A double-stranded molecular probe for homogeneous nucleic acid analysis†‡

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This paper reports the design and optimization of a double-stranded molecular probe for homogeneous detection of specific nucleotide sequences. The probes are labeled with either a fluorophore or a quencher such that the probe hybridization brings the two labels into close proximity, and this diminishes the fluorescence signal in the absence of a target. In the presence of a target, the fluorophore probe is thermodynamically driven to unzip from its hybridized form and bind with the target. An equilibrium analysis, which successfully describes all the major features of the assay without any fitting parameter, is performed to generalize the design of the probe. Several key parameters affecting the performance of the assay are examined. We show that the dynamic range and the signal-to-noise ratio of the assay can be optimized by the probe concentration, the quencher-to-fluorophore ratio, and the probe strand sequence. By proper design of the sequence, the probe discriminates single nucleotide mismatches in a single step without any separation step or measurement of melting profile.

Introduction

The ability to specifically and quantitatively detect nucleic acids is of great importance in various biomedical applications. However, the time-intensive nature of traditional techniques often creates a bottleneck in studies that require rapid quantification of the samples, such as point-of-care diagnosis, real-time PCR, genotyping, and high-throughput drug screening. Moreover, the cumbersome procedures often present challenges in implementing the techniques in microfluidic or other automated formats for high-throughput studies. An assay that requires only the addition of reagents (*i.e.* mix-and-measure) is highly desirable. Molecular beacons for rapid detection of specific oligonucleotides have been designed to realize this mix-and-measure assay type.[1] A molecular beacon is an oligonucleotide probe that self-hybridizes to form a stem-and-loop structure and can undergo a spontaneous fluorogenic conformational change upon hybridization to its complementary nucleic acid target. The molecular beacon design provides a mechanism for both molecular recognition and transduction of the hybridization events in one single step and thus dramatically accelerates the molecular detection process. The technique is capable of discriminating single nucleotide polymorphisms (SNPs) and has been applied in various biological studies.[2–8]

We are developing an alternative molecular binding scheme using two single-stranded probes for rapid quantification of specific nucleic acid molecules. In this sensing scheme, a fluorophore-conjugated nucleic acid sequence (fluorophore probe), which is complementary to a target sequence, is designed. A complementary sequence conjugated with a quencher (quencher probe) is also designed. The fluorophore probe is labeled with a fluorophore on the 5′ end and the quencher probe is labeled with a quencher on the 3′ end. In the absence of a target, the probes hybridize and bring the fluorophore and quencher into close proximity, dampening out the fluorescence signal. The existence of a target molecule thermodynamically drives the switching between the quencher probe and the target molecule, and the concentration of the target molecules can be measured quantitatively based on the fluorescence intensity (Fig. 1a). The molecular probe design allows rapid quantification of specific nucleic acid sequences in a liquid-phase, separation-free format. The double-stranded molecular probe has the same advantages of a rapid, sensitive, and simple molecular beacon. The inclusion of the quencher probe provides new opportunities for improving the dynamic range, signal-to-noise ratio, and selectivity of the assay.

Related molecular binding schemes have been studied for homogeneous DNA detection, real-time PCR, RNA polymerase activity measurement, and aptamer-based protein detection.[9–15] Nevertheless, a quantitative description of the assay for guiding the probe design is not available. The probe sequences are often designed empirically and the performance of the assay may not be optimized. Trial and error of the probe sequences is typically required to satisfy specific applications, such as tolerating genetic variations or discriminating SNPs. A generalized model that describes the major features of the assay will assist in the optimization of the operating parameters, such as the probe sequence, the probe concentration, and the quencher-to-fluorophore ratio, and will allow utilization of the full potential of the molecular assay. In this work, we show that

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‡ The HTML version of this article has been enhanced with colour images.

