MBs with [0,1] on/off output patterns. This is a notable advantage of EMS MBs over the conventional MB. The EMS probe, **16** detected 19-mer target DNAs and can discriminate the targets from their single-nucleotide mismatches at 1 nM concentration.

The EMS MBs **17a–17c** display different photophysical properties. The ODNs **17a** and **17c** show strongly red-shifted emission bands at 521 nm, in contrast, **17b** exhibits quenching spectra with λ_{em} at 435 nm. The EMS probes **17a** and **17c** function similarly to **16**, showing clear distinction in emission intensities upon hybridization with the matched and mismatched ODNs. For instance, an aqueous solution of **17a** (in suitable buffer) was green in colour (due to excimer emission) but the colour changed to blue (due to monomer emission) upon duplex formation with fully matched ODN.[26] But, **17b** did not show any colour change (wavelength shift) upon addition of the target ODN. However, it became highly fluorescent upon addition of fully matched target ODN. In **17b** the orientation of pyrenes does not allow them to form pyrene excimer. It has recently been shown that single strand linear oligodeoxyadenylates incorporated with two A^{PY}-units can be used as probes to study their self-duplex formation using time resolved fluorescence spectroscopy.[42] A unique fluorescence signal was observed for the intermolecular homo-adenine self-duplex.

The working concept of DMS MBs **18** and **19** is depicted Fig. 12. The dye, MR121 in **18** and the DCDHF-dye in **19** have sufficient dimerization tendency to form a non-fluorescent H-type dimer. Hence, the fluorescence intensity of the hairpin ODNs **18** and **19** is many-fold lower than that of respective free dyes. But in the presence of target, the MBs assume linear (stem-open) form, and the non-radiative H-dimers switch to monomers (Fig. 12), thereby exhibiting a strong fluorescence signal. For instance, **18** showed a 12-fold increase in fluorescence intensity at 635 nm, upon binding to the perfectly matched complementary ODN. Similarly, **19** showed 97% quenching and addition of complementary ODN resulted in a large increase in fluorescence intensity at 650 nm. Compared to conventional MBs the DMS MBs offer several advantages such as almost zero residual fluorescence, better S/N ratio, single-pot labelling and visible calorimetric detection of the target by two-fold signal enhancement. The MB **19** could be immobilized over a solid surface and the signal is high enough for single-molecule imaging.[29]

4.3 QF-MBs *vs.* simple fluorescent ODNs

Virtually, the design of simple fluorescent oligonucleotides like fluorescence *in situ* hybridization (FISH) probes and single strand linear QF-NAPs are the same. That is superficially both are oligonucleotides containing fluorophores. However, the FISH probes or other simple fluorescent oligonucleotides differ from the QF-NAPs (like HyBeacons, twin probes and BDF probes) in the following respects:

(a) In most of the FISH probes, the oligonucleotides are labelled with "always-fluorescent" dyes. The fluorescence intensity of FISH probes or simple fluorescent oligonucleotides remain the same before and after hybridization. But in QF-NAPs, the fluorescence intensity of the fluorophore changes (enhanced in most cases) significantly upon hybridization with the specific target. (b) The QF-NAPs identify the SNP by exhibiting different degree of fluorescence enhancement for matched and mismatched DNA sequences. Whereas simple fluorescent ODNs do not show such fluorescence intensity differences for matched and mismatched oligonucleotides. (c) In FISH probes any fluorophore can be used. But the fluorophore used in QF-NAPs should meet the basic criteria that it should be in the quenched state in the unhybridized probe. The quenching can be due to any one of the factors discussed in section 4.1. The fluorescence intensity of the fluorophore

should be enhanced many-fold upon binding to the target. Emissive nucleosides can also be used as fluorophores. Moreover, in most of the FISH probes, the dyes are attached at the strand ends (there are exceptions). Similarly, the quenched probe-based *in situ* RNA detection methods have notable advantages over the FISH probe-based methods.[41]

5. Potential applications of QF-MBs

5.1 Mono-labelled QF-MBs in PCR experiments

The HyBeacon-probes (*e.g.* **24**) have been used in real-time PCR experiments to monitor the accumulation of specific DNA amplicons.[33] HyBeacons have been used to detect and discriminate polymorphic targets present in directly amplified saliva samples. Use of similar mono-labelled QF-MBs containing different dyes as hybridization probes in target identification has already been reviewed by Brown and co-worker.[3] However, the SNP typing using HyBeacons relies on T_m differences between the matched and mismatched HyBeacon-probe duplexes (ΔT_m). Hence, two alleles having same ΔT_m cannot be differentiated.

