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Covalently Mercurated Molecular Beacon for Discriminating the Canonical Nucleobases

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Abstract: A highly nucleobase discriminating metalated nucleoside analog, 3-fluoro-2-mercuri-6-methylaniline, was incorporated into an oligonucleotide molecular beacon. Fluorescence emission spectra were measured after the addition of four different complementary strands, where the nucleobase opposite of the metalated analog varies. The fluorescence results showed a clear binding selectivity at room temperature, in the order G>T>C>A. The selectivity is based on the different affinities between the metalated nucleoside analog and the canonical nucleobases. The synthesized probe is capable of robust discrimination between the two purine as well as the two pyrimidine bases by fluorescence at room temperature, and more sophisticated temperature analysis allows clear separation of every canonical nucleobase. The probe would, hence, be a suitable method for the detection of single nucleotide polymorphisms.

Single nucleotide polymorphisms (SNP) have gained attention in medicine because of naturally appearing differences in genetic code. They have been suggested to affect the expression of genes which are related to various diseases and varying drug response.[1] Different methods have been developed for detecting single nucleotide polymorphisms. One attractive method is molecular beacons, which are hairpin forming oligonucleotides labeled with a fluorophore and a quencher at the opposite termini. In the hairpin form, fluorescence is quenched but when a complementary strand is hybridized with the loop section, the hairpin will open and fluorescence increases.[2] However, a major limitation of these methods is the recognition of only one nucleobase. Additionally, G/A and C/T base pairs are in some situations hard to detect and can lead to false results.[3] To address these issues, highly discriminating probes are needed and one way to achieve this could be by using metal-mediated base pairing. Multiple different metal mediated base pairs have been proved to increase thermal stabilities within double helical nucleic acids[4] but discrimination between all canonical nucleobases is rare.[5] Previously, Hg(II)-mediated base pairing

has been used in the stem sequence of a molecular beacon for detection of Hg(II) ions.[6] In a recently published paper, a Ag(I)-mediated base pair was used as a functional unit for discriminating pyrimidines.[7]

Our related approach shows the potential to discriminate also purine nucleobases. For this purpose, we used a recently developed organometallic 3-fluoro-2-mercuri-6-methylaniline nucleotide (**F-Hg**) (Figure 1.) because of promising results on discriminating natural nucleobases within double-helical DNA by ^{19}F NMR and UV melting temperature measurements.[8] In the present article, the nucleoside analog is part of the loop region of a molecular beacon. This newly developed mercurated molecular beacon was evaluated by fluorescence measurements and found capable of discriminating nucleobases and, hence, a promising tool for the detection of single nucleotide polymorphisms.
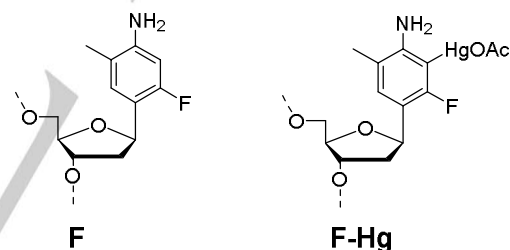

Figure 1. Structure of unmercurated and mercurated F residue.

