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A dumbbell molecular beacon for the specific recognition of nucleic acids

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ABSTRACT

A dumbbell molecular beacon (DMB) was designed and synthesized with the attachment of a fluorophore and a quencher at two ends. This DMB probe can be used to detect single mismatch of a 20mer oligodeoxynucleotide in two different buffers and discrimination factors were as high as 60 at 37 °C. Statistics of single substitutions of analytes showed that both substituted positions and substituted nucleotides have important contributions for this probe to efficiently distinguish the true analyte from mismatched ones. Hybridization kinetics of DMB with the target oligonucleotide was also studied.

Molecular beacons (MBs) are oligonucleotides with a fluorophore and a quencher connected to two ends of hairpins, which have been reported to specifically bind their complementary strands.[1] They have been extensively applied in many areas such as genetic screening, biosensors, biochips, single nucleotide polymorphism and monitoring of mRNA in vivo.[2] In order to increase the distinction between the 'closed' and 'open' states, many new versions of MBs have been reported. One strategy is to increase the sensitivity of fluorophores and/or quenchers. Dubertret and Kraemer, respectively, reported gold nanoparticle[3] and ligated copper(II)[4] as the efficient quenchers to quench fluorescence signals, while Lukhtanov et al.[5] reported fluorogenic DNA probes with the attachment of a minor groove binder at the 5′-end. These probes have provided much lower background and resulted in higher signal-to -background ratios upon hybridization with analytes. To improve the brightness of the fluorescence signal upon probe-analyte hybridization, Tyagi et al.[6] designed a wavelength-shifting molecular beacon with FRET fluorophore pair at one end. Tan et al.[7,8] demonstrated a molecular assembly of the superquenchers with multiple DABCYLs or the excimer with multiple pyrenes for probing nucleic acids, while Asanuma et al.[9] inserted multiple pyrenes and multiple quenchers as pseudo bases at two ends of molecular beacons. This design efficiently suppressed the background emission and successfully detected one-base deletion in a analyte. The other strategy is to engineer MB structures to improve the sensitivity of mispaired bases. Tyagi et al.[1] first reported that MBs can recognize a single mismatch located in the middle of 15-mer oligodeoxynucleotides. Quadruplex-based molecular beacon as a tunable DNA probe was reported by Jullien et al.[10] to diagnose a 13-mer single stranded DNA or RNA with high discrimination to the mismatched ones by independently varying the concentration of monovalent or divalent cations in the medium. Instead of normal basepairing of a duplex, a triplex-forming stem was attached to probe the target oligonucleotide. By adjusting the length of triplex forming oligodeoxynucleotides, triplex based molecular beacons were able to report the presence of DNA targets in homogenous solution by enhancement of fluorescence signals.[11] Stemless MB[12] and quencher-free MBs[13,14] were further developed to simplify MB sensing systems.

In order to develop a general strategy of the single deoxynucleotide mutation analysis for all the deoxynucleotides in the sequence of analytes, Kolpashchikov[15] developed a binary DNA probe for single deoxynucleotide substitutions of a 20mer oligodeoxynucleotide at room temperature by using two bridged oligodeoxynucleotides to sense target analyte. However, high concentrations of the bridged oligodeoxynucleotides had to be used, which was quite difficult for in vivo applications. Most recently, Soh et al. reported a single self-complementary triple-stem DNA probe which was used to distinguish the middle 5 mismatches of a 17mer oligodeoxynucleotide with relative high discrimination factors.[16]

In this Letter, we designed a new MB variant DNA probe with a dumbbell structure (DMB). This probe has a fluorophore (FAM) and a quencher (BHQ1) at the ends and forms two hairpin structures as shown in Figure 1. These two hairpins can be linked through a non-base linker or deoxynucleotides to tune the binding energy of two arms with the middle deoxynucleotides. Here, we use a guanosine