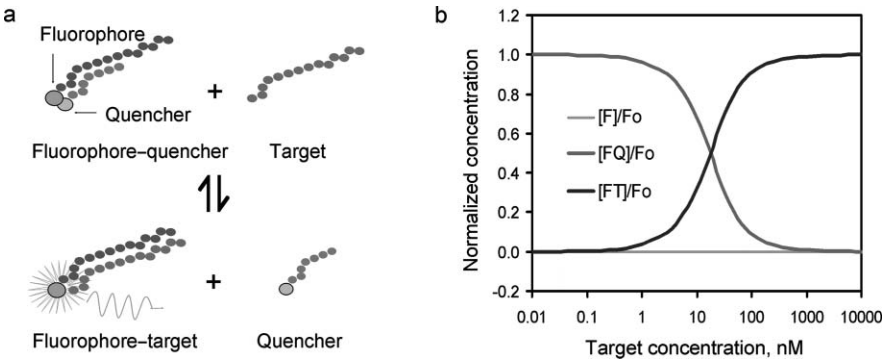

Fig. 1 Molecular probe assay for rapid quantification of specific nucleic acid sequences. (a) The molecular probe is designed such that the target spontaneously separates the quencher probe from the fluorophore probe. (b) Equilibrium concentration of the probes at different target concentrations. At low concentration of target, most of the fluorophore probes are hybridized with the quencher probe. The fluorescence response of the assay is primarily determined by the concentration of the fluorophore probe–target complex. [F], [FQ], and [FT] are the concentrations of the free fluorophore probe, fluorophore–quencher probe complex, and fluorophore probe–target complex, respectively.

the performance of the assay can be accurately described by a simple equilibrium analysis. Experimental studies are performed and compared with the prediction from the equilibrium analysis. The dynamic range and signal-to-noise ratio of the assay are optimized based on the analysis. Furthermore, discrimination of a single nucleotide mutation with a single-base-mismatch selectivity of 14 : 1 has been demonstrated in a single step without any separation step or measurement of melting profile.

Materials and methods

Equilibrium analysis

The free energy change and the binding affinity play critical roles in the success of the molecular probe design, and these parameters are primarily determined by the probe sequences. Several studies have been performed for single-stranded molecular beacons[16,17] and competitive surface hybridization.[18,19] Nevertheless, an analytical model is not available for optimizing the double-stranded molecular probe for homogeneous nucleic acid detection. To rationalize the design of the double-stranded molecular probe, we quantitatively studied the double-stranded molecular binding scheme. The molecular assay is represented by eqns (1) and (2).

$$\mathrm{F} + \mathrm{Q} \rightleftharpoons \mathrm{FQ} \tag{1}$$

$$\mathrm{F} + \mathrm{T} \rightleftharpoons \mathrm{FT} \tag{2}$$

In this analysis, F is the fluorophore probe, Q is the quencher probe, T is the target, FQ is the fluorophore–quencher probe duplex, and FT is the fluorophore probe–target duplex. At equilibrium, the molecular binding reactions lead to eqns (3) and (4). The conservation of the species results in eqns (5) and (6).

$$K_{\mathrm{Q}} = \frac{[\mathrm{FQ}]}{[\mathrm{F}][\mathrm{Q}]} \tag{3}$$

$$K_{\mathrm{T}} = \frac{[\mathrm{FT}]}{[\mathrm{F}][\mathrm{T}]} \tag{4}$$

$$F_0 = [\mathrm{F}] + [\mathrm{FQ}] + [\mathrm{FT}] \tag{5}$$

$$T_0 = [\mathrm{FT}] + [\mathrm{T}] \tag{6}$$

K_{Q} and K_{T} are the equilibrium constants of donor–quencher hybridization and donor–target binding, respectively. The equilibrium constant, K, is estimated based on the free energy change, ΔG, associated with hybridization of the complementary sequences.[20] The relationship between the equilibrium constant and the free energy change is given by $K = e^{-\Delta G/RT}$, where R is the gas constant and T is the absolute temperature. The quantities F_0, Q_0, and T_0 are the initial concentrations of the fluorophore probe, the quencher probe, and the target respectively. Assuming that the concentration of the quencher probe is large compared to the fluorophore probe, which is the typical condition in our experiment, the equilibrium concentration of the free donor, the donor–quencher, and the donor–target hybrids can be estimated by solving eqns (3)–(6). The concentrations of the molecules at equilibrium are given by:

$$[\mathrm{F}] = \frac{-\beta + \sqrt{\beta^2 + 4\alpha F_0}}{2\alpha} \tag{7}$$

$$[\mathrm{FQ}] = [\mathrm{F}] Q_0 K_{\mathrm{Q}} \tag{8}$$

$$[\mathrm{FT}] = \frac{[\mathrm{F}] K_{\mathrm{T}} T_0}{1 + [\mathrm{F}] K_{\mathrm{T}}} \tag{9}$$

$$\beta = 1 + Q_0 K_{\mathrm{Q}} + K_{\mathrm{T}} T_0 - F_0 K_{\mathrm{T}} \tag{10}$$

$$\alpha = K_{\mathrm{T}} + Q_0 K_{\mathrm{Q}} K_{\mathrm{T}} \tag{11}$$

Fig. 1b shows the results of the equilibrium analysis. At a low background level, the fluorescence response of the assay is mainly determined by eqn 9. The fluorescence response of the assay, therefore, can be predicted using eqns (7)–(11). It should be noted that the free energy change (*i.e.* the equilibrium constant) can be directly estimated using the probe sequence and the experimental conditions (*e.g.* temperature). There is no fitting parameter in the calculation. Therefore, the performance of the assay, such as the dynamic range, can be predicted for different probe sequence designs. The simplicity of the solution provides a useful tool in the design of the probe instead of optimizing the assay by trial and error. Furthermore,

the sensitivity of each experimental parameter on the overall performance of the assay can be evaluated based on the analysis.