Fluorogenic hairpin ODNs of the type **14** have been used as primers in multiplex quantitative real-time PCR experiments. The ODN **14** forms a blunt-end hairpin in the absence of the target DNA (PCR product). The fluorescence of the primers increases up to 8-fold upon formation of the PCR product. Though the linear ODN primers are equally efficient the hairpin shape is reported to provide additional specificity to the desired target DNA in PCR products by preventing primer–dimer formation and mispriming. The success of the QF-MBs in mulitiplex real-time PCR analysis has been demonstrated well in teal-time quantification of cloned IL-4 and *c-myc* cDNA target genes in the presence of closely related reference genes such as β-actin, GAPDH and 18S rRNA.[22] The target genes of 10 to 10^7 copies could be detected with precision. FAM labelled primers were used for target gene (primer set **27** and **28** in Table 2), JOE labelled primers were used for references genes (primer set **29** in Table 2). The real-time PCR amplification plots indicate that a range of 22 to 10^6 copies of the target gene (cloned IL-4 and *c-myc* cDNA) could be easily discriminated in the presence of 10^6 copies of reference (GADPH) gene. This method was also used to detect SNP of the human retinal degeneration gene by allele-specific PCR with end-point detection method using a fluorescent plate reader or a UV-trans-illuminator. Thus, the fluorogenic mono-labelled primers are shown to be an efficient and cost-effective alternative to conventional FRET-labelled oligonucleotides.

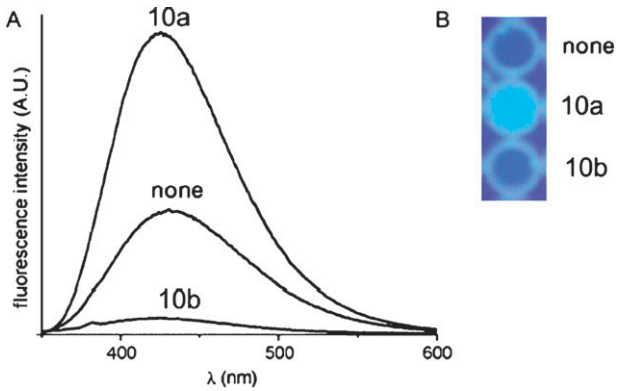

Fig. 13 (A) Emission spectra of duplexes of hairpin ODN **9a** (1.5 μM) with **10a** and **10b** recorded at 20 °C. Fluorescence spectra were recorded using an excitation wavelength of 340 nm. The spectrum labelled "none" is that of hairpin **9a** alone and in duplexes with unmodified ODNs **10a** and **10b** (2.0 μM) upon irradiation with light at 365 nm. (Reproduced with permission from ref. 16, G. T. Hwang *et al.*, *J. Am. Chem. Soc.*, 2004, **126**, 6528. Copyright 2004 American Chemical Society.)

5.2 Non-enzymatic match–mismatch discrimination using QF-MBs

Many of the mono-labelled QF-MBs discussed here can discriminate fully matched DNAs from single-base mismatched DNAs by exhibiting a sharp increase in fluorescence intensity. Hence, in principle they can all be used as fluorescent probes in non-enzymatic SNP typing. For example, the QF-MB **9a** discriminates the fully matched ODN **10a** from single-base mismatched ODN **10b** with a total discrimination factor of 14.7 (for the recognition of a single (A/C) nucleobase mismatch), in homogeneous solution as shown in Fig. 13A. From the distinct colours of **9a** and its duplexes with target DNA systems (Fig. 13B), one can visually discriminate the perfectly matched and mismatched DNAs.[16] Thus **9a** acts as a highly A-allele-specific fluorescent "on/off" switch. Similarly, hybridization of the ODN **9b** with targets (**11a** to **11d**) showed enhancement in fluorescence intensity to different extents. The ODN **11a** (matched, A) showed the maximum fluorescence intensity followed by **11b** (mismatched, C) and **11d** (mismatched, T). Similarly, the linear fluorescent ODNs **20**, **22** and **25** (twin probe) also discriminate the perfectly matched oligonucleotides from the mismatched ones with fluorescence enhancement.

