Tentacle probes: eliminating false positives without sacrificing sensitivity

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ABSTRACT

The majority of efforts to increase specificity or sensitivity in biosensors result in trade-offs with little to no gain in overall accuracy. This is because a biosensor cannot be more accurate than the affinity interaction it is based on. Accordingly, we have developed a new class of reagents based on mathematical principles of cooperativity to enhance the accuracy of the affinity interaction. Tentacle probes (TPs) have a hairpin structure similar to molecular beacons (MBs) for enhanced specificity, but are modified by the addition of a capture probe for increased kinetics and affinity. They produce kinetic rate constants up to 200-fold faster than MB with corresponding stem strengths. Concentration-independent specificity was observed with no false positives at up to 1 mM concentrations of variant analyte. In contrast, MBs were concentration dependent and experienced false positives above 3.88 μM of variant analyte. The fast kinetics of this label-free reagent may prove important for extraction efficiency, hence sensitivity and detection time, in microfluidic assays. The concentration-independent specificity of TPs may prove extremely useful in assays where starting concentrations and purities are unknown as would be the case in bioterror or clinical point of care diagnostics.

INTRODUCTION

Field-deployable biosensors require more rapid and sensitive, single-step identification methods. However, efforts to enhance assay rapidity, sensitivity and simplicity can result in an increase in false positives or in false negatives (1). Such false positives and negatives can have immense impact in biosensing for medical and biowarfare applications as even rare occurrences can have disastrous consequences. A paradigm shift in understanding and designing for the specificity–sensitivity trade-off is absolutely essential to developing field-deployable biosensors experiencing few to no false positives and negatives.

Molecular beacons (MBs) are one probe methodology used to move towards more rapid, single-step genomic sensors (2,3). A fluorescent label is attached to one end of a polynucleotide and a quencher is attached to the other. Complementary base-pairs near the label and quencher cause a hairpin-like structure, placing the fluorophore and quencher in proximity. This hairpin opens in the presence of the target producing an increase in fluorescence. The proximity of the quencher to the fluorophore can result in reductions of fluorescent intensity of up to 98% (4). The perceived efficiency can further be adjusted by altering the stem strength (length of the stem) which affects the number of beacons in the open state in the absence of the target. Accordingly, one trade-off a MB experiences is with regard to its stem strength, low strength limits fluorescent increase upon hybridization whereas high strength limits kinetics of hybridization (5).

Regardless of the detection platform or strategy, the majority of biosensors incorporate molecular recognition through a biological affinity interaction. A biosensor cannot be more accurate than this interaction (6). This interaction is used for one or more functions that include identifying the presence of a given analyte, determining changes in expression level and quantifying the agent (7). Specificity and sensitivity in biosensor research often refer to the ability of the sensor to eliminate false positives and negatives respectively for one or more of the foregoing objectives. Unfortunately, there is usually a trade-off between specificity and sensitivity (7,8) as shown in Figure 1.

In support of the limitations in improving biosensor accuracy, the numbers tell a compelling story. By way of identification of the presence of specific species, Peplies et al. tested six strains of bacteria with a 1% rate of false positives and 41% rate of false negatives (9). Diagnostic PCR, although rarely having false-negatives owing to its extreme sensitivity, experiences a reported rate of false-positives between 9 and 57% (10).

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Detectors monitoring expression level are worse having a 10–30% false negative rate for samples above the sensitivity threshold and a false positive rate of 10% (11). While this rate of false positives and negatives may be damaging for phenotypic or other biological exploration, even one error can prove lethal in clinical diagnostics and could result in loss of life (false negative) or economic harm (false positive) in homeland security applications.

In order to overcome the deficiency with these reagents and assays, we have developed a new class of reagent that performs with heightened affinity and with greater selectivity compared to single oligomer reagents. We adapted the principles of cooperativity abundantly described in cell targeting (12–16) to combat the specificity–sensitivity trade-off. Cooperativity has already been shown to enhance SNP detection and assay sensitivity (17,18). Here, we combine principles of cooperativity with a label-free hairpin in what we term tentacle probes (TPs). We discuss the effects of cooperativity on sensitivity, specificity and kinetics, demonstrating an increase in each without sacrificing the others. These faster, more sensitive and more specific probes may offer a number of advantages in many of the diagnostic applications where MBs have already been applied.

In this study, we derive a mathematical model predicting the cooperative behavior of TPs. We test theoretical descriptions of increased kinetics and specificity over MBs and demonstrate these results without a loss in sensitivity. By doing so, we introduce the first class of reagents to our knowledge to be originated as a mathematical solution to problems associated with molecular recognition in biosensors.

