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Hybridization kinetics of double-stranded DNA probes for rapid molecular analysis†

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This study reports the hybridization kinetics of double-stranded DNA probes for rapid molecular analysis. Molecular binding schemes based on double-stranded DNA probes have been developed for quantitative detection of various biomolecules, such as nucleic acids and DNA binding proteins recently. The thermodynamic competition between the target and the competitor in binding to the probe provides a highly specific mechanism for molecular detection. The kinetics of the double-stranded DNA probe, on the other hand, represent another key aspect toward its general applicability for a wide set of biomedical applications. Herein we report a systematic investigation of the kinetics of double-stranded DNA probes. The signal-to-background ratio and assay time of the double-stranded DNA probes are optimized at a high ionic strength (over 100 mM NaCl). Both the donor probe and the quencher probe sequences are shown to be important in the hybridization kinetics. A long sticky end of the probe is able to dramatically accelerate the kinetics of the assay. To provide a quantitative description of the kinetics, a two-stage binding model is developed to describe the major features of the kinetics of the assay. The sensitivity of the kinetic model and the dominant affinity constants are studied. The study provides a general guideline for the design of the probes for reducing the total assay time. With an appropriate design of the probes, the assay can be finished within minutes at room temperature.

Introduction

The ability to rapidly detect molecular biomarkers is highly desirable in various biological applications, such as point-of-care diagnostics of pathogenic agents and high-throughput screening of therapeutic and preventive agents.^{1–6} Nevertheless, the complicated protocols associated with many molecular assays often present technological hurdles in the successful implementation of these applications.⁷ Recently, several homogenous molecular binding schemes based on double-stranded DNA (dsDNA) probes have been developed for rapid detection of molecular biomarkers, such as nucleic acids and proteins, in a mix-and-measure format.^{8–15} In the dsDNA sensing scheme for nucleic acids, a DNA sequence conjugated with a fluorophore (donor probe) is designed based on the target nucleotide sequence. To enable homogeneous detection of the target nucleotide sequence without any separation step, the donor probe is hybridized to a complementary sequence conjugated with a quencher (quencher probe). As a result, the donor and

quencher are in close proximity diminishing the fluorescence signal in the absence of a target. With the existence of a target molecule, the donor probe thermodynamically separates from the quencher probe and binds to the target, which renders the donor probe fluorescent (Fig. 1). The concentration of the target nucleic acid sequences can be estimated quantitatively based on the fluorescence intensity. With an appropriate design of the probes, the assay can be finished in minutes at room temperature, which greatly simplifies the system requirement and assay protocol for point-of-care diagnostics and high-throughput drug screening.

The dsDNA probes have been applied in various biological applications. For instance, the dsDNA binding scheme provides a useful tool for quantifying the target nucleic acids in real-time polymerase chain reaction and for real-time quantification of RNA polymerase activity.^{8–10,15} By proper design of the quencher sequence, the dsDNA probe is capable of discriminating single nucleotide mismatches in a single step.¹¹ The dsDNA binding

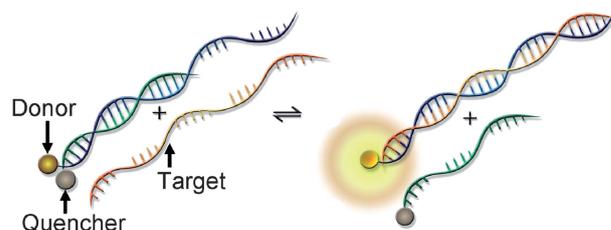


Fig. 1 Schematics of the molecular binding scheme for homogeneous detection of specific nucleic acid sequences using dsDNA probes.

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scheme can also be modified for detecting other biomolecules. By designing the donor probe sequence based on a DNA/RNA aptamer, a single-stranded (ss) oligonucleotide sequence that binds a specific target, the probe has been demonstrated for detecting proteins and other small molecules specifically.^{12–14} Furthermore, the dsDNA probe is capable of detecting dsDNA binding proteins, such as a transcription factor, by introducing a competitor probe, which competes thermodynamically with the quencher probe. In this scheme, the dsDNA binding protein stabilizes the probe and prevents the competitor from separating the donor and quencher probes. For instance, a simple, rapid molecular assay for nuclear factor kappa B without any separation or immobilization step has been demonstrated.^{16,17} This allows rapid quantification of transcription factors and other dsDNA binding proteins without any separation or immobilization step.

The kinetics of a molecular assay are a key aspect that determine its general applicability for different biological applications. The kinetics of the dsDNA probe are primarily determined by the molecular switching between the target (or competitor) and the quencher probe. The total assay times can range from minutes to hours depending on the experimental conditions.¹² While the kinetics of the dsDNA probe are a critical consideration in various biomedical applications, the parameters that govern the kinetics of the dsDNA probe have not been investigated systematically. A thorough understanding of the kinetics of the dsDNA assay will allow the maximization of the hybridization level (*i.e.* the signal) that can be archived and will facilitate the elimination of the uncertainty as a result of the transient dynamics.

Herein, we present a systematic investigation of the hybridization kinetics of dsDNA probes for homogeneous molecular analysis at room temperature. We first evaluate the dependence of the ionic strength on the kinetics of the assay. A set of dsDNA probes with different lengths of the donor and quencher sequences has been designed and tested experimentally. A two-stage binding model, which considers the competitive dynamics of dsDNA assay, has also been developed to describe the experimental data and to provide insights into the sequence design. This study will serve as an example for the design of the dsDNA probes for rapid molecular analysis.