In addition to estimating the fluorescence intensity with the presence of the target, the concentration of the free fluorophore probe can also be determined by the equilibrium analysis. The free fluorophore probe in the absence of a target represents the source of background related to the dissociation of the fluorophore–quencher duplex. To estimate the concentration of the free fluorophore probe, we considered eqn (1) without assuming that the concentration of quencher probe is large compared to the fluorophore probe. Eqn (12) considers a constant ratio between the initial concentrations of the quencher and the fluorophore probes. Eqns (13) and (14) describe the conservation of the quencher probe and fluorophore probe:

$$Q_0 = MF_0 \tag{12}$$

$$Q_0 = [\mathrm{Q}] + [\mathrm{FQ}] \tag{13}$$

$$F_0 = [\mathrm{F}] + [\mathrm{FQ}] \tag{14}$$

where M is the quencher-to-fluorophore ratio. Solving eqns (1) and (12)–(14) reveals

$$[\mathrm{F}] = \frac{-\gamma + \sqrt{\gamma^2 + 4K_{\mathrm{Q}}F_0}}{2K_{\mathrm{Q}}} \tag{15}$$

$$\gamma = \{F_0K_{\mathrm{Q}}(M-1) + 1\} \tag{16}$$

Therefore, the concentration of the free fluorophore probe [F], which contributes to the background of the molecular assay, can be determined by eqns (15) and (16). Specifically, the free fluorophore probe (*i.e.* the background level) can be minimized by adjusting the quencher-to-fluorophore ratio M.

Samples and preparation

Two sets of nucleic acid probes were designed based on the equilibrium analysis to evaluate the double-stranded molecular binding scheme. The first set of probes was designed according to an antioxidant gene, NAD(P)H:quinone oxidoreductases 1 (NQO1).[21,22] The fluorophore probe, F_{NQO1}, is complementary to the NQO1 sequence, such that it would have a high affinity for hybridization. The fluorescence detection was enabled by labeling the 5′ end of the DNA probe with a fluorescein tag (6-FAM). Two quencher probes, $Q_{NQO1\text{-}1}$ and $Q_{NQO1\text{-}2}$, were designed to be complementary to the fluorophore probe yet shorter in length. This choice was made to create a larger difference in free energy when the fluorophore probe hybridized with the target than with the quencher probe. The selection of the 6-FAM directed the choice of a high quenching efficiency quencher (Iowa Black FQ). The second set of probes, herein referred to as 'control set', was constructed around a 24 nucleotide target sequence, T_c. The fluorophore probe, F_c, is fully complementary to the target and two quencher probes were employed to hybridize with the fluorophore probe, referred to as Q_{c1} and Q_{c2}. Table 1 summarizes the probe designs in this study. All the equilibrium constants are estimated at 23 °C using the Mfold server.[20] It is known that the interaction between the fluorophore and quencher stabilizes the hybridized probe and increases its melting temperature. To account for the interaction between the fluorophore and quencher, we increase the free energy change for 2 kcal mol^{-1} for fluorophore–quencher probe hybridization in the calculation.[23]

All the experimental probes were synthesized by Integrated DNA Technologies Inc and other reagents were purchased from Sigma. Unless otherwise specified, samples were prepared in a buffer of 0.06 M Tris-EDTA and 50 mM NaCl. The probes are hybridized before mixing with the target samples. Before the experiment, the probes were heated in a dry-bath incubator at 90 °C for 10 min then slowly cooled to room temperature. For the control set, the double-stranded probes were incubated with the target for 10 min at room temperature before the measurement. For the NQO1 set, the probes and the target are heated to 85 °C for 10 min then cooled to room temperature before the measurement. All fluorescence measurements were taken in 96-well plates using a fluorescence microplate reader (BioTek, Synergy 2). Data are reported as mean ± standard deviation for at least three consecutive experiments.

Results

Dynamic range

The equilibrium analysis predicts that the molecular probe assay has a dynamic range over three orders of magnitude of target concentration (Fig. 2a). It is anticipated that the length of the quencher probe would affect the free energy change in switching the fluorophore probe between the quencher probe hybridization and the complementary target sequence. Therefore, we evaluated the effect of adjusting the quencher sequence on the dynamic range of the assay based on the equilibrium analysis.