**MATERIALS AND METHODS**

**Oligonucleotide synthesis**

MBs, TPs and target sequences were synthesized and purified through dual HPLC by Biosearch Technologies (Novato, CA). MBs were made with stem lengths from five to nine bases. Analogous TPs were created by attaching a capture region to the hairpin via a polyethylene glycol (PEG) sequence (9 mer). This required modifying the 5’0 dye in the MB to be an internal dye attached to an inert base. In order to demonstrate that the PEG linker and internally modified dye were not the source of difference between TPs and MBs, the MBs were modified at their 5’0 end to include a PEG linker attached to an internally modified dye identical to that used in the cooperative probes.

Four target sequences were synthesized representing the wild-type target (WT), a single nucleotide polymorphism (SNP) in the capture region of the TP (SNPcap), a SNP in the detection region (SNP det) and a SNP in both regions (SNP both). All probes and target sequences were suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0) with 0.18 M NaCl and 0.1% SDS and are summarized in Table 1.

**Kinetics**

Kinetics were measured on a Victor2 plate reader (Perkin Elmer) at room temperature. Due to the rapidity of the TP reactions, low concentrations were used and samples had to be run individually. For the TPs, 20 μl of each probe (100 nM) was inserted into the plate. Here, 20 μl of an excess of target (1 μM) was quickly (<5s) added to each well and the measurements were started. Ninety-nine
Table 1. The abbreviated terms for each probe and target sequence

| Name         | Sequence                                                                 |
|--------------|--------------------------------------------------------------------------|
| **Tentacle Probes** |                                                                  |
| Tp 5         | GAT TAA AAT GTC CAG ACC AG - PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1 |
| Tp 6         | GAT TAA AAT GTC CAG TGT ACC AG - PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1 |
| Tp 7         | GAT TAA AAT GTC CAG TGT ACC AG - PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1 |
| Tp 8         | GAT TAA AAT GTC CAG TGT ACC AG - PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1 |
| Tp 9         | GAT TAA AAT GTC CAG TGT ACC AG - PEG 9mer - CF560 - c c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1 |
| **Molecular Beacons** |                                                                  |
| MB 5         | PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1          |
| MB 6         | PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1          |
| MB 7         | PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1          |
| MB 8         | PEG 9mer - CF560 - c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1          |
| MB 9         | PEG 9mer - CF560 - c c TGG CGG AAA AGC TAA TAT AGT AA cgcaga - BHQ1        |
| **Targets**  |                                                                  |
| WT           | ATT ATT ACT TTA CTA TAT TAG CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GAT CAC TGG ACA TTT TAA TCA ATG TAT TC |
| SNP<sub>cap</sub> | ATT ATT ACT TTA CTA TAT TAG CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GAT CAC TGG ACA TTT TAA TCA ATG TAT TC |
| SNP<sub>det</sub> | ATT ATT ACT TTA CTA TAT TAG CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GAT CAC TGG ACA TTT TAA TCA ATG TAT TC |
| SNP<sub>both</sub> | ATT ATT ACT TTA CTA TAT TAG CTT TTC CGC CAT CTA AAA TTC TAT TTT CTG GAT CAC TGG ACA TTT TAA TCA ATG TAT TC |

The numbers in the names represent the number of bases in the stem. TP, MB, CF560, BHQ1, WT, SNP<sub>cap</sub>, SNP<sub>det</sub>, SNP<sub>both</sub> stand for tentacle probes, molecular beacons, Cal-fluor 560 fluorescent dye, Black Hole Quencher-1, wild type, SNP in the capture region SNP in the detection region and SNP in both regions respectively. Lower case bases in the probes represent bases added to help form the stem. Italics in the target represent complementary regions to the probes and bold letters are SNPs.

Measurements were taken over 10 min. The experiment was repeated three times. MB kinetics were measured identically except 10 μM target was used and fluorescence was monitored over 1 h.

Fluorescent data was plotted against time and the rate constants were fit using the kinetic equation for polynucleotide reactions in an excess of target by minimizing the sum of square errors. The rate constants from each of three trials were averaged and plotted against stem strength with 95% confidence intervals.

\[ F = F_{\text{max}} (1 - e^{-k_f T/t}) \]  

where \( F \) is fluorescence, \( F_{\text{max}} \) is the maximum fluorescence achieved at equilibrium, \( k_f \) is the effective forward rate constant, \( T \) is the target concentration and \( t \) is time.