Materials and methods

Kinetic analysis

An equilibrium analysis, which successfully describes the steady-state behavior of the dsDNA assay, has been reported previously.¹¹ This one-stage binding model can be considered to be two coupled reversible reactions (eqn (1) and (2)). In this model, the free donor probe M can bind to the quencher probe Q or the target T. Due to thermal fluctuation, a portion of the donor probe is disassociated from the quencher probe. The concentration of donor probe separated from the quencher probe (the free donor probe) can be estimated based on the equilibrium constant of the reaction. The free donor probe is available for binding to the target or the quencher probe again depending on their concentrations and association constants.



These two reversible reactions were the basis of the equilibrium analysis.¹¹ However, this model is found to be inadequate for describing the kinetics of the assay in this study. In view of this, we have developed a two-stage binding model that includes an intermediate binding step of the reaction. Generally, the quencher probe is shorter than the target such that the target is thermodynamically favorable to switch with the quencher probe. Therefore, the hybridized probe typically has an overhang of the unpaired sequence in the donor probe (the free or sticky end) and its length depends on the difference between the length of the quencher and donor probes. In the two-stage binding model, the target is transiently bounded to the sticky end. Then, the target displaces the quencher probe bound to the donor probe. This intermediate binding step can be described by two reversible reactions (eqn (3) and (4)).



In this study, all four reactions (eqn (1)–(4)) are considered for capturing different possible dynamics of the dsDNA assay. These reversible reactions can be modeled as a set of six second-order kinetic equations (eqn (5)–(10)) with eight reaction rate constants,

$$\frac{d[M]}{dt} = -k_{1+}[M][Q] - k_{2+}[M][T] + k_{1-}[MQ] + k_{2-}[MT] \quad (5)$$

$$\frac{d[Q]}{dt} = -k_{1+}[M][Q] - k_{4+}[MT][Q] + k_{1-}[MQ] + k_{4-}[MQT] \quad (6)$$

$$\frac{d[T]}{dt} = -k_{2+}[M][T] - k_{3+}[MQ][T] + k_{2-}[MT] + k_{3-}[MQT] \quad (7)$$

$$\frac{d[MQ]}{dt} = -k_{3+}[MQ][T] - k_{1-}[MQ] + k_{3-}[MQT] + k_{1+}[M][Q] \quad (8)$$

$$\frac{d[MT]}{dt} = -k_{4+}[MT][Q] - k_{2-}[MT] + k_{4-}[MQT] + k_{2+}[M][T] \quad (9)$$

$$\begin{aligned} \frac{d[MQT]}{dt} = & -k_{3-}[MQT] - k_{4-}[MQT] + k_{3+}[MQ][T] \\ & + k_{4+}[MT][Q] \end{aligned} \quad (10)$$

where k represents the reaction rate constant. The number in the subscript describes the reaction corresponding to eqn (1)–(4) and the ‘+’ and ‘-’ signs represent the forward and reverse reactions. In this work, these equations were solved numerically using

MATLAB and Gepasi. The initial probe and target concentrations were determined from the experimental conditions. In general, the reaction rate constants depend on the probe sequence and the buffer conditions. The values were determined by numerical fitting with the experimental data. The ratio of the forward and reverse rate constants (*i.e.* the equilibrium constant), K , was constrained by the free energy change of the reaction. The relationship between the equilibrium constant and the free energy change is given by $K = e^{-\Delta G/RT}$, where R is the universal gas constant, T is the absolute temperature, and ΔG is the free energy change. The free energy changes of the reactions were determined based on the probe sequences at 23 °C and 75 mM sodium chloride (NaCl) using the Mfold server.¹⁸ Further description of the kinetic model and procedures of the numerical analysis can be found in the ESI.†

Probe design

A set of DNA probes with different lengths of the donor probes and quencher probes was designed to systematically evaluate the kinetics of the dsDNA binding scheme. The probes were designed for an antioxidant gene, heme oxygenase-1 (HO-1).^{19,20} This assay can potentially be applied for high-throughput screening of agents that upregulate the endogenous antioxidant response. Three donor probes of 18–24 bases in length were designed. The donor probes were labeled with 6-FAM (fluorescein) at the 5' end. Five complementary quencher probes of 10–23 bases in length were also designed. The quencher probes were labeled with Iowa Black FQ at the 3' end. The selection of the quencher was based on its high quenching efficiency for 6-FAM. The 24-base target nucleotide sequence was complementary to the donor probe. Table 1 summarizes the probe designs in this study.

Sample preparation

DNA probes and targets were synthesized by Integrated DNA Technologies Inc. All reagents were purchased from Sigma Inc. Unless otherwise specified, the dsDNA probe was prepared in a buffer of 10 mM Tris-EDTA and 75 mM NaCl. Before the experiment, the quencher probe and the donor probe were pre-hybridized at 90 °C for 5 minutes. The probes were then allowed to cool down slowly in a dry-bath incubator for 3 h. In the ionic strength experiment, the 24-base donor probe and the 18-base quencher probe were mixed in 384-well plates at the corresponding NaCl concentration. In the kinetic experiments, all combinations of donor and quencher probes were tested. A

relative low salt concentration (75 mM NaCl) was used in order to obtain high temporal resolution of the kinetics of the assay. The concentrations of the quencher probe and donor probes were 30 nM and 60 nM, respectively. A target concentration of 312 nM, which was roughly 10 times higher than the probe concentration, was used in all experiments. To determine the titration curve, the 24-base donor probe was pre-hybridized with the 10-base quencher probe and mixed with different concentrations of the target sequence in 384-well plates. All fluorescence measurements were taken using a programmable fluorescence microplate reader (BioTek, Synergy 2) at room temperature.