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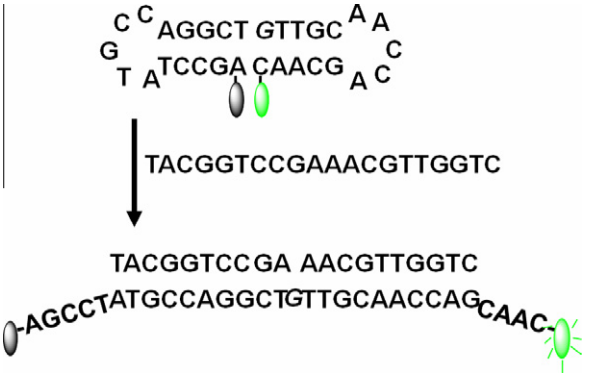

Figure 1. The strategy of dumbbell molecular beacon (DMB) for nucleic acid detection.

as the linker and stem-binding nucleobase in our DMB design. The quencher and fluorophore are close to each other and the fluorescence was efficiently quenched. Two hairpins are used to fine-tune the basepairing of the probe and target oligodeoxynucleotides, and the deoxynucleotides of two hairpin stems are base-paired with the probe itself, which kinetically disfavours hybridization of the probe and the mismatched target oligodeoxynucleotides. With this design, we can reliably discriminate a single nucleotide mismatch of a 20 mer target oligonucleotide.

A 30mer dumbbell molecular beacon (DMB: 5′-FAM-CAACGACCAACGTTGTCGGACCGTATCCGA-BHQ1-3′) was synthesized with fluorescein at 5′ end and BHQ1 at 3′ end. Five deoxynucleotides at two ends are all base-paired with the deoxynucleotides located in the middle part of the sequence of DMB instead of basepairing with each other as a traditional MB, and fluorescein and BHQ1 are closer to each other. Without the presence of the target analyte, these two hairpins were folded and the fluorescence of fluorescein was efficiently quenched by BHQ1. However, the addition of the complementary oligodeoxynucleotide (20-m0: TACGGTCCGAAACGTTGGTC) induced the unfolding of two hairpins, which promoted the separation of fluorescein and BHQ1 and triggered strong fluorescent emission. But oligodeoxynucleotide analytes with one mismatch in a 20mer oligodeoxynucleotide sequence (20-m5: TACG**C**TCCGAAACGTTGGTC) caused fluorescence increase only slightly over the background of the DMB probe and can be easily distinguished from its true analyte at the physiological temperature (37 °C), as shown in Fig. S1 in Supplementary data. We further studied whether the DMB probe could be used to reliably discriminate the true analyte from a 20mer oligodeoxynucleotide with a single mismatch at other positions. Table 1 showed all oligodeoxynucleotides with a single substitution and the discrimination factor of each substitution. As shown in Table 1, DMB distinguished all of mismatched oligodeoxynucleotides from its true analyte with reasonably high discrimination factors (DFs) at physiological temperature. At the substituted position 4th, 5th, 14th, 16th, 17th and 18th, DMB distinguished the true analyte from those mismatched ones with discrimination factors as high as 60. Even for the analyte with the substitution at two ends of oligodeoxynucleotide analytes, the discrimination factors were still higher than 2, which was usually difficult to achieve by a traditional MB. We also tested the effect of the temperature on the discrimination of single mismatched oligodeoxynucleotide analytes with the true analyte using the DMB probe. As shown in Table 1, the DMB probe could also reasonably detect the single mismatch of a 20mer oligodeoxynucleotide analyte at 20 °C with the DF as high as 13. Even though the values of DFs at 20 °C for the same mismatched oligodeoxynucleotides were smaller than those at 37 °C, the profile of DFs for base substitution of oligodeoxynucleotides was the same.