**Selecting optimal probes**

Melting curves of each TP and MB were generated for each target type on a Stratagene Mx4000 plate reader. Here, 20 μL of solutions with final probe concentration of 50 nM and target concentrations of 50 nM, 500 nM, 5 μM and 50 μM were prepared. Fluorescence was monitored from 90 to 15°C at the end of a 15-min incubation period following each 1°C increment. The experiment was also repeated from 15 to 90°C. All melting curve results presented came from this set of experiments.

The fluorescence ratio of specific (WT) to nonspecific (SNP<sub>det</sub>) targets was calculated for the 50-nM target concentration and plotted versus temperature. The TP and MB with the largest peaks were selected as the optimum probes after verifying first that their kinetic rate constants were each above 1000 M<sup>-1</sup> s<sup>-1</sup>.

**Fitting melting curves**

Melting curves were fit to an adaptation of models used by others in describing MB thermodynamics (19,20). These models were enhanced by the addition of a cooperative stem and the possibility of more configurations (Figure 2). Cooperative models are derived from collision theory, where the rate of the first binding event is proportional to the product of the reagent concentrations:

\[ (p_r \cdot v \cdot \pi \cdot R^2) \cdot P \cdot T = k_f \cdot P \cdot T \]  

where \( p_r \) is the probability of reaction, \( v \) is the average velocity, \( R \) is the sum of the radii of the two molecules, \( P \) is the probe concentration, \( T \) is the target concentration and \( k_f \) is the association rate constant. The second binding event follows the same model, but with an enhanced local probe concentration, \( P_L = 1 \) molecule/(volume swept out by linker length × Avogadro’s number), reacting with the newly formed complex, \( C \), and with an adjusted cooperative reaction probability, \( p_r, \text{coop} \), reflecting enthalpic and entropic penalties:

\[ (p_r, \text{coop} \cdot v \cdot \pi \cdot R^2) \cdot P_L \cdot C = k_f, \text{coop} \cdot P_L \cdot C \]
Assuming no cross-reactions, the following equilibrium constants for TPs can be derived:

\[
K_{\text{det}} = \frac{C_{\text{det}}}{PT} \tag{4}
\]

\[
K_{\text{cap}} = \frac{C_{\text{cap}}}{PT} \tag{5}
\]

\[
P_L F_{\text{pen}} K_{\text{det}} K_{\text{cap}} = \frac{C_{\text{both}}}{PT} \tag{6}
\]

\[
K_{\text{eff}} = K_{\text{det}} + K_{\text{cap}} + P_L F_{\text{pen}} K_{\text{det}} K_{\text{cap}} = \frac{C_{\text{both}}}{PT} \tag{7}
\]

where \( K \) is the equilibrium constant, with added subscripts, cap, det, both and eff, referring to capture probe, detection probe, both capture and detection probes, and effective, respectively, \( P = P_0 - C_{\text{cap}} - C_{\text{det}} - C_{\text{both}} \), \( T = T_0 - C_{\text{cap}} - C_{\text{det}} - C_{\text{both}} \), where \( P_0 \) and \( T_0 \) are initial probe and target concentrations respectively, \( F_{\text{pen}} \) refers to the collective enthalpic and entropic penalties (\( F_{\text{pen}} = e^{(-\Delta H_{\text{pen}}/T+\Delta \Phi_{\text{pen}})/RT} \)), and all other variables are as previously defined.

Fluorescence as a function of temperature can be adapted from models for MBs (19,20) by:

\[
F = \alpha \frac{(C_{\text{both}} + C_{\text{both}})}{P_0} + \beta \frac{(C_{\text{cap,cl}} + P_{\text{cl}})}{P_0} + \gamma \frac{(C_{\text{cap,op}} + P_{\text{op}})}{P_0} \tag{8}
\]

where \( F \) is fluorescence as a function of temperature. Alpha, beta and gamma refer to characteristic fluorescence of bound probes, closed probes (subscript cl) and random coil probes (subscript op) respectively.