Data analysis

The kinetic data were analyzed with two different approaches to compare different probe designs quantitatively. In the first model independent approach, the switching half-time, which was defined as the time that the fluorescence intensity reached 50% of the maximum value, was estimated from the time course data of the fluorescence intensity after mixing of the probe with the target. If the probe had a signal-to-background ratio less than three, the intensity change was considered to be insignificant and the half-time was discarded. In the second approach, the kinetic constants were extracted by fitting the kinetic data with the numerical result of eqn (5)–(10). A detailed description of the fitting procedure is discussed in the ESI.† All experiments were performed in triplicates and data are reported as mean \pm standard deviation.

Results

Ionic strength

The ionic strength is known to play an essential role in the hybridization kinetics of DNA molecules. We first studied the dependence of the ionic strength on the kinetics of the dsDNA probes with different NaCl concentrations. The 24-base donor probe and 18-base quencher probe were used in the experiment. Fig. 2a shows the kinetics of the assay at different salt concentrations. At room temperature, the assay finished in less than 10 minutes at 300 mM NaCl and took over two hours to finish at 5 mM NaCl. The half-time generally decreased with the salt concentration (Fig. 2b). The half-time decreased rapidly in the range of 5–50 mM NaCl and decreased slowly for NaCl concentrations over 100 mM. In addition, we measured the intensity before and after introducing the HO-1 target sequence at different salt concentrations (Fig. 2c). The background level

Table 1 Sequences of DNA probes and target in this study

Probe type	Label	Sequence	Length, bases
Donor probe	5' 6-FAM	5' TGG GCT CTC CTT GTT GCG CTC AAT 3'	24
Donor probe	5' 6-FAM	5' TGG GCT CTC CTT GTT GCG CTC 3'	21
Donor probe	5' 6-FAM	5' TGG GCT CTC CTT GTT GCG 3'	18
Quencher probe	3' Iowa Black FQ TM	3' ACC CGA GAG GAA CAA CGC GAG TT 5'	23
Quencher probe	3' Iowa Black FQ TM	3' ACC CGA GAG GAA CAA CGC 5'	18
Quencher probe	3' Iowa Black FQ TM	3' ACC CGA GAG GAA CAA 5'	15
Quencher probe	3' Iowa Black FQ TM	3' ACC CGA GAG GAA 5'	12
Quencher probe	3' Iowa Black FQ TM	3' ACC CGA GAG G 5'	10
Target/competitor	Unlabeled	3' ACCCGAGAGGAACAACGCGAGTTA 5'	24

showed only a weak dependence on the ionic strength. The steady-state signal level increased with the concentration of NaCl and displayed a sharp increase in the range of 5–50 mM. This resulted in a better signal-to-noise ratio in high ionic strength solutions (Fig. 2d).

These observations are in general agreement with other hybridization kinetic studies of ssDNA in solutions and on surfaces.^{21–23} Our results indicated that a relative high ionic strength (>100 mM) should be applied for rapid kinetics of the dsDNA assay. Since DNA molecules are negatively charged, the ionic strength has a major influence on the kinetics of DNA hybridization. In low ionic strength solutions, the large electrostatic repulsion between probe strands results in less probe binding and slower kinetics. For effective hybridization, the electrostatic repulsion has to be effectively screened by counter ions, such as using high ionic strength buffers.

Probe sequences

In addition to the ionic strength of the hybridization buffer, the probe sequence should be optimized for rapid molecular analysis at room temperature. Since the disassociation rate of the dsDNA probe depends on the hybridized sequence, the length of the quencher probe that hybridized to the donor probe is likely to play an important role in the kinetics of the assay. To elucidate the influence of the quencher probe on the kinetics of the assay, we designed quencher probes from 10 bases to 23 bases. Fig. 3a shows the kinetics of target detection of a 24-base donor probe hybridized with different quencher probes. The experiments were performed at room temperature and 75 mM NaCl. Generally,

a short quencher sequence showed faster kinetics. The half-time of the assay was approximately 4 minutes for the 10-base quencher probe and was over 40 minutes for the 18-base quencher. The intensity change for the 23-base quencher was insignificant after 3 hours and was not further analyzed. Fig. 3b shows the half-time values estimated for different quencher lengths. The result clearly indicates the dominance of the quencher probe design on the kinetics of the dsDNA assay.

We have also studied the influence of the length of the donor probe. Fig. 4 shows the kinetics of three donor probes (24, 21 and 18 bases) with the 18-base quencher probe. The half-time for the pair of the 24-base donor probe and the 18-base quencher probe was approximately 40 minutes. Interestingly, the changes in intensity were insignificant for the two short probes (21 and 18 bases). For an 18-base quencher, the 21-base donor probe did not result in a significant increase in the fluorescence intensity after 6 hours while it was only 3 bases shorter than the 24-base donor. This observation could not be explained by the one-stage binding model, since all three donor probes were hybridized to the same quencher probe (18 bases) with similar equilibrium constants and disassociation rates. As the major difference between the probes was the length of the sticky end, these data indicated that the sticky end could also play a role in determining the kinetics of the assay. The half-times for different combinations of donor probes and quencher probes were measured. For dsDNA probes with a sticky end shorter than 6 bases, the changes in intensity were insignificant after 6 hours of incubation and, therefore, were not further analyzed. Fig. 4b shows the effect of the length of the sticky end for the probes studied in this work. The half-time of the assay depended strongly on the length of the sticky end. A

