Design and applications of a novel type of Hairpin probes: Adressable Bipartite Molecular Hook (ABMH).

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ABSTRACT.

Dual labelled hairpin probes recognize target sequences with a greater degree of specificity than their linear counterpart. Due to their pre-determined secondary structure, they assume a hairpin loop-stem conformation in the absence of a target, leading to quenching of the fluorescence signal. In the presence of a target nucleic acid, the stem dissociates and separation of the fluorophore from the quencher occurs, allowing emission of fluorescence. The use of molecular beacons (MB) and tripartite molecular beacons (TMB) immobilised on solid supports have simplified microarray protocols. However, the current technologies have problems especially high background and difficulty in preserving a functional structure, extensive loss in signal and specificity upon spotting. We have developed a probe that, for the fist time, displays the solution's characteristics (specificity, sensitivity and discrimination (1 central mismatch)) of MB when ABMH are immobilised on a solid support. We will here present this novel structure with regards to their design and properties and compare these to the current technologies (especially MB).

KEYWORDS: dual-labelled hairpin probes, molecular beacons, immobilisation, addressability, microarray.

1. INTRODUCTION.

Molecular beacons (MBs) and Tripartite Molecular beacons (TMBs) recognize target sequences with a greater degree of specificity than their linear counterpart (Kostrikis, 1998; Marras, 1999; Dubertret, 2001). Molecular beacons (Tyagi and Kramer, 1996) and TMBs (Nutiu and Liu, 2002) are dual labelled hairpin probes (Figure 1A and 1B). In the absence of a target, they assume a hairpin loop-stem conformation where the fluorophore is brought into the close proximity with the quencher, leading to quenching of the fluorescence signal. In the presence of a target nucleic acid, the stem dissociates and separation of the fluorophore from the quencher occurs, allowing emission of fluorescence. The use of molecular beacons (MB) and tripartite molecular beacons (TMB) immobilised on solid supports have simplified microarray protocols by the elimination of the sample's labelling, subsequent dye purification and extensive wash steps. However, the current technologies have problems of their own, especially high background and difficulty in preserving a functional structure, extensive loss in signal and specificity upon spotting. There is therefore a need to develop new type of hairpin probes to tackle these problems.

ABMH (see figure 1C) was developped to eliminate the problems encountered by using the current technologies such as:

- Reduced sensitivity and specificity of spotted **MBs** compared to their solution counterparts (Fang *et al.,* 1999) and requirement for further modifications (Fang *et al.,* 1999; Nutiu and Li, 2002; Yao and Tan, 2004).
- Lesser specificity of **TMBs** when compared to **MBs** (Nutiu and Li, 2002). **TMB arrays** were characterized by huge background signals due to their bulky structures and unordered/random immobilization due to universal sequences.

