Cooperative Probes for Biological and Biomedical Detection: eliminating false positives

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Statement of Purpose: The majority of efforts to increase specificity or sensitivity in biosensors result in tradeoffs with little to no gain in overall accuracy. Here we develop a new class of cooperative probes that gain increases in specificity without trading off sensitivity. Tentacle Probes (TPs) have a hairpin structure similar to molecular beacons (MBs), but are modified by the addition of a capture probe. Addition of this capture region provides cooperativity in binding target DNA sequences which increases the probability of generating fluorescence from MB regions of the cooperative probes. TPs exhibit no positive signals (fluorescence is below background) at all concentrations of target with single nucleotide polymorphisms (SNPs); however, TPs exhibit similar sensitivity as MBs for wild type (WT) target sequences. Therefore, TPs can be used in detection systems for which yes/no signals are required.

Methods: MBs, TPs and target sequences are synthesized and purified through dual HPLC by Biosearch Technologies (Novato, CA). TPs are created by attaching a capture region to the hairpin via a poly(ethylene glycol) spacer (9mer). Target sequences are synthesized representing the wild type target (WT) and a SNP in the beacon detection region (SNP). Cal-fluor 560 fluorescent dye (CF560) and Black Hole Quencher – 1 (BHQ1) are the fluorophore and quencher respectively.

A Stratagene Mx4000 plate reader is used to read the fluorescence of 1 μM 9-base stem TP and 1 μM 5-base stem MB in WT and SNP targets at concentrations of 0, 2, 10, 20, 100, 1000, and 10000 nM. Concentrations of 100 μM and 1 mM SNP are used where detection limits cannot be established at the lower concentrations. Fluorescence is read upon reaching equilibrium.

Table 1. Sequences for MB5, TP9, and targets

MB5	$(PEG)_9$-CF560-CTGGCGGAAAAGCTAATATAGTAAGCCAGA-BHQ1
TP9	GATTAAAATGTCCAGTGTACCAG-$(PEG)_9$-CF560-CCCTGGCGGAAAA GCTAATATAGTAACCGCCAGGGA-BHQ1
WT	ATTATTACTTTACTATATTA**G**CTTTTCCGCCATCTAAAATTCTATTTT CTGGTACACTGGACATTTTAATCAATGTATTC
SNP	ATTATTACTTTACTATATTA**T**CTTTTCCGCCATCTAAAATTCTATTTT CTGGTACACTGGACATTTTAATCAATGTATTC

Results/Discussion: Melting curves (Fig 1) are used to extract thermodynamic equilibrium constants for binding in the detection region (K_{det}), cooperative capture region (K_{cap}), and both regions (K_{eff}). Predicted binding curves are generated using best fit thermodynamic parameters for MBs (Eqn 1) and TPs (Eqn 2) respectively:

$$\frac{C_{\det}}{P_0} = \frac{\left(P_0 + T_0 + \frac{1}{K_{\det}}\right) - \sqrt{\left(P_0 + T_0 + \frac{1}{K_{\det}}\right)^2 - 4P_0T_0}}{2P_0} \quad (1)$$

$$\frac{C_{\det} + C_{both}}{P_0} = \frac{\left(P_0 + T_0 + \frac{1}{K_{eff}}\right) - \sqrt{\left(P_0 + T_0 + \frac{1}{K_{eff}}\right)^2 - 4P_0T_0}}{2P_0} \frac{K_{\det} + P_L F_{pen} K_{\det} K_{cap}}{K_{eff}} \quad (2)$$

where P_0 and T_0 are the initial concentrations of probe and target, C_{det} and C_{both} are concentrations of probes bound in the detection region or both regions, and P_L and F_{pen} are multipliers to account for cooperativity.

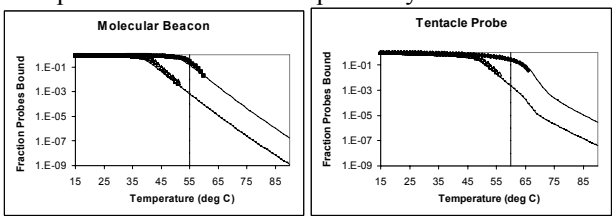

Figure 1. Log plot of fraction of probes bound to WT (closed square) and SNP (open triangle) targets in 1 μM probes as a function of temperature. Vertical line represents optimal temperature for WT:SNP detection.

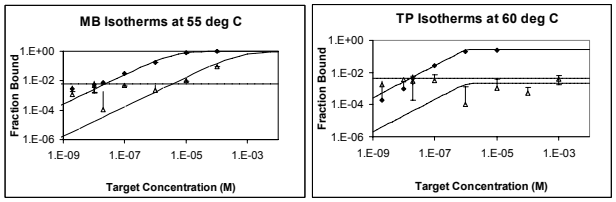

Figure 2. WT (closed diamond) and SNP (open triangle) binding as a function of target concentration. Theoretical predictions based on Eqns 1 (for MBs) and 2 (for TPs) are plotted with experiments [n=3]. Horizontal line represents detection limit above which results in a positive signal.

The fraction of probes bound as a function of target concentration is determined at optimum SNP resolution temperatures for TPs (60°C) and MBs (55°C). Theoretical predictions are graphed with experiments in Figure 2 for WT (solid line) and SNP (dashed line) targets. TP isotherms reveal a WT detection limit of 15.4 nM and no SNP detection at concentrations tested up to 1 mM. In contrast, MBs showed a positive signal for SNPs at concentrations above 3.88 μM (154 times greater than the detection limit for the WT target, 22.7 nM). The ratio of specific to nonspecific detection for TPs exceeds 53,200 and is predicted to be infinite.

Conclusions: Model predictions for cooperative probes indicate that binding to mutant targets will never cause a signal above background; thus, no false positives will result even at very high concentration. Figure 2 also shows that this enhanced specificity is achieved without sacrificing sensitivity. Therefore, cooperative probes present a general strategy for eliminating false positive detection while maintaining sensitivity.

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