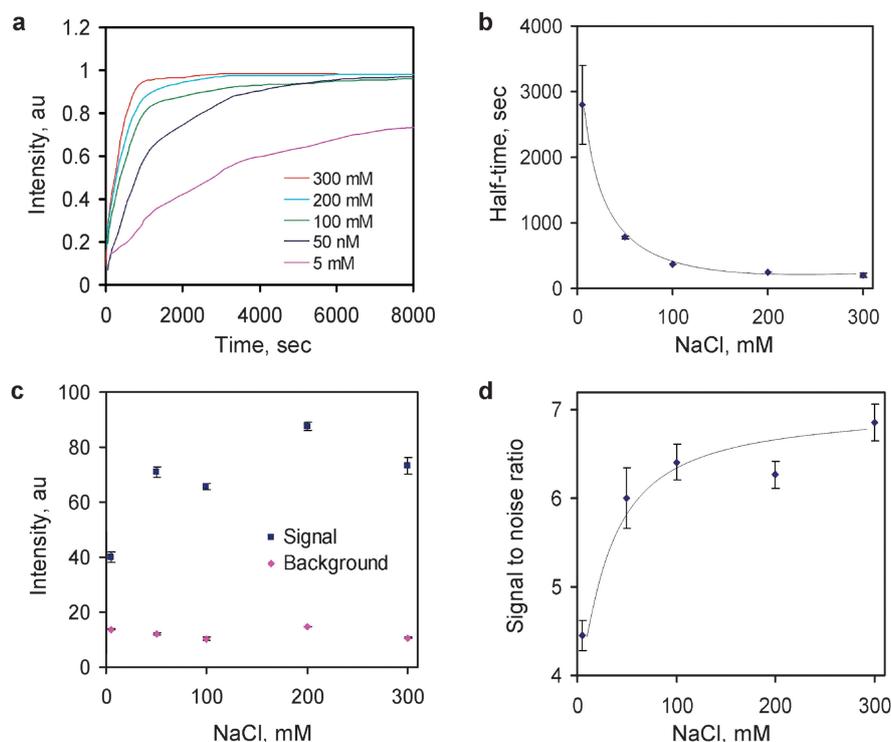


Fig. 2 Effects of the salt concentration on the dsDNA assay. (a) Representative kinetics of the dsDNA assay at different salt concentrations. (b) Switching half-time as a function of the salt concentration. (c) Fluorescence signal before and after the addition of the target. (d) Signal-to-background level as a function of the salt concentration. Data represent mean \pm standard deviation.

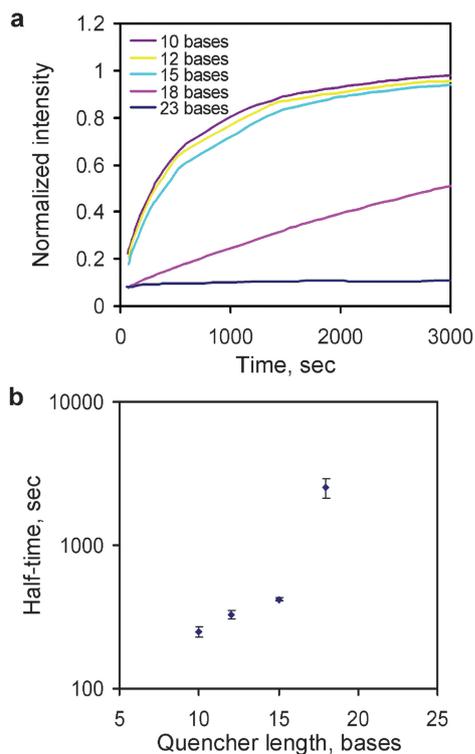


Fig. 3 Effects of the length of the quencher probe on the dsDNA assay. (a) Representative kinetics of the probe with different quencher lengths. The donor probe was 24 bases in length. (b) Switching half-time as a function of the quencher length.

longer sticky end generally had a smaller half-time. This clearly demonstrates the role of the sticky end on the kinetics of the dsDNA assay. For probes with the same sticky end length, the half-time was mainly determined by the quencher length. For a 6-base sticky end, the half-time of an 18-base quencher was over 40 minutes while the half-time of a 12-base quencher was approximately 3 minutes. These data supported that the one-stage binding model is insufficient for describing the kinetics of the dsDNA assay.

Two-stage binding model

Based on the experimental observation, a two-stage binding model is proposed to incorporate the influence of the sticky end on the kinetics of the dsDNA assay. In this model, the target first binds to the sticky end and then displaces the quencher probe (Fig. 5). Four reversible binding reactions with six reaction rate equations and eight reaction rate constants were involved. The responses were determined by solving the kinetic equations numerically (see ESI, S1†). Fig. 6a shows a representative kinetic response of the assay estimated using the two-stage binding model. Initially, the target quickly bound to the dsDNA probe, which resulted in the rapid increase in the concentration of the MQT complexes and the decay in the concentration of the MQ complexes. Then, the target slowly displaced the quencher probe as indicated by the gradual increase of the MT complexes. Fig. 6b shows a comparison between the experiment data and the numerical result, which are in reasonable agreement. Another possible scenario is that the binding reaction between the target