Table 1 Sequences of the nucleotide probes in this study

Name	Label	Sequence	ΔG/kcal mol^{-1}
F_{NQO1}	5′ 6-FAM	5′ TCC TTT GTC ATA CAT GGC AGC G 3′	—
$Q_{NQO1\text{-}1}$	3′ Iowa Black FQ	3′ AGG AAA CAG TAT GTA C 5′	−21.7
$Q_{NQO1\text{-}2}$	3′ Iowa Black FQ	3′ AGG AAA CAG TAT GTA CCG TCG 5′	−32.5
T_{NQO1}	—	3′ AGG AAA CAG TAT GTA CCG TCG C 5′	−34.1
$T_{NQO1\text{-}C}$	—	3′ AGG AAA CAG TAT C̲TA CCG TCG C 5′	−28.1
$T_{NQO1\text{-}A}$	—	3′ AGG AAA CAG TAT A̲TA CCG TCG C 5′	−28.6
$T_{NQO1\text{-}T}$	—	3′ AGG AAA CAG TAT T̲TA CCG TCG C 5′	−29.2
F_c	5′ 6-FAM	5′ TTG GGA CTT TCC C̲AA GAT AGT AAG 3′	—
Q_{c1}	3′ Iowa Black FQ	3′ AAC CCT GAA AGG 5′	−17.2
Q_{c2}	3′ Iowa Black FQ	3′ AAC CCT GAA AGG GTT C 5′	−24.1
T_c	—	3′ AAC CCT GAA AGG GTT CTA TCA TTC 5′	−34.4

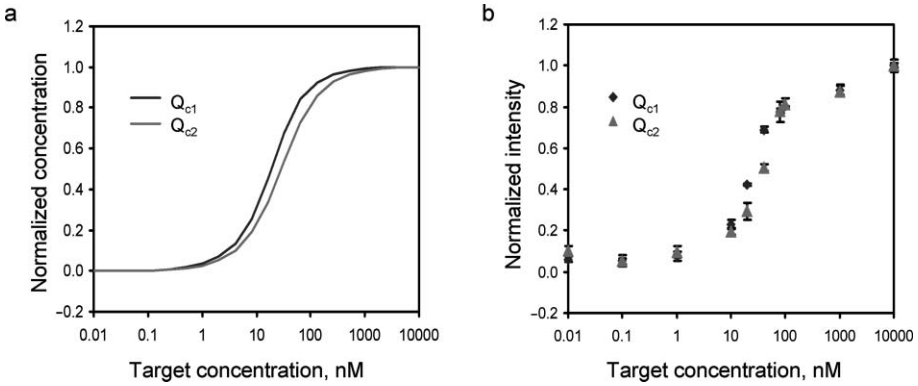

Fig. 2 Effects of the quencher length on the molecular assay shown by (a) the equilibrium analysis prediction and (b) experimental investigation. Quencher probes are of length 12 (Q_{c1}) and 16 (Q_{c2}) nucleotides respectively. The probes were employed in samples with a 60nM concentration with 20nM fluorophore probes to gauge the effect of the quencher probe design on the system sensitivity. Data represents mean ± standard deviation.

Interestingly, it was found that the quencher length has only a minimal effect on the dynamic range when the equilibrium constant for target hybridization, K_T, is large compared to the value for the quencher, K_Q.

We designed two quencher probes, Q_{c1} and Q_{c2}, with changes in free energy of −17.2 and −24.1 kcal mol^{-1} if they hybridize with the fluorophore probe F_c. The target T_c had a free energy change of −34.4 kcal mol^{-1} if it hybridizes with the fluorophore probe. The probe design followed the condition of $K_{T_c} \gg K_{Q_{c1}} > K_{Q_{c2}}$. Fig. 2a shows the response curves estimated for the two quencher probes at different target concentrations. Only a small shift of the curve to the lower concentration range is observed for decreasing K_Q. Experimentally, we tested the effects of the two quencher probes, Q_{c1} and Q_{c2} (Fig. 2b). The observation is in quantitative agreement with the equilibrium analysis. The values are within 10% for the entire range of concentration. As predicted, there is a small shift of the titration curve of quencher 1 (the shorter probe) to lower concentration, but the upper and lower limits of detection still have considerable overlap, so neither the dynamic range nor the sensitivity appear to be affected significantly by the different quencher probes under these conditions.