For the preparation of the mercurated molecular beacon Fam-**B(F-Hg)**-Dabcyl, a hairpin-forming 27-mer oligonucleotide was synthesized by an automated synthesizer (cf. sequence in table 1). The loop, connected to a six base pair long double-helical stem region, contains a sequence (**ON(F)**) that has been used for Hg(II)-mediated hybridization in our previous study.[8]
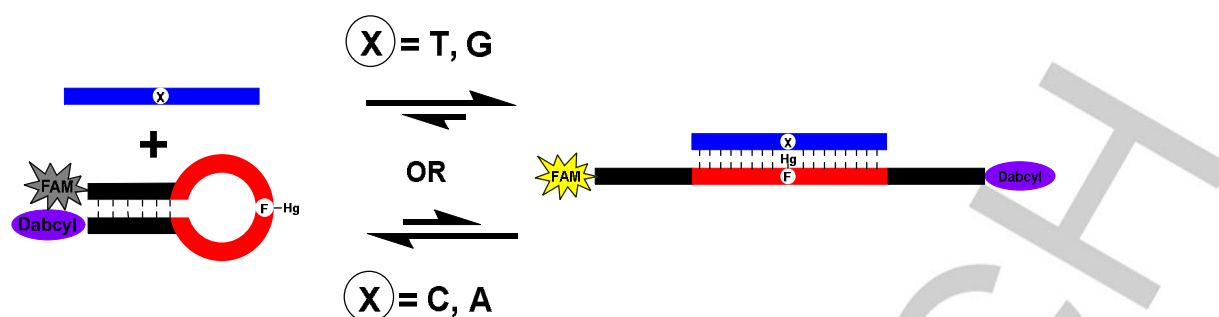
Scheme 1. Equilibria of the covalently mercurated molecular beacon after addition of target strand, where one nucleobase varies (X = A ,C ,G or T) at 25°C.
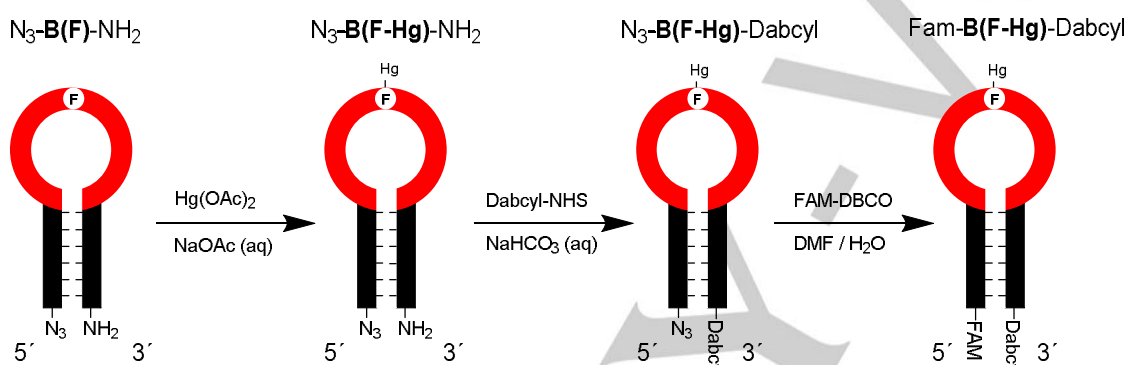
Scheme 2. Post-synthetic modifications of the molecular beacon

In the 3'- and 5'-end of the sequence, an amino- and a 6-bromoalkyl group (respectively) were incorporated for post-synthetic coupling of the quencher and the fluorophore. The post-synthetic couplings were used to prevent mercuration of fluorophore and quencher. As in the previous studies, cytosine bases were replaced by 5-methylcytosines to prevent mercuration at the 5-position.[8,9] After automated oligonucleotide synthesis, the 5´ bromo group was converted to azide on support. The oligonucleotide was then cleaved off the solid support by ammonolysis, purified by RP-HPLC and characterized by ESI-MS. Mercuration of the N_3-**B(F)**-NH_2 intermediate was conducted by treatment with an excess (10 eq) of $Hg(OAc)_2$ in 5 mM aqueous NaOAc solution at 55 °C for 24h. The reaction mixture was purified by RP-HPLC, and the mercuration to yield N_3-**B(F-Hg)**-NH_2 was confirmed by mass spectrometry.

Dabcyl-NHS ester was coupled to the 3´-amino group of N_3-**B(F-Hg)**-NH_2 in 0.1 M aqueous $NaHCO_3$ (pH = 8.4, adjusted with HCl). The reaction mixture was vortexed and aliquots (5 µl) of Dabcyl-NHS ester (3.0 mg in 50 µl DMF) were added once in a day.[10] The reaction was monitored by RP-HPLC. After completion in three days, the reaction mixture was purified by RP-HPLC. Fluorescein-DBCO was used for strain-promoted azide-alkyne cycloaddition (SPAAC) to 5´-azide of the oligonucleotide. Fam-DBCO was dissolved to DMF and 2 equivalents of it was added to an aqueous solution of N_3-**B(F-Hg)**-Dabcyl. The reaction mixture was vortexed overnight and purified by RP-HPLC and formation of the desired product was verified by ESI-MS.

Unmercurated reference beacon Fam-**B(F)**-Dabcyl was prepared as the mercurated one (all spectra and chromatograms of the intermediate products and products are presented in supporting information).

Table 1. Oligonucleotides used in this study.