By analyzing the DF value of each analyte target with single mismatched deoxynucleotide, the distribution of DFs was very interesting with two peaks among 20 deoxynucleotide substitutions. We wondered whether the type of the mismatched deoxynucleotide or the position of deoxynucleotide substitution caused the phenomenum. We drew a DF curve with corresponding substitutions of the type and position of deoxynucleotides in Figure 2. Figure 2 clearly showed that the position of a mismatch has a great contribution for the discrimination factor. With deoxynucleotide substitution in two loop domains of the DMB probe, the values of DFs were much higher comparing to other substituted positions. Figure 2 also pointed out that a G-C substitution had more effects on DF than an A-T substitution. When we compared the substitutions of C6T and C14G, both were located at the first base of two hairpin stems, but their contributions to discrimination factors were dramatically different. The substitution of T with C only caused 4.2-fold decrease of fluorescence, but replacement of G with C lost the momentum to unfold the hairpin and triggered 60-fold decrease of fluorescence. Similar results were also observed when the assay was carried at 20 °C (see Supplementary Fig. S2). Different kinds of salt in the buffer also affected the detection of mismatched oligodeoxynucleotides. We replaced 100 mM tris buffer (pH 8.0) with the buffer containing 100 mM NaCl and 10 mM tris (pH 8.0). The DMB probe in this buffer still showed good detection of the analytes with mismatches located at the loop domain. At the mismatched position 4th, 5th, 14th and 17th, the values of DFs in NaCl buffer reached as high as 32. As shown in Table 1, the values of DFs also showed the similar profile for deoxynucleotide substitutions in NaCl buffer as in 100 mM tris buffer. Comparing all mismatched analytes to the true analyte, the high DFs of analytes at the positions of loop domains may be due to the insight of hybridization of a hairpin structure and formation of the probe-target duplex, which the target analyte binds onto the loop domains of the probe to drive the unzippering of the remaining base pairs.[17] For DMB probe, the available loop domains for analyte binding are limited. To prove the advantage of DMB to conventional MB, we also designed a traditional MB (MB: 5′-FAM-TCGGAGACCAACGTTGTCGGACCGTATCCGA-BHQ1-3′) with the exactly same detection sequence except the two end-stems of MB which bind each other. In the case of MB, The DFs for each mismatched substitution were also listed in Table 1. As we can see, mismatch detection of the same substituted analytes for MB is less efficient than that for DMB, which clearly shows that the degree of constraint determines the hybridization specificity.

For the single mismatched oligodeoxynucleotides, 20-m4, 20-m5, 20-m14 and 20-m17, the different types of mismatched deoxynucleotides in the analyte were used for fluorescence study in NaCl buffer. We used an adenosine to replace a cytidine for all the four analytes (20-m4A, 20-m5A, 20-m14A and 20-m17A) at the same position of the substitution. The DFs are 7, 6, 8, and >10 at 37 °C. The relative low DFs for A-C mismatch for 20-m4A, 20-m5A, 20-m14A comparing to C–C mismatch were due to more incompatibility of the C–C mismatch in the oligodeoxynucleotide duplex.[18] RNA oligonucleotide analytes were also tested with the DMB probe. The true RNA analyte, R20-m0 (UACGGUCCGAAACGUUGGUC), induced unfolding of the DMB probe and formed a stable duplex with the probe in tris buffer. However, with a single mismatch in the analytes (R20-m5: UACGCUCCGAAACGUUGGUC and R20-m17: UACGGUCCGAAACGUUCGUC), the binding between mismatched targets (R20-m5 and R20-m17) and DMB was significantly weaker with discrimination factors of 43 (R20-m5) and 26.8 (R20-m17) at 37 °C in the same buffer, which were similar to their DNA oligodeoxynucleotide analogues (see Supplementary Fig. S3).

In order to prove the ability of the DMB probe to distinguish the true analyte from single mismatched analyte oligodeoxynucleotides, the fluorescence of the DMB probe with different concentra-

Table 1
Discrimination factors (DFs) for oligodeoxyribonucleotides with a single mismatch of 20-m0

