Fluorescence Probes

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Triplex Molecular Beacons as Modular Probes for DNA Detection**

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Detecting nucleic acid sequences is of central importance in modern life sciences. Given the rapid growth of the available sequence data, the diagnosis of DNA and RNA is likely to increase in significance over the next decade. The most useful methods employ probes that allow quantitative measurements in homogeneous solution, thereby omitting the need to separate bound from unbound probes.[1] Molecular beacons (MBs)[2] have become a class of widely used probes for nucleic acid analysis. These probes carry a fluorophore and a quencher at their termini. In the absence of a complementary target sequence, MBs form a stem–loop structure (Figure 1 A), which results in quenching of the fluorescence.

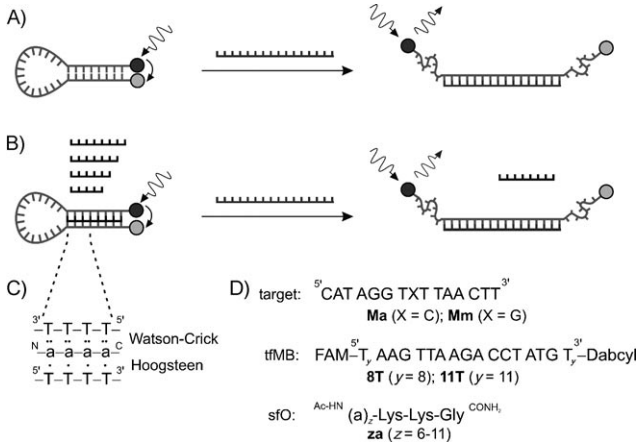

Figure 1. Comparison of a molecular beacon (MB; A) and a triplex molecular beacon (tMB, B) for the detection of complementary DNA oligonucleotides. C) Poly(a)·2poly(T) structure forming the triplex stem region. D) Sequences of DNA (target, tfMB) and PNA (sfO). Ac = acetyl, Dabcyl = 4-(4′-dimethylaminophenylazo)benzoic acid, FAM = 6-carboxyfluorescein.

Upon binding to the complementary target, the hairpin is opened, the labels are separated, and fluorescence occurs. MBs have been used in quantitative polymerase chain reactions (qPCR) to monitor the formation of the amplicon in a sequence-specific manner and in the imaging of pathogen RNA in living cells.[2]

It is a useful feature that conformationally constrained oligonucleotide probes, such as MBs, recognize their targets with higher specificity than linear probes.[3] This property is desired in the analysis of single-nucleotide alterations. The enhancement of specificity observed with structured probes implies that the selectivity of probe–target hybridization can be modulated by altering the degree of conformational constraint. For molecular beacons, this can be achieved by variation of the stem length and sequence. However, this type of modulation requires the synthesis of several different MBs. Among various conceptional modifications, replacement of the original DNA Watson–Crick base-paired stem by other pairing systems is rare, but examples include homo-DNA,[4a] LNA[4b] and G-quadruplex stems,[4c] as well as "stemless" peptide nucleic acid (PNA) molecular beacons.[4d–f] The cation-dependent G quadruplex stability was used to tune the conformational constraint of the corresponding beacon,[4c] but in applications such as real-time qPCR and measurements in vivo, the cation concentration is difficult to address.

Herein, we show that triplex-based molecular beacons (tMBs; Figure 1 B) enable a precise modulation of the conformational constraint by using a single DNA probe (triplex-forming molecular beacon, tfMB) labeled with a fluorophore and a quencher in combination with several stem-forming oligonucleotides (sfOs). In this setup, only one dual-labeled probe is needed and it is the sfO that allows tuning of the selectivity and sensitivity of the tMB towards target DNA sequences. Furthermore, the modular assembly[5] facilitates the additional incorporation of functionalities that can be easily introduced via the sfO. In an example of this concept, we prepared "superquenched"[6] tMBs by appending additional quencher moieties to the sfO.