For \( T_0 \gg P_0 \), which eliminates calculation of quadratics:

\[
F = \frac{1}{1 + K_{\text{stem}}} \tag{9}
\]

\[
K_{\text{stem}} \text{ is fit first by measuring fluorescence as a function of temperature for beacons with no target and minimizing sum of square errors as performed by Bonnet et al. and Tsourkas et al. (19,20)}:
\]

\[
F = \beta \frac{1}{1 + K_{\text{stem}}} + \gamma \frac{K_{\text{stem}}}{1 + K_{\text{stem}}} \tag{10}
\]

Next \( K_{\text{det}} \) is fit on probes with no capture probe (e.g. on an MB):

\[
F = \alpha \left( \frac{T_0 K_{\text{det}}}{1 + T_0 K_{\text{det}}} \right) + \beta \left( 1 - \left( \frac{T_0 K_{\text{det}}}{1 + T_0 K_{\text{det}}} \right) \right) \frac{1}{1 + K_{\text{stem}}} \tag{11}
\]

The thermodynamic parameters necessary for calculating \( K_{\text{cap}} \) were estimated with Mfold (21) for 0.18 M NaCl (about the same sodium concentration as 1 x SSC). Using the Mfold estimates provided, theoretical curves that diverged at ~61°C for differing concentrations of target with TP 5, while adjusting the predicted entropy from ~0.4989 to ~0.494 kcal mol\(^{-1}\) K\(^{-1}\) moved the divergence to ~65°C forming a more accurate visual fit to the data. Therefore the latter value was used for all remaining tests. Finally, \( F_{\text{pen}} \) was fit to the fluorescent curves of three different dilutions of target mixed with TPs using the original equation. All parameters necessary for calculations are summarized in Table 2.

The best fit enthalpies and entropies were used to calculate the equilibrium constants which in turn were used to calculate the amount of analyte bound to the detection probe and producing fluorescence as a function of temperature in an excess of target for MBs for specific (subscript s) and nonspecific (subscript ns) analyte:

\[
\frac{C_s}{P_0} = \frac{K_{\text{det,s}} \cdot T_0}{1 + K_{\text{det,s}} \cdot T_0} \tag{12}
\]

\[
\frac{C_{\text{ns}}}{P_0} = \frac{K_{\text{det,ns}} \cdot T_0}{1 + K_{\text{det,ns}} \cdot T_0} \tag{13}
\]
Table 2. Parameters used in theoretical calculations and source of values

| Fitted parameters | Known parameters |
|------------------|------------------|
| \( \alpha \)      | \( p_0 \)        |
| \( \beta \)       | \( T_0 \)        |
| \( \gamma \)      |                  |
| \( \Delta H_{\text{stem}} \) | Estimated parameters |
| \( \Delta S_{\text{stem}} \) | \( p_L \)     |
| \( \Delta H_{\text{det}} \) | \( \Delta H_{\text{cap}} \) Estimated by Mfold |
| \( \Delta S_{\text{det}} \) | \( \Delta S_{\text{cap}} \) Estimated by Mfold |
| \( \Delta H_{\text{pen}} \) |                  |
| \( \Delta S_{\text{pen}} \) |                  |

And for TP:

\[
C_s = \frac{(K_{\text{det}, s} + P_L F_{\text{pen}, s} K_{\text{det}, s} K_{\text{cap}, s}) \cdot T_0}{1 + K_{\text{eff}, s} \cdot T_0}
\]  

(14)

\[
C_{\text{ns}} = \frac{(K_{\text{det, ns}} + P_L F_{\text{pen, ns}} K_{\text{det, ns}} K_{\text{cap, ns}}) \cdot T_0}{1 + K_{\text{eff, ns}} \cdot T_0}
\]  

(15)

These equations were then matched with normalized fluorescent data in order to verify accuracy of thermodynamic parameters in predictions at the lowest detectable binding levels and to identify trends in binding below the level of detection. Thermodynamic values provided from best fits often fit high-level binding tightly at the expense of fitting low-level binding data due to inequalities arising from the sum of square errors. A manual adjustment to the best fit (e.g. \( \pm 0.6 \) kcal mol\(^{-1}\) for enthalpy and \( \pm 0.0016 \) kcal mol\(^{-1}\) K\(^{-1}\) for entropy) provided a fit that more thoroughly represented both high and low binding data. Once the parameters provided a perfect visual fit to low-level binding as well as high-level binding, they were kept constant in order to make predictions.

### Specificity and sensitivity

The Stratagene Mx4000 plate reader was used to read the fluorescence of 1-\( \mu \)M nine-base stem TP and 1-\( \mu \)M five-base stem MB (both probes chosen as described in Selecting Optimal Probes) in WT and SNP\(_{\text{det}}\) targets at concentrations of 0, 2, 10, 20, 100 nM, 1 and 10 \( \mu \)M. Higher concentrations of 100 \( \mu \)M and 1 mM SNP\(_{\text{det}}\) were also used where detection limits had not yet been established. Fluorescence was read at equilibrium for both probe types. The reading was performed at 60° C for the TP and 55° C for the MB. Three replicates of each type were performed.