To tune the dynamic range of the molecular probe assay, the probe concentration can be adjusted, and this is found to be a dominant factor in the dynamic range of the assay. Fig. 3a shows two titration curves of two different concentrations (20 and 200 nM) of the probe estimated by the equilibrium analysis. The probes F_{NQO1} and $Q_{NQO1\text{-}1}$ were used in the calculation. The quencher-to-fluorophore ratio is maintained at 2 : 1. Both titration curves show a sigmoid shape and have large dynamic ranges for quantifying the target DNA concentration. There is a distinct dynamic range of the fluorescence response curves for the two concentrations of probes. Varying the concentration of the probes causes the titration curve to shift, so the sensitivity of the system can be tailored by changing the probe concentration. The titration curve shifts with the probe concentration for a large range of concentrations as predicted by the equilibrium analysis (see Fig. S1, ESI†). Experimentally, serial dilutions of the target T_{NQO1} were tested to determine the titration curves at two different probe concentrations. The result shows good agreement with the equilibrium analysis (Fig. 3b). The titration curves have the same sigmoid shape. The lower probe concentration has a lower target concentration range and a lower limit of detection.

Background minimization

One of the potential sources of background in the molecular probe assay is the free fluorophore probe as a result of the disassociation of the quencher and fluorophore probe duplex. Therefore, the background level could be reduced by minimizing

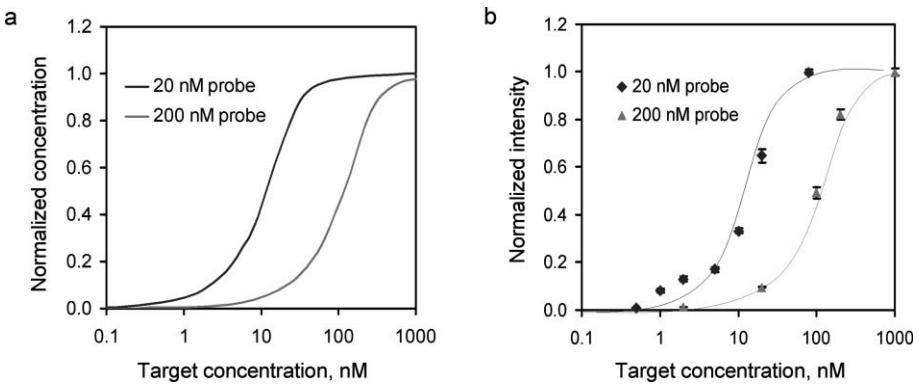

Fig. 3 Tunable dynamic range of the molecular probe assay determined by (a) the equilibrium analysis and (b) experimental investigation. Fluorophore probe concentrations of 20 and 200 nM and quencher probe concentrations of 40 and 400 nM were used, respectively. Error bars represent the standard deviation.

the amount of free fluorophore probes disassociated from the quencher probe. In the molecular probe design, the concentration of the quencher probe relative to the concentration of the fluorophore probe (quencher-to-fluorophore ratio) can be adjusted to minimize the amount of free fluorophore probes, which maximizes the signal-to-noise ratio. This presents an extra degree of freedom to improve the molecular probe design.

To determine the optimal quencher-to-fluorophore ratio, the equilibrium analysis [eqns (15) and (16)] was applied to calculate the free fluorophore probe concentration. Fig. 4a shows the intensity level estimated by the equilibrium analysis at different quencher-to-fluorophore ratios for the NQO1 set. The fluorescent intensity can be efficiently reduced to a low level at a quencher-to-fluorophore ratio of 2 : 1. It should be noted that the ratio depends on the sequence design and should be calculated for each probe design using eqn (15). We also performed experiments to compare the optimal quencher-to-fluorophore ratio using the NQO1 set probes. Consistent with the equilibrium analysis, a quencher-to-fluorophore ratio of 2 : 1 was able to effectively quench the fluorescence intensity to a low level (approx. 3% of the maximum value) (Fig. 4b). Further increase in the quencher concentration did not further reduce the intensity (up to a ratio of 10 : 1). This is consistent with other experimentally optimized values reported in related studies.[9,10] It indicates that the equilibrium analysis can facilitate the optimization of the signal-to-noise ratio. On the other hand, an excessive amount of quencher probe could potentially reduce the sensitivity of the assay and shift the titration curve to a higher target concentration range. We estimated the effect of the quencher-to-fluorophore ratio on the titration curve using the equilibrium analysis. Only a minor shift of the titration curve was observed at a 2 : 1 quencher-to-fluorophore ratio (see Fig. S2, ESI†). Therefore, we determine that a 2 : 1 ratio is able to minimize the background level without affecting the signaling level significantly.

Detection of a single nucleotide mismatch

The selectivity of the assay arises from the competition between the binding of the target and the quencher probe. Therefore, non-specific binding can be greatly reduced by designing a quencher probe with a strong affinity for the probe. It is possible to design a quencher probe such that the fluorophore probe is thermodynamically favorable to bind to the perfect match target while it is not favorable to bind to a single nucleotide mismatch target. Mathematically, this condition can be formulated as $K_T > K_Q > K_M$, where K_M is the equilibrium binding constant for the mismatch target with the fluorophore probe. In such a condition, the probe is capable of directly detecting SNPs without any extra separation procedures, as predicted by the equilibrium analysis.