Table 2 Characteristics of primer sets used in PCR

ODN	Gene target	Dye	Forward primer sequence (5′ → 3′) Reverse primer sequence (5′ → 3′)
27	IL-4	FAM	d(GAGTTGACCGTAACAGACATCTT) d(*CTACAG*TCCTTCTCATGGTGGCTG**T**AG)
28	*C-myc*	FAM	d(GACGCGGGGAGGCTATTCTG) d(*GACTCG*TAGAAATACGGCTGCACCGAG**T**C)
29	GAPDH	JOE	d(*CACGAC*TGGCGCTGAGTACGTCG**T**G) d(ATGGCATGGACTGTGGTCAT)

The dye is conjugated to the bold T; italicized nucleobases denote non-specific sequence built onto the primer to allow the hairpin conformation.

The smart probes with terminal fluorophores could also be used for target identification and match–mismatch discrimination. The sensitivity can be increased to nanomolar or even picomolar range using single-molecule fluorescence spectroscopy (SMFS) technique. The SMFS is a highly sensitive multi-parameter analytical technique, where the fluorescence bursts from a single molecule (smart probe) is measured accurately in the absence and presence of large excess of target DNA.[43] Due to availability of the 3′ -end for further modification, the smart probes could be immobilized on a solid surface after suitable functionalization. For example, the 3′-biotinylated hairpin ODN **30** was immobilized over streptavidin-coated silica surface.[31] The fluorescence intensity of the immobilized smart probe is very low. But in the presence of the complementary oligonucleotide (oligo $(dA)_{30}$) in micromolar concentration the fluorescence intensity increases to 4-fold within a short time (about 5 min) at neutral pH (7.4). Therefore, the method is ideally suitable for the identification of specific DNA or RNA sequences using very low concentrations of target.

The smart probes have also been used for specific and highly sensitive detection of pathogenic DNA sequences in microsphere based heterogeneous assays.[44] In this method short biotinylated target DNA of *Mycobacterium xenopi* (ODN **31**) was immobilized on streptavidin-coated silica microspheres in different concentrations (10^{-5}–10^{-11} M). The fluorescence intensity images of these microspheres were measured in the presence of 10^{-8} M complementary probe ODN **32** prior to washing step (Fig. 14). A comparison of intensities of these images and that of probe-unspecific target DNA (10^{-5} M) mixture shows that target DNAs can be easily identified even if their concentration is down to 10^{-11} M. It may be recalled that in the earlier report[31] the probe was immobilized, whereas, in this heterogeneous assay the target (ODN **31**) is immobilized. This heterogeneous assay can be used for quantitative analysis of immobilized PCR amplicons.[44]

30: MR121-C6-5′-d(CCCCT $(T)_{20}$ AGGGGTTT)-3′-Biotin

31: 5′-d($(A)_{15}$CCCCTTGTGAGGAACTACT)-3′-Biotin
32: 3′-d(GGGGAACACTCCTTGATGATTCCCC)-5′-C6-MR121

33: 5′-d(ATTO655-CCCTCTGG (T) CCATGAATTGAGGG)-3′
34: 5′-d(CCCTCAATTCATGG (N) CCA)-3′
34a: N = A; **34b**: N = T; **34c**: N = C; **34d**: N = G