The predicted binding curves as a function of target concentration were generated using best fit thermodynamic parameters for MBs:

\[
C_{\text{det}} = \frac{(P_0 + T_0 + (1/K_{\text{det}})) - \sqrt{(P_0 + T_0 + (1/K_{\text{det}}))^2 - 4P_0 T_0}}{2P_0}
\]  

(16)

These predictions were compared to experimental data plotted with 95% confidence intervals. Before plotting, data was normalized by subtracting the background fluorescence measured at 0 nM target concentration and dividing by the maximum intensity experienced at 100 \( \mu \)M WT target at room temperature. The background fluorescence level plotted was the average plus one standard deviation of the signals of experiments run below and including the highest concentration that could not be statistically confirmed as having fluorescence greater than the preceding concentrations (\emph{t}-test, \( P > 0.05 \)).

The detection limits were determined as the analyte concentration at which the theoretical binding curve intersected with the background fluorescence level and were validated by experimental data. The ratio of detection limits was also calculated. Accordingly, the ratio of detection limits can be viewed as a measure of diagnostic accuracy, giving the total range of concentrations over which an assay is expected to achieve 100% true positives and negatives. Throughout this article, this ratio is referred to as the molecular or absolute accuracy of an assay.

### RESULTS

#### Kinetics

TPs have measured rate constants ranging from 100 to 200 times larger than their MB counterparts (Figure 3). In comparison with literature, the TP rates are several times faster than those reported for standard linear DNA-probe-binding kinetics and stand in contrast to the MB rates which are slightly slower than literature values (20). The faster rates of TP over linear probes can be explained by the presence of two probes, a capture and a detection
probe, providing for increased probability of reaction. In addition, these probes are longer than the linear probes used by Tsourkas et al. (20). The MB’s slower reaction than what was reported in literature can be explained by their longer stems and the fact that the reactions were monitored at room temperature instead of 37°C. Although molecular beacons are not expected to perform well at room temperature, this setting was selected to better contrast TPs with MBs. The rate constants indicate that TPs react in ~1% of the time that MBs with the same stem strength require. The room temperature rates of TPs are still >10-fold faster than those reported by Tsourkas et al. using MBs with short stems and higher reaction temperatures (20).

Fitting melting curves

Melting curves were used to extract thermodynamic constants (Figure 4, Table 3). As was expected, the enthalpy of the stem increased with stem length, whereas the enthalpy of the hybridization reaction decreased. The enthalpic and entropic penalties also decreased with increasing stem strength. The only unexpected finding was that for the shorter stems (five and six bases), the stem melting temperature varied greatly between TP and MB. Since these two probe types were constructed identically including choice of dyes and even the attachment of a PEG linker, the only other explanation for this difference in melting temperatures appeared to be that the proximity of the capture probe allowed for some interaction with the stem-loop structure.

Tsourkas et al. have performed the most extensive work on thermodynamics in MBs (20). Their data for MB enthalpies of reaction ranged from −80 to −221 kcal mol⁻¹ and entropies of reaction ranged from −0.21 to −0.61 kcal mol⁻¹ K⁻¹. While these numbers appear similar to what was recorded in our experiments, it should be noted that our beacons were larger, which should produce larger enthalpies, and had stronger stems, which should result in lower net enthalpies for probe–target interactions. Therefore a direct comparison cannot be made. However, we feel confident in the accuracy of the thermodynamic parameters recorded inasmuch as adding the energetic losses due to the stem to the probe enthalpy (−ΔHstem + ΔHdet) produces consistent values near the predicted enthalpies for binding to linear probes of −175.3 kcal mol⁻¹ and similarly for the predicted entropies of −0.4936 kcal mol⁻¹ K⁻¹ (21).

The capture probe parameters were calculated on Mfold. The predicted enthalpy and entropy of reaction were −175.8 kcal mol⁻¹ and −0.4989 kcal mol⁻¹ K⁻¹, respectively (21). It should be noted that the design criterion for both the detection probe and capture probe affinities was a melting temperature 10°C above the desired reaction temperature. The optimal TP stem strength was designed to have a melting temperature 30°C above the reaction temperature. In practice, in order to achieve specificity, we had to increase the reaction temperature such that the predicted melting temperatures for the detection and capture probes were 5°C below the reaction temperature, whereas the predicted stem melting temperature was only 15°C above the reaction temperature.