	Sequence (5´-3´)
B(F)	6-Fam-C^{m}C^{m}TAGC^m**TTC^{m}GAGC^{m}FC^{m}TGGC^{m}TT**GC^{m}TAGG-dabcyl
B(F-Hg)	6-Fam-C^{m}C^{m}TAGC^m**TTC^{m}GAGC^{m}F^{Hg}C^{m}TGGC^{m}TT**GC^{m}TAGG-dabcyl
B(X)[a]	6-FAM-CCTAGCTT**GCCAGXGCTCG**TTGCTAGG-dabcyl
ON(X)[a]	**AAGCCAGXGCTCGAA**
ON(F)	**C^{m}GAGC^{m}FC^{m}TGGC^m**
ON(F-Hg)	**C^{m}GAGC^{m}F^{Hg}C^{m}TGGC^m**

[a] (X = A,C,G,T)

The potential of the mercurated oligonucleotides for detecting SNPs was first evaluated by exposing four non-metalated commercially available beacons (**B(X)**) to one mercurated complementary sequence ON(F-Hg) (1 equiv). The assay was

designed to pair the organomercury nucleobase of **ON(F-Hg)** with each of the canonical nucleobases. After addition of the mercurated oligonucleotide **ON(F-Hg)**, fluorescence emission spectra of the beacons (**B(X)**) were recorded as a function of time (Figure S34–37). The hybridization process was sluggish and fluorescence increased slowly over time ($t_{1/2}$ = 170–240 min depending on **X**). The rate-limiting steps of the molecular beacon are the target binding (conformational changes of the target to match the loop geometry) and the beacon opening (the newly formed duplex overcomes the energy barrier of dissociation of the stem), therefore the combination of the relatively short loop and long stem has a rate-retarding effect on the hybridization kinetics.[11] In addition, an additional rate-limiting step might be caused by a high energy barrier of dissociation of the pre-formed intra- or intermolecular **F-Hg-X** base pairs in the 11mer target sequence which has been studied previously in more detail in simple double helix models of **ON(F-Hg)** using ^{19}F NMR.[8] Similar slower hybridization kinetics has been observed with **T-Hg-T** base pairs.[6b,12] Despite the relatively slow process, clear differences in the fluorescence intensities of **B(X)•ON(F-Hg)** at equilibrium were observed in the order of **B(T) > B(G) > B(C) ≥ B(A)**, suggesting that a metalated beacon could be capable of SNP detection. The order of the fluorescence intensities is in line with previously published affinities based on the UV melting temperatures of corresponding oligonucleotide duplexes.[8] Reference measurements were carried out with the unmetalated **ON(F)** as the complementary strand. No marked increase in fluorescence was observed, which highlights the effect of covalently bound mercury. The impact of covalently bound mercury in the oligonucleotide sequence was crucial since fluorescence intensity changes were very small or nonexistent in the case of all **B(X)•ON(F)**.

Encouraged by the results obtained, we next set out to test the applicability of the mercurated beacon **B(F-Hg)** for detecting SNPs. Fluorescence measurements were repeated using **B(F-Hg)** or **B(F)** and 5 equiv. of **ON(X)**. The longer target sequences (15mer) was used to push adjust the equilibrium to favor duplex formation over the hairpin, and higher target sequence concentration (250 nM, 5 equiv.) was used to speed up the hybridization kinetics. As previously, the hybridization process was slow ($t_{1/2}$ = 20–80 min depending on **X**) compared to beacons containing canonical base pairs only[10] but faster compared to measurements where the target strand (rather than the beacon) contained covalently bound mercury (**B(X)•ON(F-Hg)**). The measurements were carried on until fluorescence emission had stabilized (for at least 2 half-lives). In the case of **B(F-Hg)•ON(T)** and **B(F-Hg)•ON(G)** eight- and nine-fold fluorescence intensity (respectively) compared to the initial fluorescence was observed. (Figure 2.) In the case of **ON(C)** and **ON(A)**, increase of the fluorescence was moderate. The difference in the fluorescence intensities is sufficient for a robust discrimination of **ON(T)** and **ON(G)** from **ON(A)** and **ON(C)** and, with a more careful analysis, for complete discrimination of the canonical nucleobases. The intensity varied depending on the opposite nucleobase (**G > T >> C > A**). Reference measurements were done using the unmercurated beacon **B(F)**. Fluorescence was not changed on addition of any of the complementary strands **ON(X)**, which indicated a low affinity between unmercurated **F** and canonical nucleobases. Interestingly, the initial fluorescence of **B(F)** was much higher than that of **B(X)** or **B(F-Hg)**. The high initial level seems to be in line with previous studies.[7] Control measurements