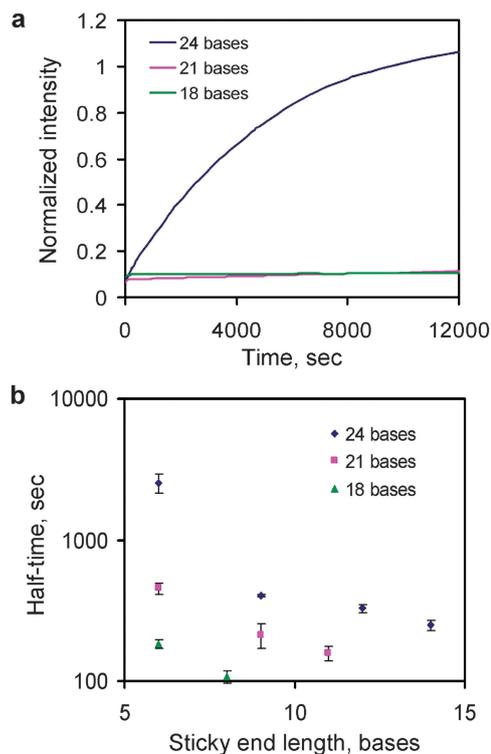


Fig. 4 Effects of the lengths of the donor probe and the sticky end. (a) Representative kinetics of the probe with different donor probes. The quencher probe was 18 bases in length. (b) Switching half-time as a function of the sticky end length.

and the dsDNA probe (eqn (3)) is slower than the displacement reaction (eqn (4)). In this situation, the intensity data would display a significant delay in the initial fluorescence response, which was not observed for probes with long sticky ends (see also ESI S2†). Therefore, the total assay time was likely dominated by the displacement reaction in our experimental conditions (eqn (4)).

A sensitivity analysis was performed to identify the key parameters in the two-stage binding model (see ESI S2†). The analysis showed that the kinetics of the assay were primarily determined by two of the reaction rate constants. These reaction rate constants represented the binding of the target to the sticky end (k_{3+} , the binding rate) and the displacement of the quencher probe (k_{4-} , the displacement rate). These constants were applied to fit all the kinetic data of different combinations of quencher and donor probes (ESI S3†). In the fitting procedure, the binding rate (k_{3+}) was assumed to depend only on the length of the sticky end, and the displacement rate (k_{4-}) was assumed to depend only on the length of the quencher. For instance, an 18-base quencher probe would have the same displacement constant for 21-base and 24-base donor probes as the sequence involving in the displacement reaction was the same. This actually presents a constrain for testing the capability of the two-stage binding model in predicting the kinetics of the assay. The two-stage binding model was in good agreement with the major features of the experimental data (see ESI Fig. S3†). Fig. 7 shows the kinetic constants as a function of the sticky end length and the quencher length determined from the fitting procedure. Generally, the

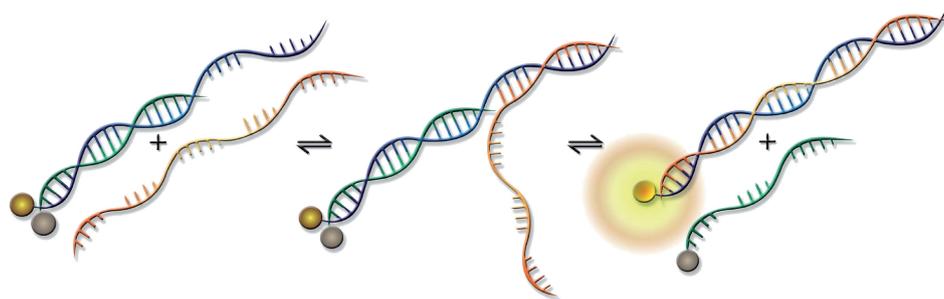


Fig. 5 Schematics of the two-stage binding model.

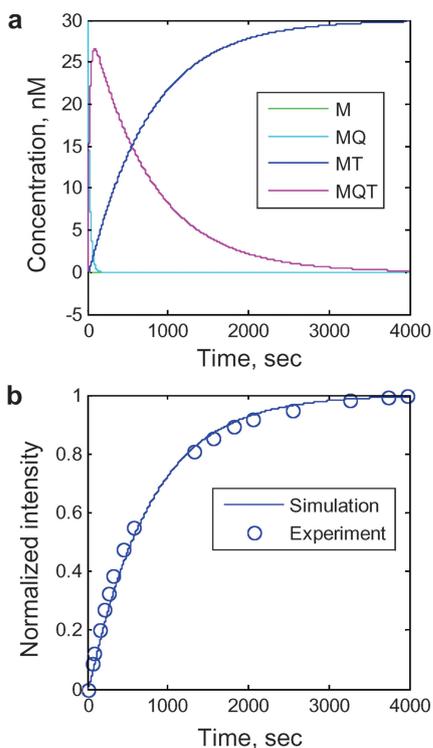


Fig. 6 Two-stage binding model. (a) Kinetic responses of the assay simulated using the two-stage binding model. (b) Comparison of the experimental and numerical results.

binding rate increased with the sticky end length while the displacement decreased with the quencher length. It should be noted that the two-stage binding kinetic model also successfully predicted the titration curve using these reaction rate constants (ESI S4†). In addition, we have also investigated the dependence of the salt concentration on the kinetics of the assay. Fitting the experimental data with the model reveals that the displacement constant increases with the salt concentration (ESI S5†). The data appeared to follow a power law with a scaling constant of 0.66 in the range tested. This provided an empirical relationship to predict the rate constants (see ESI Fig. S6†).

Discussion

In this study, the kinetics of dsDNA probes in homogeneous solution were investigated. Previously, several experimental and

theoretical investigations have been performed to study the binding of single-stranded nucleic acid targets on solid substrates.^{24,25} For dsDNA probes coupled on a solid substrate,²⁶ the reaction is often diffusion limited and the model should be modified to incorporate the mass transport kinetics. The current study, on the other hand, focuses primarily on the kinetics of dsDNA probes in a homogeneous, reaction rate limited regime. A related kinetic study has been reported earlier for molecular beacons, which are dual label molecular probes with a stem-loop structure.²⁷ The reaction rate was observed to be highly sensitive to the length of the stem sequence, which played a similar role to the sticky end of the dsDNA probe. An addition of one base in the probe length was found to result in a 10-fold increase in the rate constant. This is in qualitative agreement with our results.