The tMB is designed in a way that the stem region is formed by a poly(a)·2poly(T) (a is the PNA base) structure (Figure 1 C).[7] The sfO poly(a) segment is composed of peptide nucleic acid (PNA), a DNA analogue bearing an uncharged *N*-(2-aminoethyl)glycine backbone. PNA is capable of forming more-stable duplexes and triplexes with complementary DNA than corresponding DNA.[8] The sfO was designed to bind two poly(T) arm segments of the tfMB through Watson–Crick and Hoogsteen base pairing.

We decided to investigate two tfMB systems (**8T** and **11T**, Figure 1 D). Both tfMBs consist of a central DNA sequence that is complementary to the matched target sequence (**Ma**). This central sequence is flanked by poly(T) regions labeled with 6-FAM/Dabcyl as the fluorphor/quencher. The sfO lengths varied from six to 11 adenine residues. In initial UV denaturation experiments, we studied the stability of duplexes formed between tfMB and the target (tfMB·target) in the absence of sfOs as well as the stability of triplexes (tfMB·sfO)

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in the absence of the target. Table 1 shows the melting temperatures (T_m) of the nonlabeled tfMBs (**8T-nl** and **11T-nl**) complexed with targets or sfOs. We observed the expected lower stability ($\Delta T_m = -11\,°C$) of the single-base-mismatched duplexes tfMB·**Mm** compared with the perfectly matched duplexes tfMB·**Ma**. As expected, melting temperatures of tfMB·sfO triplexes increased with increasing sfO length. This should allow the precise modulation of the conformational constraint of the tMB.

Table 1: Melting temperatures (°C) of nonlabeled tfMBs in complex with a perfect complementary target (**Ma**), a single mismatched target (**Mm**), and with the different sfOs.[a]

	tfMB·Target		tfMB·**sfO**					
	Ma	**Mm**	**6a**	**7a**	**8a**	**9a**	**10a**	**11a**
8T-nl	47	36	38	44	49	–	–	–
11T-nl	48	37	44	48	53	57	59	60

[a] Conditions: 1 μM oligonucleotide; buffer solution: 10 mM Tris-HCl, 30 mM KCl, 2 mM $MgCl_2$, pH 9.

To compare the ability of each tfMB·sfO triplex beacon to discriminate between matched (**Ma**) and single-mismatched (**Mm**) targets, we performed denaturation experiments monitored by FAM fluorescence in the absence of the target and in the presence of the matched (**Ma**) and mismatched (**Mm**) targets. Denaturation curves were reversible and no hysteresis was observed (see the Supporting Information). To correct for small pipetting variations and the intrinsic temperature dependence of fluorescence, normalized match/mismatch selectivities were calculated (see the Supporting Information). Figure 2A shows the highest normalized match/mismatch selectivity determined for each tfMB·sfO complex. It became apparent that the selectivity improved with increasing triplex stability as higher selectivities were achieved with **11T** than with **8T**. Longer sfOs also led to higher selectivities. For **11T**, a plateau in terms of selectivity was reached at an sfO length of nine adenine bases. The maximal normalized selectivities of **11T** with **9a** (16.4), **10a** (13.4), and **11a** (17.2) were observed at temperatures between 42 °C and 43 °C. Furthermore, we performed real-time kinetic measurements at 43 °C with the most selective tMB, **11T·11a**. Figure 2B gives the time course of normalized FAM fluorescence before and after the addition of two equivalents of target DNA. Although almost no signal increase occurred in the presence of mismatched target (**Mm**, orange), we observed a significant increase in the presence of the matched target (**Ma**, red).