were also done by adding Hg(II) ions to the **B(F)•ON(X)** samples, which did not increase the fluorescence (S42−S45). Comparison of the B(F-Hg) between B(X) and previously published beacons by Müller *et al.*[7] were done to highlight the much higher fluorescence.(Figure 3.)
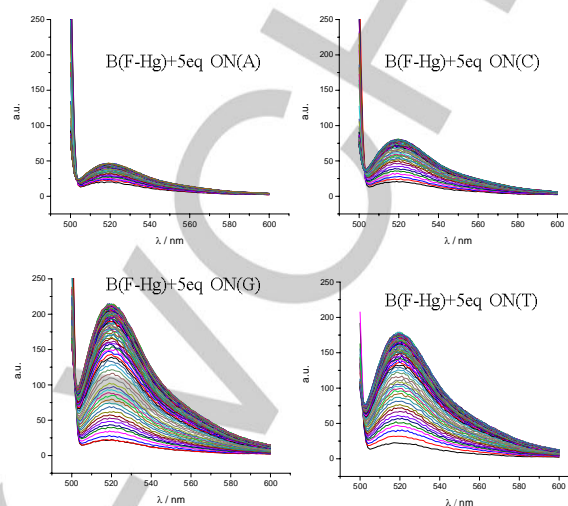

Figure 2. Fluorescence intensities of **B(F-Hg)** + 5 eq **ON(X)**. Measuring times: 0, 1, 3, 5, 7, 10, 13, 16, 20-60 in every 5min and after 60min in every 10 minutes up to 16 hours at 25°C.
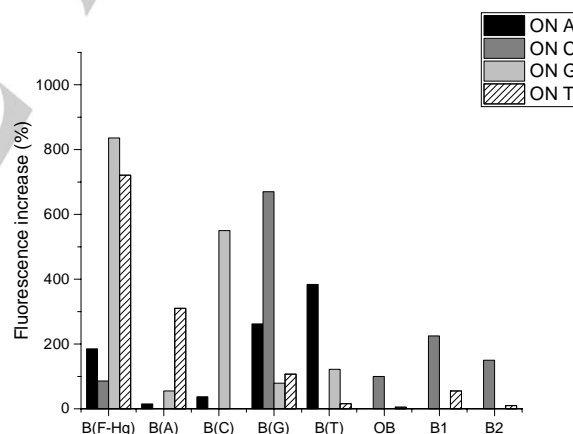

Figure 3. Comparison of **B(F-Hg)** between **B(X)** and previously published **OB**[7a], **B1** and **B2**[7b].

Fluorescence intensities of all **B(F-Hg)•ON(X)** samples at 520 nm were also recorded as the function of temperature (Figure 4.). Normalization of the curves was done between the initial fluorescence level and the highest measured fluorescence of each sample. Behavior of beacon fluorescence as the function of temperature was similar with all **ON(X)**s.(Scheme 3.) The increased intensity in the beginning of the temperature ramps might be linked to partial hybridization of stems, resulting in partial quenching at low temperatures. The drop of fluorescence intensity stands for dissociation of the beacon and the target and

subsequent regeneration of the hairpin (**B(F-Hg)•ON(X)**) → **B(F-Hg)**), and the second increase represents the opening of the hairpin and formation of a random coil structure. The cooling cycle represents the formation of the hairpin and only a small portion of the duplex. The huge difference between heating and cooling cycle is consistent with the slow hybridization of metal-mediated duplex.(Figure 4 and Scheme 3) The minimum level (i.e. the turning point) of **B(F-Hg)** fluorescence was at 45 °C with **ON(A)**, at 50 °C with **ON(C)**, at 60 °C with **ON(G)**, and at 65 °C with **ON(T)**, which are in line with the results of our previous study on a simple double-helical structure. In the case of **ON(C)**, **ON(G),** and **ON(T),** the turning point of fluorescence is higher than initial fluorescence which indicates the simultaneous denaturation of the duplex and hairpin, or incomplete hairpin formation after duplex denaturation at high temperatures. The fluorescence-based discrimination of the different target nucleobases improved at higher temperatures. For example at 35°C, the **ON(A)** gave 20% of the maximum fluorescence versus 75-85 % with other **ON(X)**s, at 45 °C, the **ON(G)** and **ON(T)** gave 70% of the maximum fluorescence versus 20% with **ON(C)** and almost 0 % with **ON(A)** and finally at 60 °C, the **ON(T)** gave a bit over 50% of the maximum fluorescence versus c.a 25% with other **ON(X)** (Figure 5.).