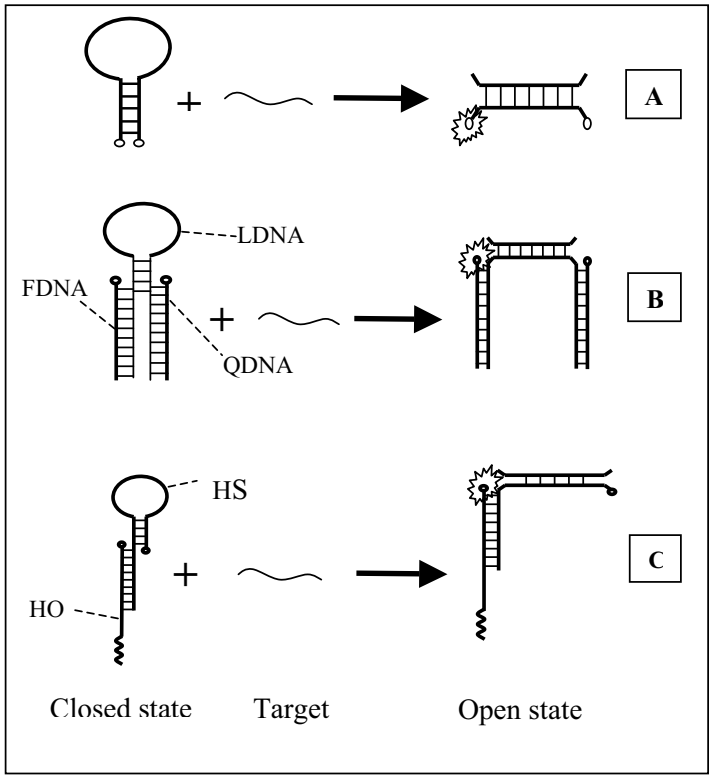

Figure 1: Closed and open conformation of **A:** classic molecular beacons, **B:** tripartite molecular beacon, **C:** addressable bipartite molecular hook. **FDNA:** TMB strand that bears the fluorophore, **QDNA**: TMB strand that bears the Quencher, **HO:** ABMH strand that bears the Fluorophore and the address sequence, **HS:** ABMH strand that bears the Quencher and the address complementary sequence.

2. ADVANTAGES/DISADVANTAGES OF MB AND TMB WITH REGARD TO IMMOBILISATION.

2.1 MOLECULAR BEACONS.

With regard to MB application within DNA microarray format, the features of this class of probes eliminate the need to label the analysed nucleic acid samples prior to hybridisation to DNA microarrays, therefore reducing the systematic errors related to the incorporation efficiency, purification yield and hybridization impact upon fluorophore labelling. Moreover, since free MB probe is internally quenched, no special removal step of the probe is required and, thus, represents an ideal "gain in signal" assay with low final background. Another significant advantage of MBs is that they are readily capable of discriminating between targets that differ by only a single nucleotide due to a competition of the unimolecular hairpin forming reaction with bimolecular probe-target hybridization.

Several modifications of the classic MB (= triple modified oligonucleotide) for attachment purposes to diverse solid surfaces have been reported in the literature (Steemers *et al.,* 2000; Brown *et al.,* 2000; Wang *et al.,* 2002). However, there are important drawbacks associated with this technology:

1) Triple modifications are needed for classic MBs to be immobilised resulting in a complex synthesis and exorbitant costs. One new MB must be synthesized for every additional target under investigation.

2) Solid support assays with MBs modified by the introduction of an appropriate linker/spacer and spotted directly as folded molecules or molecules to be refolded after the immobilization processes have proven to be difficult. This, primarily due to the initial background level upon immobilization and, thus, low dynamic range of resultant data. The reasons, obviously, relate to the strong conformational requirements for MB molecules and correspondingly, to the sensitivity of folding process to high ionic strength/salt content of spotting buffers and sterical and/or electrostatic hindrances from the solid support. These problems, thus, require a search for optimized compositions of spotting buffers and corresponding post-spotting surface proceedings as well as further modifications of the linker/spacer part of MBs.

3) Spotted MBs have a reduced sensitivity and specificity compared to their solution counterparts(Fang *et al*, 1999).

2.2 TRIPARTITE MOLECULAR BEACONS.

Nutiu and Li introduced, in 2002, another class of MB (see figure 1 B) called tripartite molecular beacons (TMBs) to resolve some of the above mentioned issues, in particular with regard to the modification of MBs to suit new targets and their immobilisation procedures. TMBs are composed of three oligonucleotide sequences forming a classic "stem and loop" structure flanked by two extra double stranded structures (one is termed FDNA, the other QDNA). The FDNA structure carries the fluorophore and the QDNA the quencher. The complementary parts of these three different oligonucleotides form the whole TMB structure via base pairing. The mode of action of the TMB is identical to the one of classic MBs i.e. upon target binding, a conformational change occurs bringing the beacon's stem to dissociate and separate the fluorophore from the quencher, leading to fluorescence.

Advantages of TMBs are:

1) easily modified to fit new targets (i.e. a change into any new target(s) would only require redesigning and synthesizing the central part(s));
2) a cost effective production ?of MB. Due to the separate strands to which either the fluorophore or the quencher are coupled, these remain the same and stock solutions can be manufactured.
3) more flexible approach to changing the fluorophore, therefore facilitating multiplex experiments, however due to the 5'-3' orientation of QDNA, restriction in the choice of possible fluorophore or quencher occur (e.g. BODIPY630/650 chemistry does not allow its coupling to QDNA).