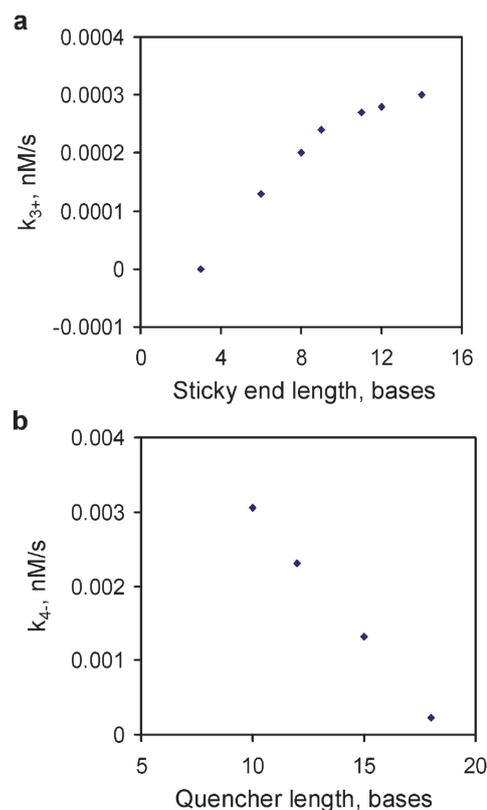


Fig. 7 Dependences of the probe design. (a) The binding rate, k_{3+} , as a function of the sticky end length. (b) The disassociation rate, k_{4-} , as a function of the quencher length.

Strictly speaking, the reaction rate constants should depend on the specific DNA sequence instead of the sequence length.²⁴ This study will serve as a design example and provide useful guidelines for designing of dsDNA probes, especially for molecular analysis at room temperature.

Our results indicate the importance of the free sticky end on the kinetics of dsDNA probes. Since the quencher sequence is often designed for optimizing the signal-to-noise ratio, the inclusion of the sticky end provides a simple way to optimize the dsDNA sequences independently. To interpret the role of the sticky end, the displacement reaction can be considered to involve two separate sub-steps: (sub-step 1) disassociation of the quencher probe from the donor probe and (sub-step 2) binding of the remaining portion of the target sequence to the donor probe. With a long sticky end, the target is able to hybridize stably with the sticky end. This can be considered as a local increase of the target concentration. In this situation, the target is able to hybridize efficiently to the remaining portion of the donor sequence when the quencher probe thermally disassociates from the donor probe. This provides an effective mechanism for accelerating the kinetics of the assay. A related concept has also been demonstrated to increase the kinetics for detecting dsDNA binding proteins by physically linking two sets of probes with a tether.²⁸ Without a long sticky end, the target is not able to stably hybridize to the probe and the disassociation of the quencher probe represents the time limiting step of the reaction. In this situation, such as in single nucleotide mismatch detection with a relatively long quencher sequence, the reaction should be performed at elevated temperature to increase the chance of the disassociation of the dsDNA probe.¹¹ If the dsDNA probe is designed based on an aptamer sequence for detection of small molecules, this intermediate binding with the sticky end is unlikely to be effective. Similarly, a relatively short quencher sequence and elevated temperature should be used to increase the reaction rate. The half-time of a dsDNA probe with an 11-base quencher probe for ATP detection was reported to be 830 minutes at 22 °C and 6.8 minutes at 37 °C.¹² These results are consistent with our study.

Conclusion

The flexibility of the dsDNA binding schemes has already been demonstrated for the detection of various biomolecules for different biomedical applications. In this study, we investigated the kinetics of the dsDNA probes. The two-stage binding model can serve as a general guideline for implementing dsDNA assays for rapid molecular analysis at room temperature. With an appropriate design of the probe sequences and buffer conditions, the assay can be finished within minutes without any heating or separation step. The simplicity of dsDNA probes and the rapid kinetics available can potentially facilitate intracellular measurement of gene expressions, rapid quantification of 16s rRNA for point-of-care diagnostics of pathogenic microorganisms, and high-throughput drug screening with cell-based assays.

Acknowledgements

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SUPPLEMENTARY INFORMATION

Hybridization kinetics of double-stranded DNA probes for rapid molecular analysis

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S1. Kinetic model

A two-stage binding model is developed to describe the kinetics of the double-stranded (ds) DNA probe. In this model, a free target molecule first binds to the sticky end of the dsDNA probe and displaces the quencher probe subsequently (Figure S1). This binding scheme is modeled as four reversible binding reactions (equations 1-4).

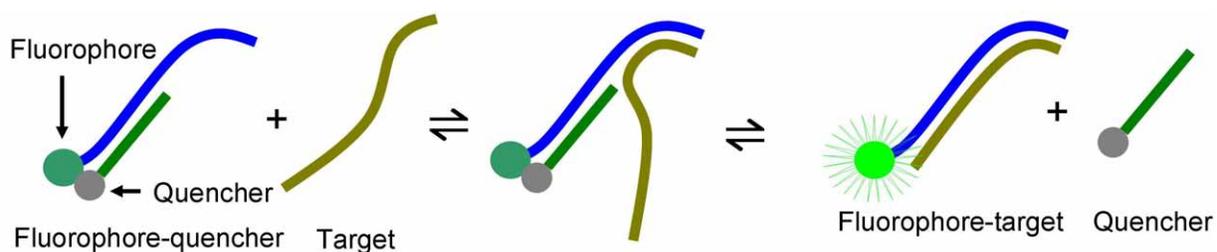


Figure S1. A two-step binding model for describing the kinetics of dsDNA probes.