Once the parameters were fitted, they could be used to calculate binding curves and be double checked for accuracy by comparing theoretical curves against normalized data in a semi-log plot (Figure 5). These predictions reveal a slightly different binding pattern for TP than for MB. TP exhibit a slight bend in the binding curves ~70°C. This is mathematically due to the melting of the capture probe, causing an instant loss in the cooperative interaction and consequent signal.

![Figure 3](image-url)  
**Figure 3.** Rate constants are shown for TP (dark bars) and MB (light bars) for each stem strength with 95% confidence intervals.

![Figure 4](image-url)  
**Figure 4.** Example of fitted melting curves. (A) shows MB (50 nM probe with seven-base stem in 500 nM SNP target) and (B) shows TP (50 nM probe with eight-base stem in 5 μM wild type target) fitted curves with data (squares with target, triangles probe only).
Selecting optimal probes

Optimal candidates were selected by looking at the specific to nonspecific signal ratio for each stem at 50 nM probe and target concentrations (data not shown). The optimal candidates were then verified with the kinetic results in order to assure that the kinetics were ideal as well. Since TP 9 had the greatest signal differentiation and its kinetics were still much faster than the fastest MB, it was selected as the optimal TP. MB 5 was the fastest and most selective of the MBs, so it was chosen as the best MB.

Application in SNP detection

Negligible differences exist between the melting curves of WT and SNP_{cap} and between SNP_{det} and SNP_{both} for both the TP and MB (Figure 6, data not shown for MB). This is most likely due to the relatively high affinity of the capture probes. By utilizing a capture probe which does not respond to SNPs, the location of a SNP can be pinpointed to the region of the detection probe. However, if greater selectivity is required, it may be possible to design the capture probe to respond to SNPs by reducing the probe length. This also indicates that the design of the capture region is probably not as significant as the choice of the detection probe.

Table 3. Enthalpies and entropies for beacon stems, capture probes and detection probes in addition to kinetic data for the wild-type target

| Name   | $\Delta H_{\text{stem}}$ | $\Delta S_{\text{stem}}$ | $\Delta H_{\text{det}}$ | $\Delta S_{\text{det}}$ | $\Delta H_{\text{cap}}$ | $\Delta S_{\text{cap}}$ | $\Delta H_{\text{pen}}$ | $\Delta S_{\text{pen}}$ | Rate |
|--------|-------------------------|--------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|------|
| MB 5   | -70.03                  | -0.2030                  | -93.43                  | -0.2596                 | NA                      | NA                      | NA                      | NA                      | 1000.1 |
| MB 6   | -72.20                  | -0.2070                  | -85.44                  | -0.2373                 | NA                      | NA                      | NA                      | NA                      | 562.4 |
| MB 7   | -76.13                  | -0.2161                  | -83.27                  | -0.2323                 | NA                      | NA                      | NA                      | NA                      | 362.4 |
| MB 8   | -84.60                  | -0.2392                  | -76.56                  | -0.2118                 | NA                      | NA                      | NA                      | NA                      | 219.3 |
| MB 9   | -93.91                  | -0.2639                  | -70.26                  | -0.1947                 | NA                      | NA                      | NA                      | NA                      | 205.0 |
| TP 5   | -56.59                  | -0.1654                  | -93.43                  | -0.2596                 | -175.80                 | -0.4940                 | 69.32                   | 0.1993                  | 90.233.9 |
| TP 6   | -62.42                  | -0.1804                  | -85.44                  | -0.2373                 | -175.80                 | -0.4940                 | 67.49                   | 0.1948                  | 79.300.5 |
| TP 7   | -76.69                  | -0.2188                  | -83.27                  | -0.2323                 | -175.80                 | -0.4940                 | 57.28                   | 0.1657                  | 72.428.2 |
| TP 8   | -78.78                  | -0.2237                  | -76.56                  | -0.2118                 | -175.80                 | -0.4940                 | 52.33                   | 0.1500                  | 58.422.9 |
| TP 9   | -91.02                  | -0.2568                  | -70.26                  | -0.1947                 | -175.80                 | -0.4940                 | 39.79                   | 0.1129                  | 55.861.1 |

Units are in kcal mol$^{-1}$ for enthalpic parameters, kcal mol$^{-1}$ K$^{-1}$ for entropic values and in M$^{-1}$ s$^{-1}$ for rate constants. TP detection probes were assumed to have the same thermodynamic parameters as the molecular beacons. Sign convention is for hairpin closing then binding with target.