For the NQO1 set, the change in free energy for the perfect match target is -34.1 kcal mol^{-1} and the values for the mismatch targets are -28.1, -28.6 and -29.2 kcal mol^{-1}. Therefore, we designed the quencher probe $Q_{NQO1\text{-}2}$ to have a change in free energy of -32.5 kcal mol^{-1}. Fig. 5a shows the prediction of the equilibrium analysis, which indicates high selectivity between targets with a perfect match and single nucleotide mismatches for the NQO1 probe set. Fluorescence intensity only increases with the perfect match target. Experimentally, we have tested the ability of the molecular probe for detecting an SNP of the NQO1 sequence. Both quencher probes $Q_{NQO1\text{-}1}$ and $Q_{NQO1\text{-}2}$ were tested. $Q_{NQO1\text{-}1}$, which does not satisfy the condition, shows little selectivity between the perfect and mismatch targets (data not shown). $Q_{NQO1\text{-}2}$, which was designed according to the condition $K_T > K_Q > K_M$, shows excellent selectivity between the targets. Fig. 5b shows the experimental result of the SNP detection experiment. We observed little increase in the fluorescence intensity even at 100-fold excess concentration of the mismatch target relative to the fluorophore probe. The selectivity for single-base-mismatch (the ratio of the intensities between the perfect match and mismatch targets) is 14 : 1. It should be noted that a typical molecular beacon has a single-base-mismatch selectivity of 4 : 1.[24] Our result indicates that the equilibrium analysis is able to predict the condition for SNP detection and the double-stranded molecular probe is able to detect single nucleotide mismatches in a single step without any separation step or measurement of melting profile.

Discussion

We present the design and optimization of a double-stranded molecular probe biosensor. Our results are generally consistent

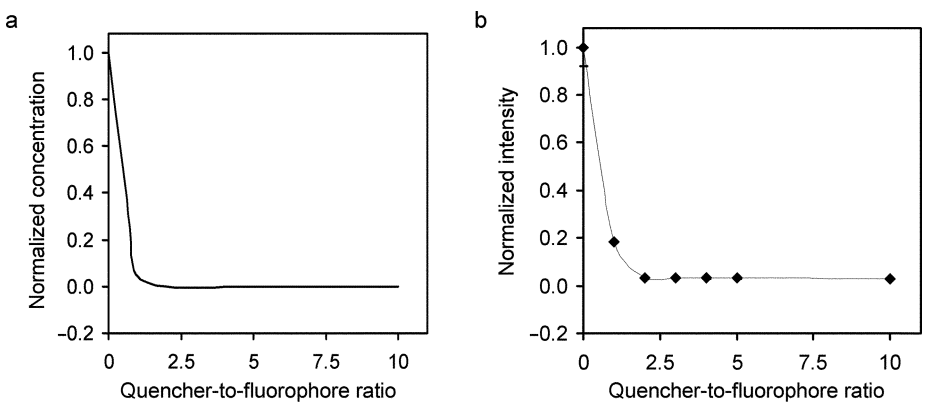

Fig. 4 Optimization of the quencher-to-fluorophore ratio. The equilibrium concentration of the free fluorophore probe determined by (a) the equilibrium analysis and (b) experimental investigation. The concentration of the fluorophore probe is 40 nM and the concentration of the quencher probe is adjusted to obtain different quencher-to-fluorophore ratios. Error bars representing the standard deviation are not shown as they are smaller than the symbols themselves.