Moreover, in a recent report it is shown that along with blocking oligonucleotides, the smart probes can be used to detect different SNPs through time resolved SMFS in homogeneous solution.[45] The ODN **33** containing a fluorophore (ATTO655) and 13-nucleotide loop region, which is complementary to part of the target, was used for sensitive SNP typing in the target ODNs **34a–34d**. The blocking ODN prevents the hybridization of probe (**33**) to the DNA containing specific mismatch. For instance the blocking ODN, *block-A* prevents the hybridization of the target ODN 34b (where N = T) with the probe. Thus addition of *block-A*, *block-G* and *block-C* prevent the hybridization of targets **34b**, **34c** and **34d** with the probe (**33**). Hence the fluorescence enhancement is only due to the **33**·**34a** duplex. This method enables the detection of specific DNA sequences among a huge excess (up to 100- to 1000-fold) of target oligonucleotides differing by a single nucleotide mismatch. The detection method is highly sensitive (10^{-9} to 10^{-11} M). However, this method is complex and not cost-effective as it requires many ODNs.

35: 5′-d(CATAGGT (N) CTAACTT)-3′
35a: N = T; **35b**: N = A; **35c**: N = G; **35d**: N = C

The end-labelled QF-MB **13a** showed good match (**35a**)–mismatch (**35b–35d**) discrimination factor (7.1).[20] The matched duplex has higher T_m value (53 °C) as compared to that of single-nucleotide mismatched ODNs **35b–35d** (all in the range of 45–47 °C). These parameters indicate that ODN **13a** can also be used in identifying the matched ODN in the presence of mismatched ones at suitable temperature (around 50 °C).

5.2.1. Direct detection of mutated nucleobase using BDF probes. The "mutation-specific" fluorescence enhancement of

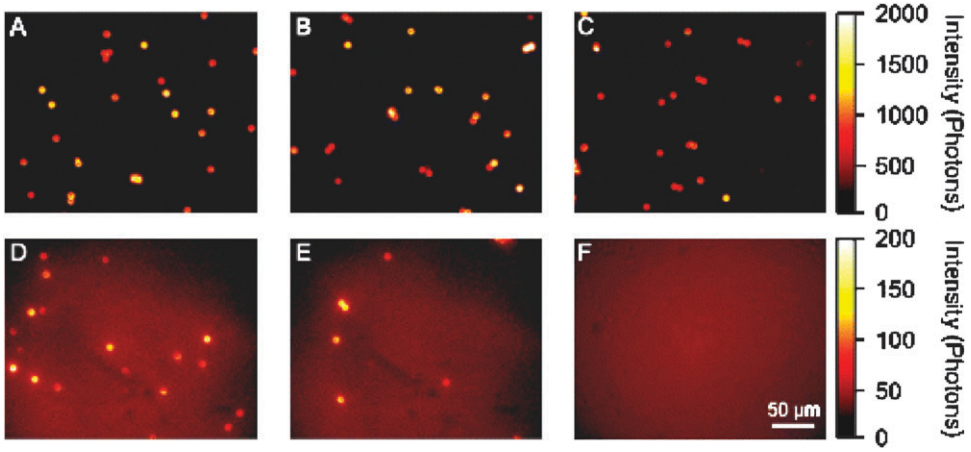

Fig. 14 Fluorescence intensity images of 5 µm microspheres modified with single stranded target ODN **31** in different concentrations upon addition of a 10^{-8} M solution of smart probe **32** in 10 mM PBS, pH 7.4, at 25 °C. Biotinylated target DNAs were immobilized on streptavidin-coated microspheres using concentrations of 10^{-5} (A), 10^{-7} (B), 10^{-8} (C), 10^{-10} (D), and 10^{-11} M (E). (F) Fluorescence intensity image measured for microspheres modified with unspecific DNA (5′-TGGTG GAAAG CGTTT GGTAG CGGTG TGGGA TGGGC CCGC-Biotin-3′, 10^{-5} M) in the presence of 10^{-8} M smart probe. (Reproduced with permission from ref. 44, K. Stohr *et al.*, *Anal. Chem.*, 2005, **77**, 7195. Copyright 2005 American Chemical Society.)