Selecting optimal probes

Optimal candidates were selected by looking at the specific to nonspecific signal ratio for each stem at 50 nM probe and target concentrations (data not shown). The optimal candidates were then verified with the kinetic results in order to assure that the kinetics were ideal as well. Since TP 9 had the greatest signal differentiation and its kinetics were still much faster than the fastest MB, it was selected as the optimal TP. MB 5 was the fastest and most selective of the MBs, so it was chosen as the best MB.

Application in SNP detection

Negligible differences exist between the melting curves of WT and SNP_{cap} and between SNP_{det} and SNP_{both} for both the TP and MB (Figure 6, data not shown for MB). This is most likely due to the relatively high affinity of the capture probes. By utilizing a capture probe which does not respond to SNPs, the location of a SNP can be pinpointed to the region of the detection probe. However, if greater selectivity is required, it may be possible to design the capture probe to respond to SNPs by reducing the probe length. This also indicates that the design of the capture region is probably not as significant as the choice of the detection probe.

Because of the cooperative interaction of TP, their melting curves do not change significantly over a wide range of concentrations (Figure 7). A reaction temperature can be chosen for TPs (vertical line in Figure 7A) such that there is 100% accuracy in SNP determination based merely on whether or not a signal is detected. This would be ideal for real-time PCR in SNP discrimination.
Additional mismatches, such as are common in organism detection, should allow for greater resolution. In contrast, the melting temperatures of MB change with concentration making it difficult to find a reaction temperature which allows for discrimination over a wide range of concentrations.

**Specificity and sensitivity**

We performed an experiment at the optimum SNP resolution temperatures for both TP (60°C) and MB (55°C). Theoretical predictions were graphed alongside the experimental results for binding as a function of target concentration (Figure 8). The theoretical predictions appeared to describe experimental data well. TP isotherms reveal wild-type detection limit of 15.4 nM and no SNP detection at concentrations tested up to 1 mM. The model predictions indicate that binding to mutant targets possessing a SNP will never be sufficient to cause a signal above the background resulting in false positives regardless of how high the concentration is. In contrast, mutant targets resulted in false-positive signals for MB at concentrations above 3.88 μM, 154 times greater than the detection limit for the wild-type target of 22.7 nM. The ratio of specific to nonspecific detection limits for the TP was tested in excess of 53 200 and is predicted to increase indefinitely. In contrast with Figure 1, TP specificity was improved without sacrificing sensitivity.

**DISCUSSION**

Due to the thermodynamic principles that govern molecular interactions, it is extremely difficult to develop an assay that increases specificity without sacrificing sensitivity. Likewise, sensitivity is not easily increased without a loss in specificity. We have developed a new class of reagents that does not improve specificity or sensitivity via a trade-off, but that improves overall assay accuracy as indicated by the ratio of detection limits by utilizing cooperativity.
The difficulty in increasing sensitivity or specificity without sacrificing the other lies in the fact that efforts to address assay performance are often directed at instrumentation and buffers. For example, the temperature is raised to increase specificity or lowered to increase sensitivity. Salt concentrations are raised and lowered to optimize binding. Detection volumes are decreased and filter sets are optimized to increase sensitivity (22,23). However none of these methods addresses the fundamental issue of how affinity reagents recognize and bind an analyte. Altering instrumentation does not affect thermodynamic parameters governing binding and thus only affects the threshold of detection. Altering the threshold merely results in a sensitivity–specificity trade-off as demonstrated by receiver operating curve analysis (1). Raising the temperature or decreasing salt concentrations lowers the equilibrium constant for both specific and nonspecific binding, leading to greater specificity at the expense of sensitivity. Likewise decreasing the temperature or increasing salt concentrations increases the equilibrium constant for specific and nonspecific binding, resulting in increased sensitivity at the expense of specificity. None of these methods decreases the nonspecific equilibrium constant while maintaining or increasing the specific equilibrium constant. Accordingly, trade-offs in specificity and sensitivity can be achieved but the ability of the affinity reagent to discriminate between specific and nonspecific targets remains largely unchanged.

TPs represent a novel innovation inasmuch as they are mathematically engineered to manipulate thermodynamic principles that govern affinity reagent recognition of a biomolecule. Collision theory dictates that a second binding event from a molecule already bound to a substrate and held at an enhanced local concentration will typically occur at a faster rate than it would in free solution. This principle was used to develop mathematical models that predicted both enhanced kinetics and specificity of cooperative probes. For a homovalent probe pair with no penalty term, the effective equilibrium specificity of cooperative probes. For a homovalent models that predicted both enhanced kinetics and solution. This principle was used to develop mathematical

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in SNP discrimination that is matched by no other probe set to our knowledge.