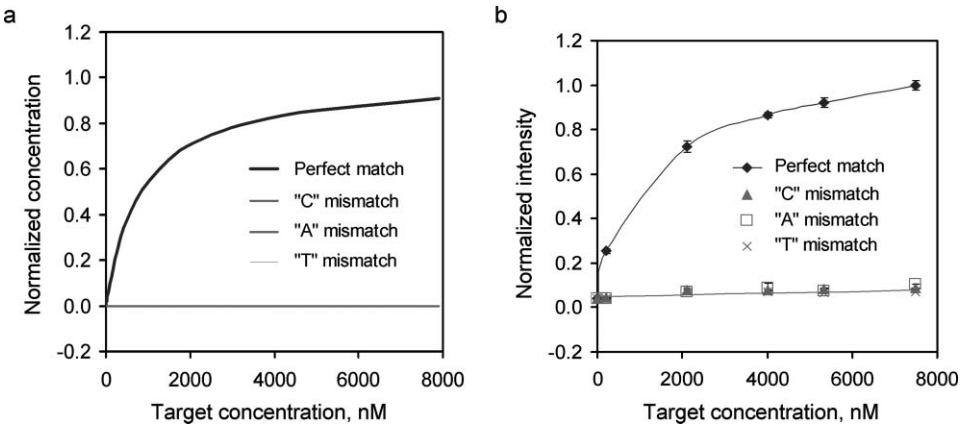

Fig. 5 Detection of single-base-mismatches using the molecular probe biosensor. The fluorescence response of the assay determined by the (a) equilibrium analysis and (b) experimental investigation. The three mismatch targets show insignificant increases in the equilibrium analysis and cannot be distinguished in the graph. The overall signal-to-noise ratio of the assay is 24 and the selectivity for single-base-mismatch is 14. Error bars represent the standard deviations.

with other experimental studies.[9,10] Despite the simplicity of the analysis, the model successfully predicts the major features of the molecular binding scheme without any fitting parameter. In order to estimate the performance of a probe design, the free energy change should first be estimated based on the probe sequences[25] and the equilibrium constant can be determined using $K = e^{-\Delta G/RT}$. This allows evaluation of the probe design before the actual experiment. In this study, DNA is used as a model to evaluate the equilibrium analysis. It should be noted that the model does not restrict the type of binding as long as the equilibrium constant is known. The quantitative analysis should also be applicable to the detection of RNA molecules and proteins.[15,26] If the target molecule has other secondary structures, the change in free energy associated with the unfolding of the structure should be subtracted to determine the total change in free energy. In general, the performance of the assay including (1) the dynamic range, (2) the signal-to-noise ratio, and (3) the selectivity can be adjusted by the probe concentration, the quencher-to-fluorophore ratio, and the quencher length.

Firstly, the probe concentration can be adjusted to tune the dynamic range of the assay. Our data indicate that the equilibrium analysis is able to accurately predict the dynamic range of the assay and that the detectable range can be adjusted by the probe concentration. The detectable range of the assay can, therefore, be estimated according to eqns (7)–(11). The probe concentration can be selected based on the target concentration range of interest and the equilibrium analysis provides a guide to select the probe concentration of the assay. Another important implication of the analysis is the accuracy of the assay for quantitative measurement. In typical fluorescence assays, it is commonly assumed that the intensity is directly proportional to the target concentration. In the double-stranded molecular probe, the target concentration has to increase over three orders of magnitude in order to change the fluorescence intensity from 1 to 99% as determined by the equilibrium analysis and experimental verification. It could introduce a significant amount of uncertainty in the measurement to simply assume that the intensity is proportional to the concentration. This is especially important for using the molecular probe for direct quantification of RNA or other molecules. Furthermore, the molecular assay is capable of quantitative measurement at a dynamic range of concentrations spanning over three orders of magnitude. For a given target, the intensity–target concentration correlation can be directly calculated using eqns (7)–(11). Therefore, the equilibrium analysis can assist quantitative assessment of the data. The range of highest sensitivity (slope of the titration curve) is at intermediate concentrations of the titration curve. If a small difference in concentration has to be measured, the equilibrium analysis can provide useful guidelines for choosing the probe concentration in order to maximize the resolution.

The quencher-to-fluorophore ratio can be adjusted to facilitate the minimization of the background due to the disassociation of the double-stranded probe. In this study, we have shown that the equilibrium analysis can facilitate the optimization of the signal-to-noise ratio. For a given probe design, the background level can be estimated by eqns (15) and (16). Since the concentration of the free fluorophore probe in the experimental conditiosn is minimized, the background is likely to be predominantly contributed by the imperfect quenching efficiency of the fluorophore–quencher pair. For a typical fluorophore–quencher pair, the efficiency of contact-mediated quenching is approximately 95%,[23] which is equivalent to a signal-to-noise ratio of 20. To further improve the molecular assay, other energy transfer mechanisms, such as gold nanoparticle-mediated quenching, fluorescence resonance energy transfer, and quantum dot-based sensing, can be incorporated to the molecular probe to enhance the signal-to-noise ratio.[6,27,28]

Lastly, the selectivity of the assay can be adjusted by the quencher length. We have shown that the performance of the assay is not sensitive to the quencher design when $K_T \gg K_Q$. On the other hand, single nucleotide mismatches can be distinguished with high selectivity when $K_T > K_Q > K_M$. Since the free energy change (and the equilibrium constant) can be calculated based on the probe sequences, the analysis can guide the design of the quencher probe sequence for a specific application. For instance, a short quencher sequence can be designed to tolerate genetic variation of the target probe

sequence (with $K_T > K_{T\text{-with-variation}} > K_Q$). On the other hand, highly specific probes can also be designed for SNP detection (with $K_T > K_Q > K_M$).