	Sequences	DMB 37 °C (100 mM Tris)	DMB 20 °C (100 mM Tris)	DMB 37 °C (100 mM NaCl + 10 mM Tris)	MB 37 °C (100 mM NaCl + 10 mM Tris)
20-m0	TACGGTCCGAAACGTTGGTC	1	1	1	1
20-m1	**C**ACGGTCCGAAACGTTGGTC	2.6 ± 0.3	1.9 ± 0.3	1.3 ± 0.01	1.0 ± 0.2
20-m2	T**C**CGGTCCGAAACGTTGGTC	2.1 ± 0.3	1.8 ± 0.3	1.5 ± 0.02	0.9 ± 0.2
20-m3	TA**A**GGTCCGAAACGTTGGTC	2.7 ± 0.3	1.3 ± 0.1	1.6 ± 0.02	0.9 ± 0.2
20-m4	TAC**C**GTCCGAAACGTTGGTC	27.7 ± 2.4	6.5 ± 0.7	28.2 ± 0.3	3.8 ± 0.5
20-m5	TACG**C**TCCGAAACGTTGGTC	47.3 ± 2.6	7.3 ± 0.5	19.7 ± 1.5	5.8 ± 0.1
20-m6	TACGG**C**CCGAAACGTTGGTC	4.2 ± 0.4	3.0 ± 0.3	2.7 ± 0.07	1.6 ± 0.06
20-m7	TACGGT**T**CGAAACGTTGGTC	3.8 ± 0.3	3.3 ± 0.4	2.3 ± 0.24	1.1 ± 0.2
20-m8	TACGGTC**G**GAAACGTTGGTC	5.8 ± 0.6	2.0 ± 0.3	2.7 ± 0.2	1.0 ± 0.03
20-m9	TACGGTCC**T**AAACGTTGGTC	3.7 ± 0.8	2.0 ± 0.1	1.7 ± 0.09	1.0 ± 0.1
20-m10	TACGGTCCG**T**AACGTTGGTC	1.9 ± 0.2	2.0 ± 0.1	1.1 ± 0.04	0.9 ± 0.2
20-m11	TACGGTCCGA**T**ACGTTGGTC	1.7 ± 0.4	1.8 ± 0.07	1.3 ± 0.02	0.9 ± 0.2
20-m12	TACGGTCCGAA**T**CGTTGGTC	4.1 ± 0.3	1.8 ± 0.08	1.9 ± 0.2	0.9 ± 0.2
20-m13	TACGGTCCGAAA**A**GTTGGTC	6.9 ± 0.7	1.6 ± 0.08	2.3 ± 0.09	0.9 ± 0.2
20-m14	TACGGTCCGAAAC**C**TTGGTC	60.2 ± 6.2	13.1 ± 1.7	32.3 ± 1.6	6.2 ± 0.09
20-m15	TACGGTCCGAAACG**C**TGGTC	7.7 ± 0.9	2.5 ± 0.2	3.5 ± 0.15	1.2 ± 0.03
20-m16	TACGGTCCGAAACGT**C**GGTC	28.7 ± 3.9	7.2 ± 0.6	21.1 ± 0.34	2.6 ± 0.1
20-m17	TACGGTCCGAAACGTT**C**GTC	32.6 ± 1.8	12.6 ± 0.7	>20	10 ± 0.06
20-m18	TACGGTCCGAAACGTTG**C**TC	28.4 ± 1.2	13.3 ± 0.3	20.9 ± 0.37	4.1 ± 0.3
20-m19	TACGGTCCGAAACGTTGG**C**C	4.9 ± 0.8	5.8 ± 0.9	2.1 ± 0.15	0.9 ± 0.1
20-m20	TACGGTCCGAAACGTTGGT**G**	3.6 ± 0.5	2.1 ± 0.15	1.3 ± 0.02	0.9 ± 0.2

DFs were calculated as the ratio of fluorescence intensity of the DMB at 518 nm in the presence of 20-m0 to the presence of each mismatched oligodeoxynucleotide after MB background fluorescence correction. The mismatches are bold and underlined. The date is the average of three independent measurements.

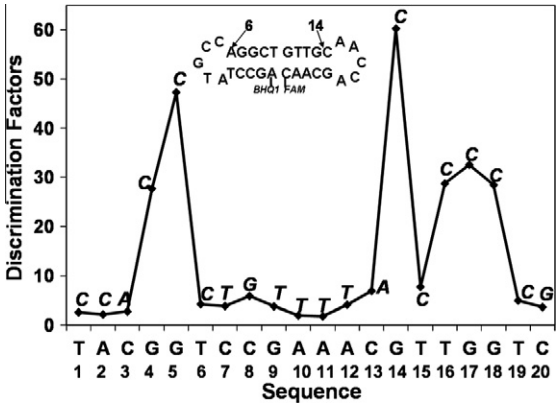

Figure 2. The relationship of discrimination factors (DFs) with substitution position and nucleobase of an analyte at 37 °C. The sequence of 5′-TACGGTCCGAAACGTTG GTC-3 is the true target and the number is the base position of the target analyte. The A, T, C, G on the curve is the substituted deoxynucleotide of each single mismatched oligodeoxynucleotide analyte.

