

STRUCTURE-FUNCTION RELATIONSHIPS OF MOLECULAR BEACONS

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INTRODUCTION

Molecular beacons are dual-labeled oligonucleotide probes that fluoresce upon hybridization with a complementary target sequence [1]. As shown in Figure 1, the oligonucleotide is labeled at one end with a fluorescent reporter dye (D) and at the opposite end with a fluorescence quencher (Q). Molecular beacons are designed to form a stem-loop hairpin structure in the absence of target, forcing the fluorescence reporter group in proximity with the quencher group. In this conformation, fluorescence is quenched. In the presence of a complementary target molecule, the molecular beacon opens due to the formation of the more stable probe-target duplex, increasing distance between the reporter and quencher, and restoring fluorescence. The competing reaction between hairpin formation and target hybridization improves specificity of molecular beacons compared with linear probes, while the transition between quenched and fluorescent states allows for the differentiation between bound and unbound probes [2]. Molecular beacons are capable of having >200-fold increase in fluorescence intensity upon hybridization [1]. The clear advantages of molecular beacons over linear oligonucleotide probes have led to their use in numerous applications [3], ranging from quantitative PCR [4], to the study of protein-DNA interactions [5], to visualizing RNA expression in living cells [6].

To fully realize the potential of molecular beacons, it is necessary to optimize their structure. Here we report a systematic study of the thermodynamic and kinetic parameters that describe the molecular beacon structure-function relationship [7-8]. Our results show that both probe and stem lengths have a significant impact on the binding specificity and hybridization kinetic rates of molecular beacons; these findings have significant implications on the design of molecular beacons for various applications.

MATERIALS AND METHODS

Design Parameters of Molecular Beacons

To quantify the effect of molecular beacon structure (probe and stem length) on probe-target hybridization thermodynamics and

kinetics, a series of molecular beacons were designed and synthesized which are in antisense orientation with respect to exon 6 of the human GAPDH gene. Conventional molecular beacons are designed such that the target-specific probe domain is located between short self-complementary stems that are independent of the target-specific domain. In this study, however, we consider molecular beacons with one stem complementary to the target sequence (shared-stem molecular beacons) [7]. The molecular beacons have stem lengths of 4, 5, and 6 bases and probe lengths of 17, 18, and 19 bases (Fig. 1), are labeled with a Cy3 fluorophore at the 5' end and a dabcyI quencher at the 3' end. Five target oligonucleotides were synthesized, one wild-type and four with mismatches at assorted locations [7].

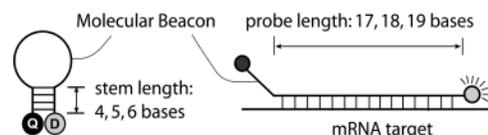


Figure 1. The design and structural parameters of molecular beacons considered in the study

Hybridization Assays

Thermal denaturation profiles and bind kinetic rates were obtained for molecular beacons alone and for beacon-target duplexes. To obtain the thermal profile for each molecular beacon hairpin and molecular beacon-target duplex, 200 nM of MB was mixed with 0 to 20 μ M of target in a buffer containing 10 mM KCl, 5 mM MgCl₂, and 10 mM Tris-HCl and excited with a 488 nm laser using a spectrofluorometric thermal cycler (Applied Biosystems). The rate constants that determine the hybridization kinetics for each molecular beacon-target pair were obtained by instantaneously mixing 250 nM of molecular beacons with 2.5 μ M of each target using a stopped-flow accessory (Hi-Tech SFA-20) with a SFA-12 temp/trigger module. Fluorescence as a function of time was obtained by exciting the sample at 545 nm and recording the emission at 570 nm using a

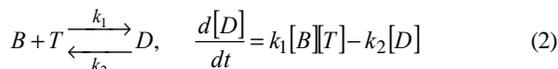
spectrofluorometer (SPEX fluorolog-2). All assays were performed in the buffer described above at 37°C and repeated five times.

Equilibrium and Kinetic Analysis

The dissociation constant K_{12} describing the equilibrium state between unbound molecular beacons in the stem-loop conformation and probe-target duplexes was calculated for molecular beacons with various stem-loop structures. All calculations assumed a temperature of 37°C and utilized the thermodynamic parameters, enthalpy change ΔH_{12} and entropy change ΔS_{12} , obtained from the thermal profiles for each probe-target duplex:

$$R \ln K_{12} = -\Delta H_{12} \frac{1}{\theta} + \Delta S_{12} \quad (1)$$

where θ is temperature in Kelvin and R is the gas constant. To analyze the hybridization kinetics a second-order reaction was assumed:



where $[B]$, $[T]$ and $[D]$ are the concentrations of MB, target, and MB-target duplex, respectively, k_1 is the on-rate and k_2 the off-rate constants of molecular beacon-target hybridization. An exact solution of equation (2) was obtained and used to fit the experimental data in determining k_1 and k_2 ($= K_{12}k_1$).