M , Q , and T represent the free donor probe, quencher probe and target. MQ , MT , and MQT are molecular complexes of the donor probe bound to the quencher probe, target, and both quencher probe and target simultaneously. In this two-stage binding model, these reactions are modeled by second-order reaction kinetics. Four reversible reactions, six molecular species, and eight kinetic rate constants are involved to capture the dependences of the kinetics on the quencher probe and the sticky end of the dsDNA probe (equation 5-10).

$$\frac{d[M]}{dt} = -k_{1+}[M][Q] - k_{2+}[M][T] + k_{1-}[MQ] + k_{2-}[MT] \quad (5)$$

$$\frac{d[Q]}{dt} = -k_{1+}[M][Q] - k_{4+}[MT][Q] + k_{1-}[MQ] + k_{4-}[MQT] \quad (6)$$

$$\frac{d[T]}{dt} = -k_{2+}[M][T] - k_{3+}[MQ][T] + k_{2-}[MT] + k_{3-}[MQT] \quad (7)$$

$$\frac{d[MQ]}{dt} = -k_{3+}[MQ][T] - k_{1-}[MQ] + k_{3-}[MQT] + k_{1+}[M][Q] \quad (8)$$

$$\frac{d[MT]}{dt} = -k_{4+}[MT][Q] - k_{2-}[MT] + k_{4-}[MQT] + k_{2+}[M][T] \quad (9)$$

$$\frac{d[MQT]}{dt} = -k_{3-}[MQT] - k_{4-}[MQT] + k_{3+}[MQ][T] + k_{4+}[MT][Q] \quad (10)$$

where k is the reaction constant. The subscript number represents the reaction in equations 1-4 and “+” and “-” signs denote the forward and reverse reactions.

In this study, the kinetic responses were simulated using MATLAB R2008a (version 7.6) and Gepasi 3. The initial concentrations of the species and the kinetic rate constants were required for simulating the kinetic responses. The initial conditions were determined based on our experimental conditions. In the experiment, 30 nM donor probes and 60 nM quencher probes were hybridized at 90°C and allowed to cool down slowly in the dry bath incubator for three hours. The initial concentrations of M , Q , and MQ applied in the numerical simulation were 0, 30, and 30 nM assuming all the donor probes were hybridized to the quencher probes initially. Similarly, the initial concentrations of T , MT , and MQT were 312, 0, and 0 nM. The rate constants were estimated based on reported values and further optimized by fitting with experimental data. The ratio of the forward and reverse rate constants (i.e., the equilibrium constant), K , was constrained by the free energy change of the reaction. The equilibrium constant is given by $K = e^{-\Delta G/RT}$, where R is the universal gas constant, T is the absolute temperature, and ΔG is the free energy change. The free energy changes of the reactions were estimated based on the probe sequences at 23°C using the Mfold server. The free energy changes for M-T and M-Q binding were estimated to be -33 kcal/mol and -19.3 kcal/mol, respectively. The kinetic rate constants optimized for the dsDNA pair of the 21-base donor probe and the 15-base quencher probe is shown in Table 1 (see Figure 6).

Table S1. Kinetic rate constants for the 21-base donor-15-base quencher pair.

	k_{1+}	k_{1-}	k_{2+}	k_{2-}	k_{3+}	k_{3-}	k_{4+}	k_{4-}
Rate	4.0×10^{-3}	2.2×10^{-17}	7.5×10^{-3}	1.9×10^{-25}	1.3×10^{-4}	1.3×10^{-10}	3.1×10^{-5}	1.3×10^{-3}
unit	$\text{nM}^{-1}\text{s}^{-1}$	s^{-1}	$\text{nM}^{-1}\text{s}^{-1}$	s^{-1}	$\text{nM}^{-1}\text{s}^{-1}$	s^{-1}	$\text{nM}^{-1}\text{s}^{-1}$	s^{-1}

S2. Sensitivity analysis

Eight kinetic rate constants were involved in the two-stage binding model. To determine the sensitivity of these parameters in the overall kinetic responses, we performed a sensitivity analysis by varying a rate constant while holding others unchanged. Based on the parameters determined in Table S1, a rate constant was multiplied by a factor of 0.01, 0.1, 1, 10, and 100 in the sensitivity analysis. This changed the rate constant for four orders of magnitude. The overall kinetic responses of MT , which primarily determined the fluorescence response, were studied (Figure S2). Generally, the kinetic responses were not sensitive to the rate constants k_{1+} , k_{1-} , k_{2+} , k_{2-} , k_{3-} , and k_{4+} in the range tested. The kinetic data were overlapped on each other and can not be distinguished in the graphs. For k_{3+} , increasing the binding rate constant showed negligible effect on the kinetic responses and decreasing the value induced significant delay on the kinetics (Figure S2e). Mathematically, the kinetic response was only slow down when the displacement rate was small compared to the target binding rate (i.e., $k_{3+} \ll k_{4-}$). Physically, this indicated that the reaction kinetics was dominated by the displacement reaction. In such a situation, the kinetic response curve would display a slow growing period initially. This slow growing period was not observed for probes with long sticky ends (> 6 bases). Therefore, the time limiting step was likely the displacement reaction for a probe with a long sticky ends. In fact, the kinetic response was highly sensitive to the displacement rate constant k_{4-} (Figure S2h). The time scale for the dsDNA assay ranged from seconds to hours. This further indicated the importance of the displacement reaction. Based on this sensitivity analysis, we concluded that the kinetics of dsDNA assays were mainly determined by the binding rate constant (k_{3+}) and the displacement rate constant (k_{4-}). These parameters were used for the curve fitting with the experimental data.