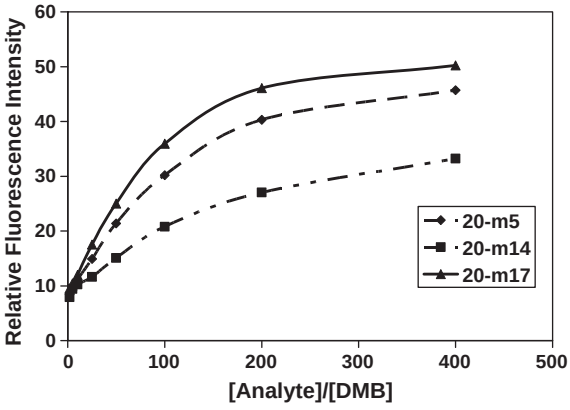

Figure 3. Concentration dependence of DMB fluorescence intensity on single mismatched oligodeoxynucleotide analytes 20-m5, 20-m14 and 20-m17; final concentration of DMB is 40 nM, and *X*-axis is the ratio of mismatched analytes to DMB.

tions of single mismatched analytes was recorded at 518 nm. When the concentration of the DMB probe was fixed at 40 nM in tris buffer (pH 8.0), the relative fluorescence intensity reached 125 with the addition of 80 nM true analyte. However, for the mismatched ones, even though the concentration of mismatched analytes reached 16 μM, the relative fluorescent intensity was still less than half of the true analyte, as shown in Figure 3. This suggests that single base mutants could be detected against high (99.5%) wild-type background.

In addition to the binding studies of the fluorescence probe with the analyte, hybridization kinetics was also examined. Fluorescence increase at 520 nm was recorded at 37 °C as a function of time with the addition of the analyte by exciting the samples at 490 nm. The on-rate constants that determine the hybridization kinetics for the DMB probe with the true analyte were obtained by instantaneously mixing 40 nM of the DMB probe with 200 nM of 20-m0. Second-order rate constants, k_h (M^{-1} s^{-1}), for the hybridization of the structured dual molecular beacon to the analyte, were then calculated by using the linearization procedures for a second-order reaction with non-stoichiometric proportions of reactants. By fitting the data, the constant, k_h (M^{-1} s^{-1}) for the addition of 200 nM true analyte (20–0 m) in 100 mM Tris buffer (pH 8.0) was 4839 M^{-1} s^{-1}. Changing the buffer to NaCl buffer containing 100 mM NaCl and 10 mM tris (pH 8.0), the constant, k_h (M^{-1} s^{-1}), for addition of 200 nM true analyte was 4863 M^{-1} s^{-1}, which showed the similar on-rate constant under our experimental conditions.(see Supplementary Fig. S5). Comparing to traditional MB, the hybridization for DMB is slower with the addition of its target. This is probably due to the high stability of DMB itself (T_m = 57 °C), and T_ms of DMB and DMB with 20-m0, 20-m5, and 20-m14 are 57, 59, 55, 54, while T_ms of MB and MB with 20-m0, 20-m5, 20-m14 are 49, 59, 49, 48 °C (Fig. S6). The high stability of DMB provides a kinetic barrier for the rearrangement required upon formation of the probe-target duplex. Further studies on bal-

ancing the specificity and kinetics of DMB design with the optimization of relative loop size and binding arms are on the way.

In summary, a dumbbell molecular beacon (DMB) was designed to provide a versatile strategy for probing a single mismatch in oligodeoxynucleotide analytes and demonstrate the specificity of oligodeoxynucleotide target recognition. In regard to the mismatch recognition, the nucleotide substitution in the loop domains and GC substitution, both had large effects on mismatch recognition. The results also showed that the analyte started to bind the nucleotides in the loop domain, which triggered the unzipping of the middle paired domain.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.047.

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