Table 3 The sequences of the BDF probes (**36**, **37**, **38** and **40**) and the nucleobases recognized in the target ODNs (**39**, **41** and **42**)

	Type of Gene	Sequences[a] (5′ → 3′)	Nucleobase recognized (SNP typing)
36	Ha-*ras*	d(GGCGCCG ^{PY}U CGGTGTG)	Adenine
37	*BRCA-2*	d(GCAGCCT ^{PY}C AGGCAGC)	Guanine
38	*BRCA-1*	d(GGTACCA ^{PY}A TGAAATA)	Thymine[c]
39	*BRCA-1*	d(ATTTCA (N)[b] TGGTACC)	
40	*abl*	d(TGAAGGGCT $^{2-ANT}$U CTTCCAGATA)	Adenine
41	*abl*	d(TATCTGGAAG (N)[b] AGCCCTTCA)	
42		D(GCGTTG (N)[b] GTTGCG)	Cytosine[cd]

[a] See Fig. 8c for structure of BDF nucleosides; ^{PY}U, ^{PY}C, ^{PY}A and $^{2-ANT}$U. [b] (N) denotes nucleoside analogue containing any of the four nucleobases, namely, A (**a**), C (**b**), G (**c**) or T(**d**). [c] Marked by sharp decrease in fluorescence intensity. [d] The nucleobase in this target is recognized by BDF probe **26d**, given in Fig. 8c.

BDF probes can be used for direct SNP typing of genes. The BDF probes also act as on/off switches in reporting the presence or absence of a specific nucleoside in the target strand. The utility of BDF probes in direct SNP typing has been demonstrated well using different gene fragments (Table 3) like human breast cancer 1 and 2 genes (*BRCA-1*[39] and *BRCA-2*[35]), human interferon-γ (IFN-γ), c-Ha-*ras* sequence[35] and 20-mer DNA strand of *abl* genes,[37] as models.

The $^{2-ANT}$U-containing ODN **40** was synthesized for SNP typing in the target *abl* DNA sequences **41**. The fluorescence change of the 5′-amino modified derivative of ODN **40**, (**amino-40**) upon hybridization with target ODNs **41a–41d** was studied by simply mixing it (**amino-40**) with different solutions containing the target ODNs **41a**, **41b**, **41c** and **41d**. A strong emission band was observed only for the fully matched complementary strand **41a** (where N = A). The mismatched duplexes (**41b–41d**) showed negligible fluorescence (Fig. 15). The BDF probe **40** was shown to be more effective for sensing A on a target DNA by a drastic fluorescence change at longer wavelength than that observed for pyrenecarboxamide-labelled BDF probe.[37] Also, the ODN **amino-40** can be immobilized on a DNA microarray *via* the free terminal amino group.

It has recently been shown that ODNs containing hydrophobic perylene substituted deoxyuridine (^{PER}U) can recognize ^{PER}U–C mismatches by exhibiting enhanced fluorescence. The hydrophobicity of the perylene is reported to be crucial for the function of ODN **26d** (Fig. 8C).[38] Also, both G–C and A–T flanking nucleobase-pair stabilize the duplex containing mismatched ^{PER}U–C nucleobase-pair. Thus, the ODN **26d** showed enhanced fluorescence upon binding to the one base mismatched ODN **42b** (Table 3). The SNP typing method using BDF probes is a single step homogeneous method. Technically, this method involves a simple mix-and-read SNP typing assay without involving any tedious probe-designing or washing processes for exclusion of hybridization error or any addition of DNA-modifying enzymes. So, this method is different from conventional methods. Also, simultaneous analysis of a number of samples could be achieved with ease, and with a accuracy using BDF probes containing different BDF nucleosides.

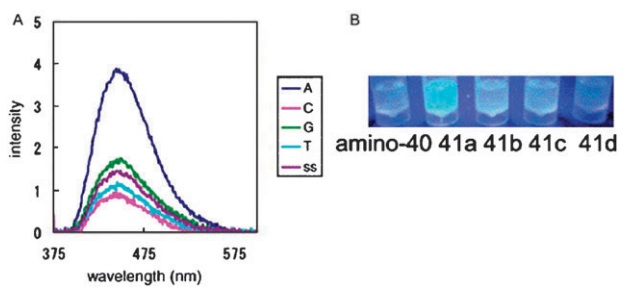

Fig. 15 (A) Fluorescence spectra of ODN **amino-40** hybridized with 2.5 μM ODN **41a–41d**. Excitation wavelength was 371 nm. (B) Comparison of the fluorescence intensity changes upon addition of ODNs **41a–41d** $^{2-ANT}$U (50 mM sodium phosphate, 0.1 M sodium chloride, pH 7.0, room temperature). The sample solutions were illuminated with a 365 nm transilluminator. (Reproduced with permission from ref. 37, Y. Saito *et al.*, *Bioorg. Med. Chem.*, 2008, **16**, 107–113. Copyright 2006 Elsevier.)