However, these structures still display major disadvantages.

1) Solution experiments showed TMBs to have a diminished closed/open form ratio when compared to classical MBs (Nutiu and Li, 2002) due to obvious structural features:

a) Exceeding the Foerster radius of the fluorophore/quencher pairs (usually 1-10 nm) necessary for the FRET to occur given that this pair is separated by three DNA double helices, the radius of each being 2.3 nm;

b) Along with the above problem, there are difficulties with the forming of a base pair at the outside edge of the beacon's stem due to the overcrowding effect and base-stacking forces at the location where all three DNA duplexes forming the TMB meet and convert into each other. This extends the distance between the fluorophore and the quencher even further and poses the problem of optimizing the conditions for the proper TMB folding; additionally, the problem of optimized TMB design becomes of great importance.

2) Immobilisation of TMBs to the solid support via the central part has been suggested, evoking that the spotting protocol would be improved (Nutiu and Li, 2002). However, assessment of this process is impossible as there is only one report in the literature describing, exclusively, experiments performed in solution. Nevertheless, the following difficulties related to the above experimental system are foreseeable and deduced from our own experiments:

a) The loop has to perform both functions, being an attachment site and a target hybridization site. This can will lead to folding problems of the TMB's central part (false positives) as well as loop accessibility for the target molecules due to sterical hindrance from the stem and sequences complementary to F- and QDNA elements.

b) Attempts in changing TMB immobilization procedure via coupling through aminolink-modified FDNA, QDNA or both (i.e. FDNA + QDNA), performed by our group, led to complications of pre- (performing a folding reaction for the TMB to form prior to spotting), spotting (a composition of spotting buffers have to be carefully designed) and post-spotting (harsh conditions in regard to ionic strength, detergent presence and temperature have to be avoided) procedures due to the need to form and preserve the TMB structure. Prior to hybridization, the fabricated TMB arrays were characterized by huge background signals. As possible reasons, those given above and describing disadvantages of MBs and TMBs upon solid support immobilization, will apply. In particular for preformed TMBs, upon their immobilization via FDNA, QDNA or FDNA + QDNA as opposed to the process described by Nutiu et al., the bulky structures will pose a crowding effect problem, namely a problem of accessibility to the surface active groups for efficient immobilization reactions.

c) The presence of universal complementary strands can lead to intermolecular recombination and, as a result, to unordered/random immobilization. This is especially true for FDNA+QDNA immobilisations, given that the density of surface active groups greatly exceeds the maximal density of preformed TMBs that appear above the unit surface of each time period unit. Moreover, we could not exclude completely the possibility that the central part of the structure dissociates at any stage of the experiment and binds at other locations due to the universality of FDNA and QDNA sequences.

It is common knowledge within the field of MB technology that the need for novel probe formats remains mainly unmet. TMB have only very insufficiently answered this need with regard to design flexibility and cost. However, this format is impractical for microarray applications. To this end we developed ABMH (see figure 1 C).

3. ABMH: STRUCTURAL DESIGN, OPTIMISATION AND COMPARISON WITH MB PERFORMANCES.

3.1. DESIGN.

The novel bipartite hairpin probe designed in our laboratory is called **A**ddressable **B**ipartite **M**olecular **H**ooks (ABMH; see figure 1C) and is being composed of an oligonucleotide (HO) having a unique address sequence that will hybridised only to the Hook (HS) having the corresponding complementary sequence. The potential TMB leakage problem was addressed in our novel probe design using an unique and specific sequence recognition motif (i.e. address sequence)zip code? for each spot as well as simplifying the immobilisation procedure (i.e. the spotting of HO can be performed following classical oligo-/poly-nucleotide/cDNA immobilisation techniques). This system also offers flexible procedures for the hybridisation process:

1) Spotting of HOs is followed by addressed hybridization of HS to corresponding HO with simultaneous folding of the hairpin configuration in the appropriate buffer. In subsequent steps, a target will be hybridized and fluorescent signals quantified.

2) Spotting of HOs with independent hybridization in solution (allowing higher specificity than on solid surfaces) between HS and target, pre-formed hybrids will subsequently hybridise to their respective spotted HO.