Scheme 3. Suggested behavior of the **Beacon (F-Hg)** + target strand at different temperatures. Black line represents heating cycle and grey line cooling cycle.
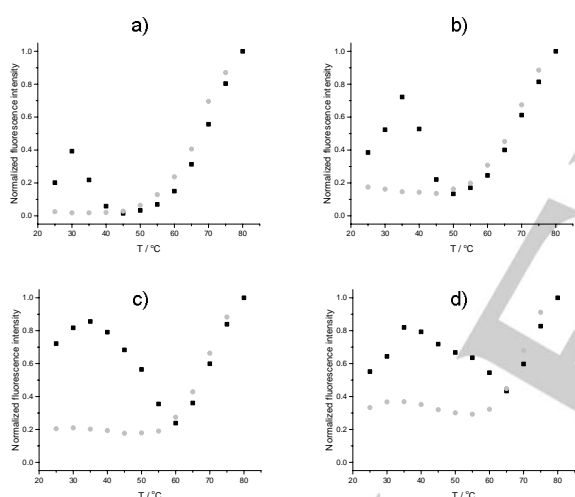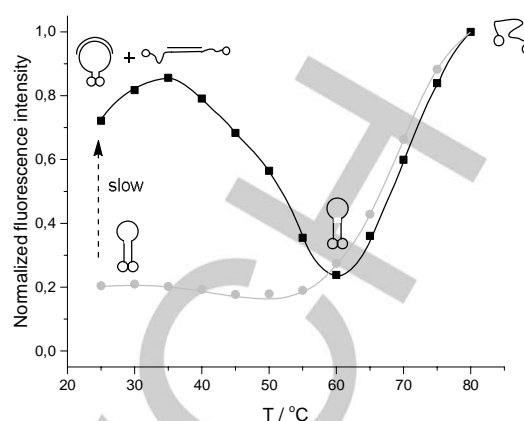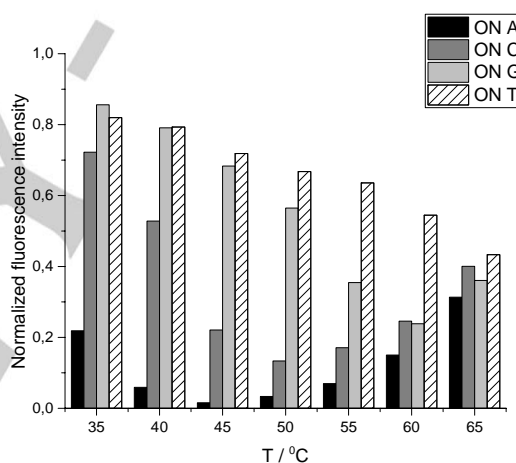

Figure 4. Temperature ramps of the **B(F-Hg)•ON(X)** where fluorescence intensities at 520 nm are plotted as function of temperature. a) X = A, b) X = C, c) X = G and d) X = T. Black squares represent heating cycle and grey circles represent cooling cycle.

Figure 5. Selected differences in fluorescence intensities of **B(F-Hg)•ON(X)** as a function of temperature.

In this paper, we present the synthesis of a metalated molecular beacon bearing covalently mercurated nucleoside analog and its properties to detect canonical nucleobases by fluorescence. The developed molecular beacon is capable of discriminating conventional purine bases and pyrimidine bases from each other based on fluorescence differences at various temperatures. Since molecular beacons are sensitive for the length and base composition of the stem and loop as well as changes in the fluorophore and quencher, further optimization to enhance the fluorescence properties and the suitability for different target sequences would make the probe even more potential. However, the applicability of **F-Hg** as a functional unit in the molecular beacon is well proven and overall metalated SNP probes would offer one interesting way to detect SNPs.

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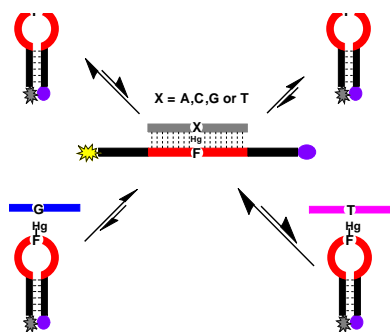
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