5.3 Miscellaneous applications of QF-MBs and QF-NAPs

Abasic sites are a common form of DNA damage; they are generated during base excision-repair process of damaged DNAs by enzymes or due to spontaneous hydrolytic loss of purine units. Several methods have been developed for detecting the presence of these cytotoxic abasic sites; most of them require irreversible modifications of isolated DNA. The fluorescent method is an attractive option as it is non-invasive and does not involve harsh chemical treatments. Fluorescent ODNs have been used to detect such abasic sites.[46] Recently, the quencher-free ODN **9a** has been shown to exhibit a sharp decrease in fluorescence intensity upon binding ODN **43** (Table 4) containing an abasic site, as shown in Fig. 16. The presence of abasic sites at different parts of the ODNs **44** and **45** (Table 4) did not show notable fluorescence change. It is also noteworthy that the T_m of the duplex **9a**·**43** is much higher (55 °C) than other abasic ODNs (46 °C). Thus the QF-ODN **9a** can also be used for detection of abasic sites in the target ODNs.

46: 5′-d(A^{PY} GCGCGCGCG)-3′
47: 5′-d(CGCGCGCGC)-3′
48: 5′-d(CGCGCGCGC U^{PY})-3′
49: 5′-d(GCGCGCGCG)-3′

The quencher-free G,C-alternating ODNs **46** and **48** have been used as fluorescent probes to study the B to Z DNA transition as shown in Fig. 17.[47] These ODNs can be used as optical sensors. The helicities of the B and Z DNA duplexes are opposite to each other, hence the end-stacking also differs in the B and Z structures. Pyrene-modified adenosine (A^{PY} in **46**) and uridine (U^{PY} in **48**) units are incorporated in the

Table 4 Optical properties and T_m of the abasic site (ϕ)-containing DNA duplexes relative to those of ODN **9a**

ODNs	T_m/°C[a]	λ_{em}/nm[b]	RFI[c]		Sequences of (ϕ)-containing ODNs (**43–45**)
9a	48	429	1.00	**43**	5′-d(TTCTGAAAG ϕ GAGTCAGAA)- 3′
9a·43	55	433	0.16	**44**	5′-d(TTCTGAAA ϕ A ϕ AGTCAGAA)- 3′
9a·44	46	432	1.17	**45**	5′-d(TTCTGAAA ϕ C ϕ AGTCAGAA)- 3′
9a·45	46	430	1.16		

[a] All experiments were conducted in 10 mM Tris–HCl buffer (pH 7.2; 100 mM NaCl, 20 mM $MgCl_2$); [a] melting temperature. [b] Emission wavelength when excited at 340 nm [c] Fluorescence intensities relative to that of ODN **9a**.

dangling positions of ODNs **46** and **48**. The A^{PY} and U^{PY} units undergo terminal π-stacking in their B-DNA duplexes with respective complementary ODNs **47** and **49**, and so the fluorescence intensity of the solution decreases. But, in case of their Z-DNA duplexes, due to difficulty in terminal end-stacking, the fluorophore remains unquenched. The fluorescence change (transition) can be seen with naked eye as shown in Fig. 17A.

The quencher-free mono-labelled fluorescent oligonucleotides have also been used as DNA-aptamers to study the protein–DNA interactions. Aptamers labelled with different types of dyes such as texas-red, fluorescein and TMR were used as fluorescent probes to study IgE–DNA interactions.[48] The picosecond time-scale fluorescence dynamics of the dye-labelled DNA-aptamers were measured under different conditions. The data obtained gave significant information about the structure and dynamics of the protein, IgE and its interaction with DNA.