Irrespective of which from the above mentioned methods is used for hybridisation, this novel bipartite system offers an additional dimension of flexibility in the design of the ABMH along with the folding/detection method allowing the introduction of two ABMH tools:

1) ABMH with a fluorophor/quencher pair coupled to HO and HS, respectively and vice versa;

2) Two different fluorophores (FRET) present on HO and HS, respectively leading to a more sensitive and controlled system where one fluorophore represent the AMBH "closed" state and the other the "open" state.

3.2 CHARACTERISTICS IN SOLUTION.

Thermal denaturation profiles for MB, ABMH with and without perfect as well as various mismatched targets were performed in various buffers to determine their specificities (TMB were omited from all these studies due to their known lower specificity). Both with pure target and in a complex nucleic acid mixture, MB thermal denaturation profiles showed a low single mismatch discrimination,. Moreover, MB limit of sensitivity could be determined at 10 nM target in both conditions. ABMH thermal denaturation profiles characteristics were similar to those of their MB counterpart (see figure 2). Our novel structure ABMH displayed even a greater mismatch discrimination (i.e. 1 central mismatch) compared to its MB equivalent both in presence of a pure target or when target was part of a complex nucleic acid mix. With regard to sensitivity, MB and ABMH displayed a similar detection limit.

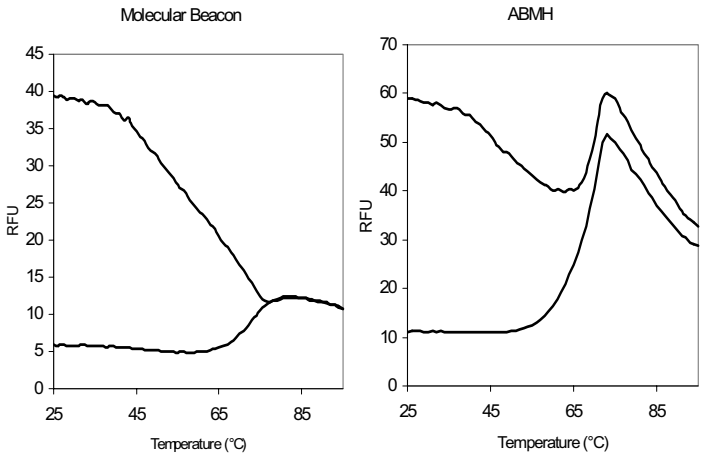

Figure2: Thermal denaturation profile of **A:** MB with and w/o targets and **B:** AMBH with and w/o targets

3.3 CHARACTERISTICS ON SOLID SUPPORTS.

We opted to use the second hybridisation procedure to take advantage of the increased specificity and sensitivity of hairpin probes in solution, followed by localised hybridisation of the resulting complex. We obtained good signals at spotted concentration of 1 and 0.2 µM which is 25-100 fold and 50-200 fold less than for oligonucleotide and molecular beacon (Ramachandran *et al.,*2003)arrays respectively. This could lead to considerable savings when considering the price of their synthesis (especially molecular beacons) and elimination of dye-labelling. Immobilisation of ABMH on solid support lead to a reduce loos in signal when compared to MB (2,5 for ABMH vs 10 for MB (Fang *et al.,*1999)). ABMH bound to a surface could still discriminate between a perfect match target and one with a central mismatch (i.e. the most disruptive) both in presence of a pure target or when target was part of a complex nucleic acid mix. The sensitivity of our ABMH array (100 fM) was up to 10 fold more sensitive than what is reported in the literature for Oligonucleotide arrays (Stemmers et al., 1999), 100-1000 more sensitive than most MB arrays (Stemmers et al., 1999), and up to 10^4 time more sensitive than the latest report on spotted MB (Yao *et al., 2004*).

CONCLUSION

We here present a novel dual labelled hairpin structure that displayed all the solution characteristics of classical MB. Furthermore, ABMH were shown to be more specific than MB both in solution and on solid support. The unique design allow the use of a sensitive and specific target/loop hybridisation in solution followed by an addressable and leakage free localisation of the resulting target/hook complex on a solid support. This advantage channels the structure to become an interesting tool for the detection of nucleic acid ,especially for the microarray-format.

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