RESULTS

Melting Behavior

To elucidate the effect of molecular beacon structure on the stability of the probe-target duplex, the melting temperatures θ_m for conventional and shared-stem molecular beacons with a probe length of 19 bases and stem lengths ranging from 4 to 7 bases were compared, as shown in Figure 2. It was found that conventional molecular beacons had lower melting temperatures than shared-stem molecular beacons for each of the stem lengths considered; however, both types of molecular beacons exhibited similar trends. Specifically, the melting temperature progressively decreased as the stem length increased. In fact, it appears that the melting temperature would be quite low for conventional molecular beacons with a probe length of 19 bases and a stem length of 7 bases or greater. This may be because that with long free arms of the stem a bound molecular beacon is very easy to dissociate from the target and form a stable hairpin structure even at low temperatures

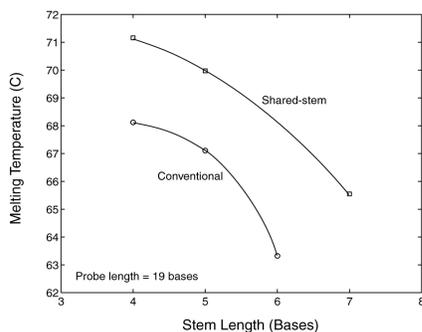


Figure 2. Comparison of melting temperatures as a function of stem length for conventional and shared-stem molecular beacons

Hybridization Kinetics

While both the stability of the hairpin and the specificity of the target-probe hybridization increase with increasing stem length, it is accompanied by a decrease in hybridization on-rate. For example, molecular beacons with a 4-base stem had an on-rate up to 100 times

greater than molecular beacons with a 6-base stem. Dual-labeled linear probes (stem = 0) hybridized on slightly faster (~2 times) than molecular beacons with a 4-base stem. Hybridization kinetics was studied at 37°C, which is close to the melting temperature for the 4 base hairpin employed in these probes. It is possible that greater differences in hybridization rates between linear and 4-base stem probes would be seen if the reaction kinetic rates were studied at a lower temperature.

CONCLUSIONS

This study has demonstrated clearly that in designing molecular beacons for a specific application, both stem and probe lengths must be carefully chosen. For example, when high probe specificity is required, as in the case of detecting point mutations or polymorphisms, molecular beacons will offer improved discrimination when relatively more stable (longer) stems are matched with shorter probe domains. Conversely, when studying RNA expression in living cells in real-time, it may be more important to have fast hybridization kinetics. In this case, molecular beacons with less stable (shorter) stems and longer probe domains would be preferred. Mismatch discrimination is further improved if the mutation is positioned centrally within the probe domain. Finally, since molecular beacons with a 4 base stem length may have high background fluorescence, the use of longer stems would be preferred in most applications. In summary, the quantitative studies of structure-function relationship of molecular beacons can provide guidance to the design of molecular beacons to achieve an optimal balance among specificity, signal to background ratio and kinetic rates desirable for a specific application.

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REFERENCES

1. Tyagi, S. and Kramer, F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat. Biotechnol.*,14, 303-308.
2. Tyagi, S., Bratu, D.P. and Kramer, F.R. (1998) Multicolor molecular beacons for allele discrimination. *Nat. Biotechnol.*, 16, 49-53.
3. Tsourkas, A. and Bao, G. (2002) Shedding light on health and disease using molecular beacons, *Briefings in Functional Genomics & Proteomics*, in press, Volume 1, Issue No. 4.
4. Vogelstein, B. and Kinzler, K.W. (1999) Digital PCR. *Proc. Natl. Acad. Sci. USA*, 96, 9236-9241.
5. Li, J.J., Geyer, R. and Tan, W. (2000) Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA. *Nucleic Acids Res.*, 28, E52.
6. Sokol, D.L., Zhang, X., Lu, P. and Gewirtz, A.M. (1998) Real time detection of DNA:RNA hybridization in living cells. *Proc. Natl. Acad. Sci. USA*, 95, 11538-11543.
7. Tsourkas, A., Behlke, M.A. and Bao, G. (2002) Structure-function relationship of shared-stem and conventional molecular beacons, *Nucleic Acids Res.*, 30:4208-4215.
8. Tsourkas, A., Behlke, M.A. and Bao, G. (2002) Hybridization of 2'-O-methyl and 2'-deoxy molecular beacons to RNA and DNA targets, *Nucleic Acids Res.*, 5168-5174.