Terminal "guanine-quenching" in G-quadruplex forming oligonucleotides carrying a fluorophore at the 3′- or 5′-end have been used to monitor the G-quadruplex formation, in the presence of different metal cations. The fluorescent human telomeric 21-mer ODN **50** (5′-$(GGGTTA)_3$GGG-3′-TMR) carrying 3′-TMR was prepared for this purpose.[49] The G-quadruplex formation of ODN **50** in the presence of metal cations like, Li^+, Na^+, K^+ and Mg^{2+} was studied. The fluorescence intensity of **50** was enhanced only in the presence of Na^+, and partially in the presence of K^+ cations. The

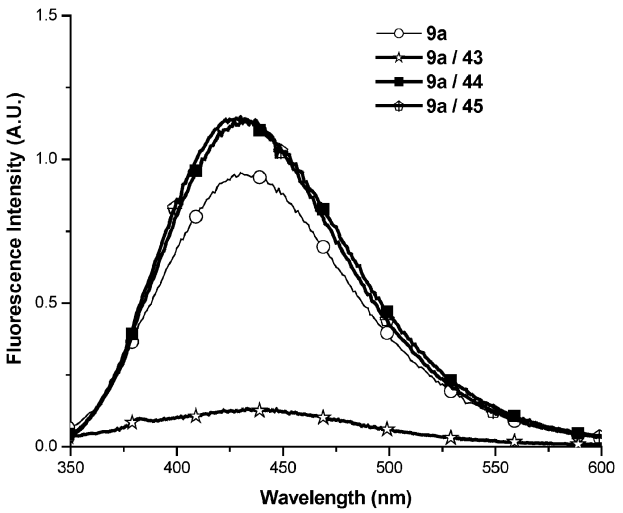

Fig. 16 Fluorescence spectra of ODN **9a** (1.5 μM) and its duplexes with abasic ODNs (**43–45**), recorded in 10 mM Tris-HCl buffer (pH 7.2; 100 mM NaCl, 20 mN $MgCl_2$).

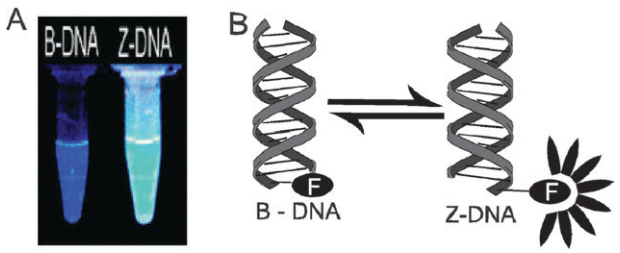

Fig. 17 (A) Visual detection of B and Z forms of **46·47** duplex DNA using the photo image. (B) The B to Z transformation of duplexes. (Reproduced with permission from ref. 47, Y. J. Seo *et al.*, *Chem. Commun.*, 2006, 150. Copyright 2006 Royal Society of Chemistry.)

presence of other metal ions did not show fluorescence enhancement. This phenomenon has been explained as due to structural variation of ODN **50** in the presence and absence of metal ions as shown in Fig. 18. In the absence of metal ions **50** forms a hairpin structure (A; Fig. 18), while in the presence of Mg^{2+} (and partially K^+) ions a "chair-like" G-quadruplex (B; Fig. 18) is formed. In both these structures the TMR is quenched due to its close proximity to terminal guanines. But, in the presence of Na^+ (and partially K^+) ions, **50** forms fluorescent intramolecular antiparallel "basket-type" G-