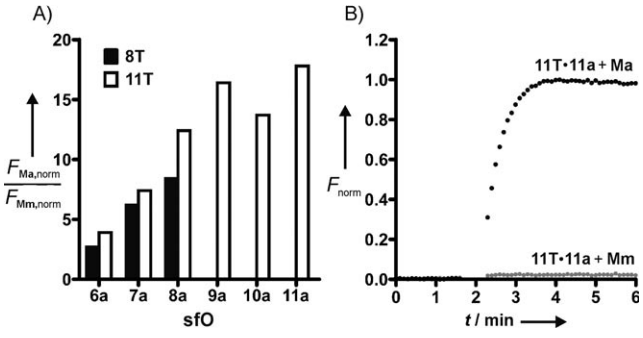

Figure 2. A) Maximal normalized match/mismatch selectivities of both tfMBs with different sfOs, as calculated from thermal denaturation experiments (see the Supporting Information). B) The kinetics were monitored at $\lambda_{ex} = 492$ nm and $\lambda_{em} = 525$ nm at 43 °C. **Ma** or **Mm** were added after 2 min. Conditions: 0.2 μM **11T**, 0.3 μM **11a** and 0.4 μM target (**Ma** or **Mm**); buffer solution: 10 mM Tris-HCl (Tris = tris(hydroxymethyl)aminomethane), 30 mM KCl, 2 mM $MgCl_2$, pH 9.

Our next aim was to investigate the sfO length dependence of the fluorescence increase upon addition of a matched target (**Ma**). From the denaturation experiments described above, we calculated the fluorescence increases at 20 °C (Figure 3A). With an increasing restoring force upon triplex elongation, going from **7a** to **11a**, we observed a lower tendency for opening of the tMB, which was indicated by a reduced fluorescence increase. A maximum in the fluorescence increase was reached with sfO **7a**, presumably owing to the incapability of the shorter sfO **6a** to close the tMB quantitatively. The long-stem triplex beacons (**11T**·sfO) gave a larger fluorescence increase than the shorter-stem beacons (**8T**·sfO). This is due to more-efficient fluorescence quenching in **11T**·sfO–tMBs. Figure 3B shows real-time measurements of the addition of three equivalents of **Ma** to **8T·7a** and **11T·7a**. Under these conditions, we achieved a sixfold and eightfold fluorescence increase, respectively. Notably, fluorescence signaling occurred even with the most stable tMB (**11T·11a**). This is surprising when considering the high stability of the triplex stem ($T_m = 60\,°C$, Table 1) and the comparably low stability of the **11T**·target complex ($T_m = 48\,°C$). We speculate that hybridization of the target firstly disrupts Hoogsteen interactions. The affinity of the long sfO **11a** for the Watson–Crick strand of **11T** may be sufficient to keep both target **Ma** and **11a** complexed to **11T**.[9] In contrast, the shorter sfO **7a** should have a higher tendency for dissociation.

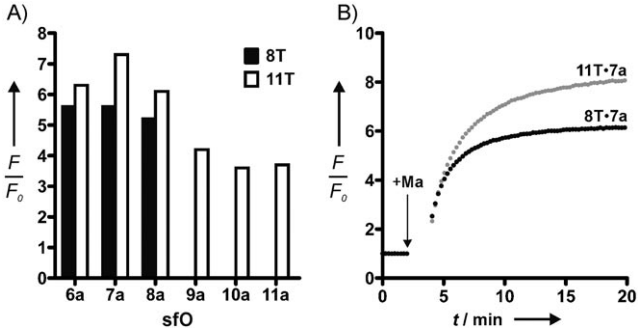

Figure 3. A) Thermal denaturation experiments were used to calculate increases in fluorescence upon binding of different tfMBs to the matched target (**Ma**) at 20 °C (see the Supporting Information). B) Kinetics monitored at $\lambda_{ex} = 492$ nm and $\lambda_{em} = 525$ nm at 20 °C. Conditions: 0.2 μM tfMB (**8T** or **11T**), 0.25 μM **7a** and 0.6 μM **Ma**; buffer solution: 20 mM NaH_2PO_4, 100 mM KCl, 2 mM $MgCl_2$, pH 9.0.