MBs, on the other hand, are known to possess greater specificity than linear probes due to the competition from stem formation (19). Discrimination between wild-type and mismatched targets is expected to increase with stem strength (20). However, utility of stronger stems in MBs is compromised by reduced sensitivity from lower affinity and by slower kinetics. By combining the unique secondary structure of MBs with the idea of cooperative probes, we have created a label-free, highly specific reagent that has greater specificity than a MB, but that reacts up to 200 times faster. Again TPs show no sign of a trade-off. This may make the use of TPs in continuous flow detection where reagents passing by only have a short time to react an ideal candidate over MB.

The sensitivity loss from stronger stems in MBs has been studied by Tsourkas et al. (20). The loss in fluorescence experienced by MBs is replaced by gains in TPs with identical stem strengths because of the lower free energy available from cooperative binding. We collected thermodynamic parameters in order to compare with the results of Tsourkas et al. In order to get their thermodynamic parameters, they fit a line through the fluorescence at the melting temperature of six different concentrations of target. We deviated from this method because we felt it was statistically more relevant to use the entire set of fluorescent data for each concentration rather than a single point. Also, their method did not directly apply to fitting thermodynamic parameters for TPs.

In addition to a melting curve which does not shift significantly with target concentration, Figure 5 reveals a difference between TP and regular probes in the amount of specific and nonspecific binding resulting in fluorescence as a function of temperature. Data points are plotted to where fluorescent levels are no longer distinguishable from the background. Since the actual data could not be observed at low amounts of binding, the fitted thermodynamic parameters were used to calculate binding as a function of temperature beyond where data could be collected. Since the models and fitted parameters account for the majority of the TP behavior, and because these models have been derived for other applications as previously discussed, we feel justified in their use to examine the thermodynamic patterns of cooperative binding.

As a further justification of the use of the models in describing TP behavior, the models were used to predict binding as a function of concentration (Figure 8). Theoretical predictions estimated that a target molecule possessing a SNP in the detection region would never be detected by TPs regardless of target concentration, virtually eliminating false positives. We were able to test this hypothesis to 1 nM SNP concentrations with no false detections. We note that the models were sufficiently accurate to predict this trend. We also note that the models predict the ability to concentrate nonspecific analyte to levels that are not physically possible without false positives. This is supported by the fluorescent signals of TP 9 at 60°C, which seem to be completely independent of target concentration (Figure 7). The fact that we were unable to create SNP analyte concentrations sufficiently high for TP detection without sacrificing the sensitivity of wild-type detection illustrates the point of this article. This stands in stark contrast to any other probe set of which we are aware. By creating cooperative probes, we have been able to effectively reduce false positives without sacrificing sensitivity.

This high level of specificity may find utility in a number of research and diagnostic applications. Real-time PCR, for example, is often required to perform SNP discrimination. While comparative analysis of a multiplexed assay often yields good results in a laboratory, field tests do not always have the same options of sample purity or multiplexing and those running the tests do not always have the capacity to interpret the results. Rather many field test units resolve the complexity of analysis by resorting to a signal/no signal report. Few probes possess the capacity to distinguish SNPs by the presence or absence of a signal. This problem is compounded in homeland security where a number of select agents like *Bacillus anthracis* and *Yersinia pestis* have near neighbors with large portions of similar genetic content (34,35). TPs may again provide an answer to this real-world need.

Although TPs have shown benefits in several areas, there is still room for improvement. This set of experiments only examined the effect of stem length on binding. Other options for adaptation include linker length and composition, capture and detection probe affinities, the distance between capture and detection regions on the target polynucleotide, and the interactions of each of these features. Given the large enthalpic and entropic penalties, there may be a great deal of room for improvement, potentially producing a much sharper slope in the melting curves. This in turn could lead to higher signal-to-noise ratios and greater detection limit ratios, while maintaining the incredible specificity.

Six years ago, Iqbal et al. called for the creation of new affinity reagents as the most important means of improving biosensor accuracy (6). We have created a new class of label-free affinity reagents, TPs. These special beacons manipulate thermodynamic principles in order to increase kinetics up to 200-fold and molecular accuracy in SNP detection by at least 345-fold, with predicted enhancements of near infinite improvement. Because of their cooperative interaction, they truly allow significant enhancements in sensitivity, specificity and kinetics without a trade-off.

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