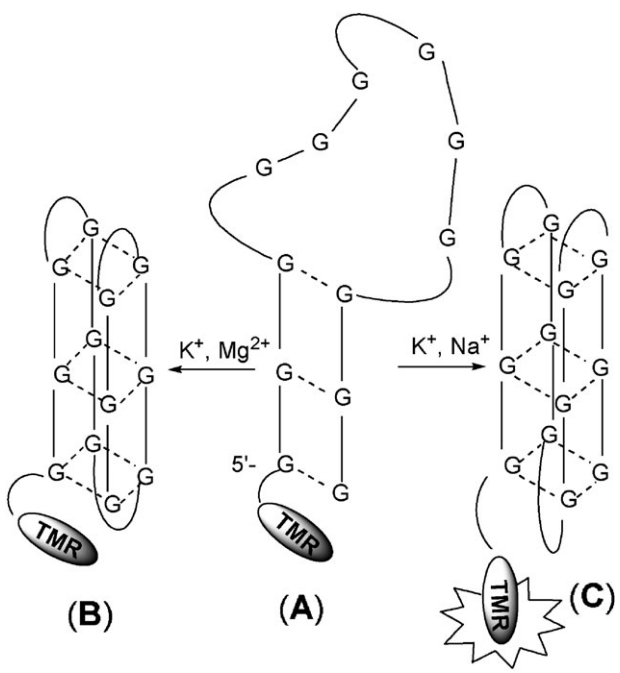

Fig. 18 Schematic representation of G-quadruplex structures of **50** showing possible interactions of TMR with terminal guanine planes.

quadruplex (C; Fig. 18) in which a diagonal loop prevents the dye from interactions with terminal guanines. These ODNs (in quadruplex structure) can also be used as ion sensors.

6. Conclusions and outlook

In summary, we have illustrated that quencher-free molecular beacons (QF-MBs) play a significant role in nucleic acid analysis. The QF-MBs are designed mainly for sensitive identification of the fully matched target sequences in presence of even large excess of mismatched DNAs. The QF-MBs have several advantages over the quencher–fluorophore MB systems. For example, mono-labelled QF-MBs can be immobilized over solid surface easily through the free end. The smart probes are shown to identify the target DNA in homogeneous as well as heterogeneous assay formats. The single-molecule fluorescence spectroscopic (SMFS) technique is used to enhance the sensitivity of smart probes. Due to the presence of one free end the mono-labelled QF-MBs can be used as primers in real-time quantification of PCR products with high sensitivity. HyBeacons have also been used in real-time PCR experiments. SNP typing using BDF probes is relatively simple. Some of the QF-NAPs are versatile enough to be used as fluorescent probes in specialized applications like detection of abasic sites and B–Z transition of DNA. As pointed out in Section 4, functioning of QF-MBs is controlled by simple factors. Nucleobase quenching, specifically, ''guanine quenching'' and environment sensitive fluorescence change of the fluorophore are the important factors behind the successful working of QF-MB systems. In predominant QF-MBs discussed here the nucleobase (guanine) present in close proximity to the fluorophore (from the same or opposite strand), acts as an in-built quencher.

However, the QF-MB systems have the following drawbacks. (a) There is higher similarity in fluorescence signals between the unhybridized QF-MBs and the mismatched duplexes. Hence quantitative estimation of matched ODNs in presence of mismatched ODNs is very difficult. (b) The non-specific guanine quenching limits the universal applications of mono-labelled QF-MBs and QF-NAPs. For example, in certain BDF probes direct identification of single-base alterations is limited by the fluorescence quenching from the G–C nucleobase-pairs flanking the SNP site. (c) Though the use of single molecule spectroscopy surely enhances the performance, it is highly unlikely that it will become a mainstream technique in near future. (d) Finally, in QF-MBs design only those fluorophores, which can be quenched by nucleobases can be used. For example, in ODNs of the type **14** the dyes such as texas-red, BODIPY and Cy5 did not show hybridization induced fluorescence intensity changes.[21]

In future, efforts should be aimed at minimizing the above mentioned drawbacks. Though the nature of linker is reported to play a crucial role in smart probe (increasing quenching efficiency) and BDF probes (minimizing the non-specific quenching), its role should be studied further. The *in vivo* applications of the QF-MBs should also be explored. The disadvantages associated with conventional MBs, like enzymatic digestion before binding to the target, are still carried over in the QF-systems. Designing QF-MBs based on modified ODNs could redress this problem to a large extent. For better activity, the electron donating ability of nucleobases can be enhanced by suitable chemical modification. Similarly, new fluorophores with better quantum yields, emission maxima and ability to interact better with nucleobases should be developed. Thus studies on QF-MBs and QF-NAPs should be pursued further to realize their full potential in nucleic acid analysis, especially in SNP typing.

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