Conclusion

The current work generalizes the double-stranded molecular probe design using an equilibrium analysis. The flexibility of the design allows the probe to be applied in a wide spectrum of applications. The specificity of the molecular assay provides a useful tool in different biomedical applications. For instance, the molecular probe can be adopted for detecting mutation of genes and quantification of mRNA. The simplicity of the assay is also beneficial for microfluidic-based point-of-care diagnostic systems and the high-throughput screening of drugs.

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References

1 S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303–308.
2 P. Santangelo, N. Nitin, L. LaConte, A. Woolums and G. Bao, *J. Virol.*, 2006, **80**, 682–688.
3 G. Bao, A. Tsourkas and P. Santangelo, *Abstr. Pap. Am. Chem. Soc.*, 2003, **225**, U982.
4 X. H. Fang, J. W. J. Li, J. Perlette, W. H. Tan and K. M. Wang, *Anal. Chem.*, 2000, **72**, 747a–753a.
5 T. H. Wang, Y. H. Peng, C. Y. Zhang, P. K. Wong and C. M. Ho, *J. Am. Chem. Soc.*, 2005, **127**, 5354–5359.
6 B. Dubertret, M. Calame and A. J. Libchaber, *Nat. Biotechnol.*, 2001, **19**, 680–681.
7 S. Tyagi, D. P. Bratu and F. R. Kramer, *Nat. Biotechnol.*, 1998, **16**, 49–53.
8 T. J. Huang, M. S. Liu, L. D. Knight, W. W. Grody, J. F. Miller and C. M. Ho, *Nucleic Acids Res.*, 2002, **30**, e55.
9 S. Huang, J. Salituro, N. Tang, K. C. Luk, J. Hackett, P. Swanson, G. Cloherty, W. B. Mak, J. Robinson and K. Abravaya, *Nucleic Acids Res.*, 2007, **35**, e101.
10 R. H. Blair, E. S. Rosenblum, E. D. Dawson, R. D. Kuchta, L. R. Kuck and K. L. Rowlen, *Anal. Biochem.*, 2007, **362**, 213–220.
11 Q. Q. Li, G. Y. Luan, Q. P. Guo and J. X. Liang, *Nucleic Acids Res.*, 2002, **30**, e5.
12 R. Nutiu and Y. F. Li, *J. Am. Chem. Soc.*, 2003, **125**, 4771–4778.
13 A. O. Crockett and C. T. Wittwer, *Anal. Biochem.*, 2001, **290**, 89–97.
14 R. A. Cardullo, S. Agrawal, C. Flores, P. C. Zamecnik and D. E. Wolf, *Proc. Natl. Acad. Sci. U. S. A.*, 1988, **85**, 8790–8794.
15 N. Li and C. M. Ho, *J. Am. Chem. Soc.*, 2008, **130**, 2380–2381.
16 A. Tsourkas, M. A. Behlke, S. D. Rose and G. Bao, *Nucleic Acids Res.*, 2003, **31**, 1319–1330.
17 G. Bonnet, S. Tyagi, A. Libchaber and F. R. Kramer, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 6171–6176.
18 J. Bishop, S. Blair and A. M. Chagovetz, *Biophys. J.*, 2006, **90**, 831–840.
19 J. Bishop, C. Wilson, A. M. Chagovetz and S. Blair, *Biophys. J.*, 2007, **92**, L10–L12.
20 N. R. Markham and M. Zuker, *Nucleic Acids Res.*, 2005, **33**, W577–W581.
21 D. Ross, J. K. Kepa, S. L. Winski, H. D. Beall, A. Anwar and D. Siegel, *Chem.-Biol. Interact.*, 2000, **129**, 77–97.
22 Z. Sun, S. Zhang, J. Y. Chan and D. D. Zhang, *Mol. Cell. Biol.*, 2007, **27**, 6334–6349.
23 S. A. E. Marras, F. R. Kramer and S. Tyagi, *Nucleic Acids Res.*, 2002, **30**, e122.
24 N. L. Rosi and C. A. Mirkin, *Chem. Rev.*, 2005, **105**, 1547–1562.
25 M. Zuker, *Nucleic Acids Res.*, 2003, **31**, 3406–3415.
26 S. X. Su, R. Nutiu, C. D. M. Filipe, Y. F. Li and R. Pelton, *Langmuir*, 2007, **23**, 1300–1302.
27 D. J. Maxwell, J. R. Taylor and S. M. Nie, *J. Am. Chem. Soc.*, 2002, **124**, 9606–9612.
28 H. C. Yeh, Y. P. Ho, I. M. Shih and T. H. Wang, *Nucleic Acids Res.*, 2006, **34**, e35.