The modular assembly of triplex molecular beacons enables a very facile introduction of further functionalities. As an example, we constructed "superquenched"[6] tMBs by

attaching additional Dabcyl quencher moieties to sfO **7a** (**7a*** and **7a****, Figure 4A). The aim was to increase the fluorescence enhancement upon target addition by reducing the fluorescence of the closed tMB. Indeed, the use of the Dabcyl-

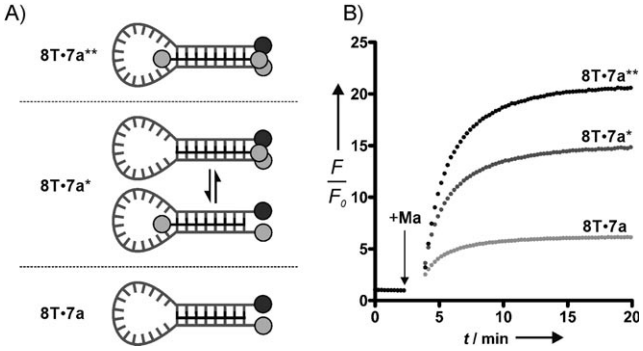

Figure 4. A) Concept of "superquenched" triplex molecular beacons (sequences: **7a**** $^{Ac\text{-}HN}$Lys(Dabcyl)-a_7-Lys(Dabcyl)-Lys_2-Gly^{CONH_2}; **7a*** $^{Ac\text{-}HN}$Lys(Dabcyl)-a_7-Lys_2-$Gly_2^{CONH_2}$). B) Kinetics monitored at λ_{ex} = 492 nm and λ_{em} = 525 nm at 20°C. Conditions: 0.2 μM **11T**, 0.25 μM sfO (**7a**, **7a***, or **7a****) and 0.6 μM **Ma**; buffer solution: 10 mM Tris-HCl, 30 mM KCl, 2 mM $MgCl_2$, pH 9.

labeled sfOs **7a*** and **7a**** resulted in dramatic increases in the quenching efficiency from 77.6% (**8T·7a**) to 98.3% (**8T·7a****) and from 86.0% (**11T·7a**) to 96.9% (**11T·7a****). Real-time kinetic measurements of the **8T** beacon system with Dabcyl-labeled sfOs (Figure 4B) revealed significantly enlarged fluorescence increases upon hybridization to **Ma** (**7a**: 6.2-fold; **7a***: 14.9-fold; **7a****: 20.8-fold).

In summary, triplex molecular beacons are able to report the presence of DNA targets in homogeneous solution by enhancements of fluorescence signals. The addition of a target induced fluorescence enhancements up to eightfold when using nonlabeled sfOs and up to 21-fold enhancements when using Dabcyl-labeled sfOs. The use of improved fluorophore/quencher pairs should lead to even higher signal increases.[10] The obtained specificity of fluorescence signaling as far as match/mismatch discrimination is concerned, compares well with the specificity achieved with conventional duplex molecular beacons. The central hallmark of tMBs is the feasibility to adjust the specificity and the sensitivity of a given dual-labeled oligonucleotide by choosing the appropriate stem-forming oligonucleotide. This property may be of advantage in optimization studies, for example in real-time qPCR. In contrast, optimization of alternative DNA detection probes usually requires resynthesis.[11] The modular assembly of two oligonucleotides (tfMB and sfO) allows for a very facile introduction of further functionalities without increasing the complexity (and cost) of the beacon synthesis. We demonstrated the attachment of fluorescence quenchers to the sfO, which resulted in increases of fluorescence signaling. Other conjugation opportunities, such as additional fluorphores, handles for immobilization, etc., can be envisioned. The extension with chemically or biologically active moieties that trigger a signal upon release of the sfO may add to the versatility of tMBs as a new class of easily tunable DNA detection probes.

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