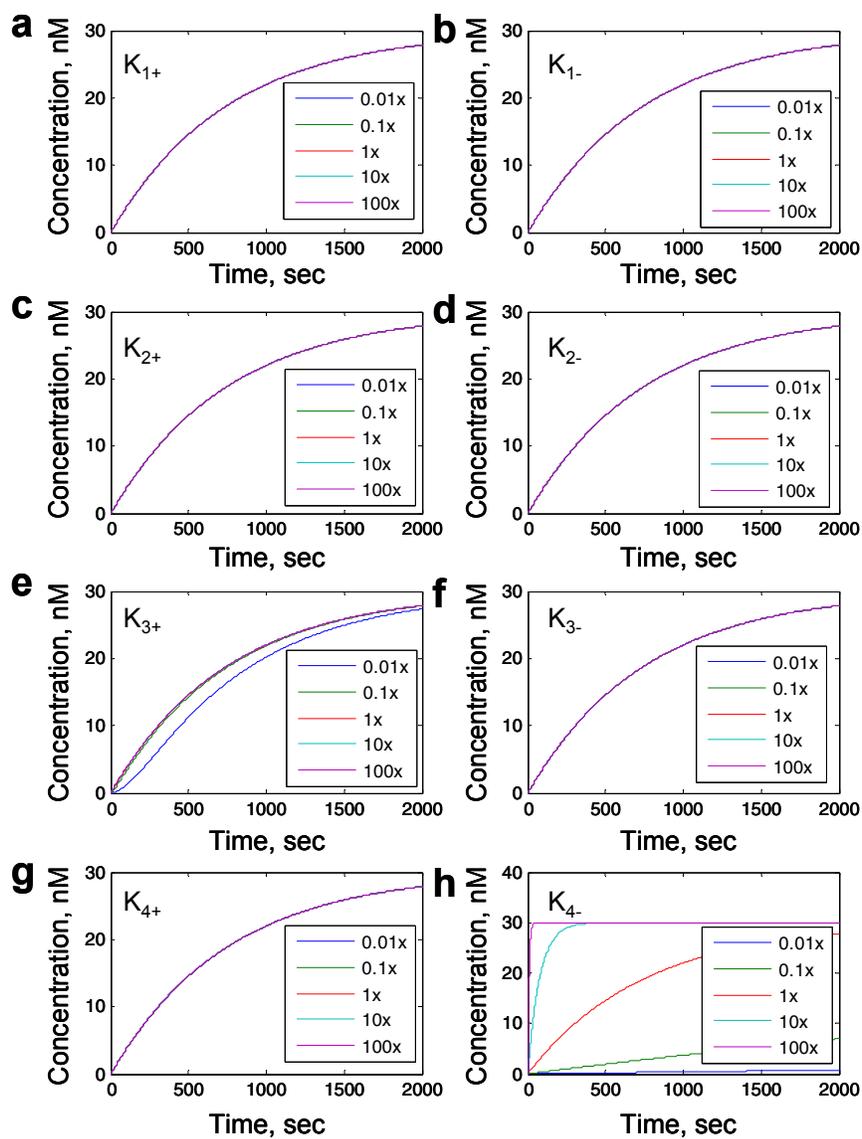


Figure S2. Sensitivity analysis of the kinetic rate constants in the two-stage binding model.

S3. Model fitting for estimating rate constants k_{3+} and k_{4-} .

The rate constants k_{3+} and k_{4-} , which were found to be most sensitivity to the kinetics responses, were determined based on numerical fitting with the experimental data. These constants were applied to fit all the kinetic data of different quencher lengths and donor lengths using Matlab. To fit the data, the displacement constant, k_{4-} , was assumed to be only determined by the length of the quencher. For instance, an 18-base quencher probe would have the same displacement constant when pairing with 21-base or 24-base donor probes, as the sequence involved in the displacement reaction was the same. Similarly, the binding constant, k_{3+} , was assumed to depend on the length of the sticky end, i.e., the same length of the sticky end has the same binding constant k_{3+} . Strictly speaking, the binding constant depends on the sticky end sequence, instead of the length. This simplifies the fitting procedure and will provide a general guideline in the design of the dsDNA assay. This leads a set of seven binding constants and four displacement constants in our experiments. Tables S2 and S3 show the kinetic rate constants determined by the fitting procedure. Figure S3 shows the comparison of the kinetic responses determined by experiments and the two-stage binding model. The two-stage binding is able to describe the general trend observed in our experiment. As determined in the sensitivity analysis, the kinetic response is only sensitive to the binding constant, k_{3+} , when it is small compared to the displacement rate, k_{4-} . The value k_{3+} is likely to be more accurate for probes with short sticky ends.

Table S2. The binding constant k_{3+} for different sticky end lengths

Sticky end, bases	3	6	8	9	11	12	14
k_{3+} , nM ⁻¹ s ⁻¹	2.0×10^{-7}	1.3×10^{-4}	2.0×10^{-4}	2.4×10^{-4}	2.7×10^{-4}	2.8×10^{-4}	3.0×10^{-4}

Table S3. The displacement constant k_{4-} for different quencher lengths

Quencher probe, bases	10	12	15	18
k_{4-} , s ⁻¹	3.1×10^{-3}	2.3×10^{-3}	1.3×10^{-3}	2.3×10^{-4}

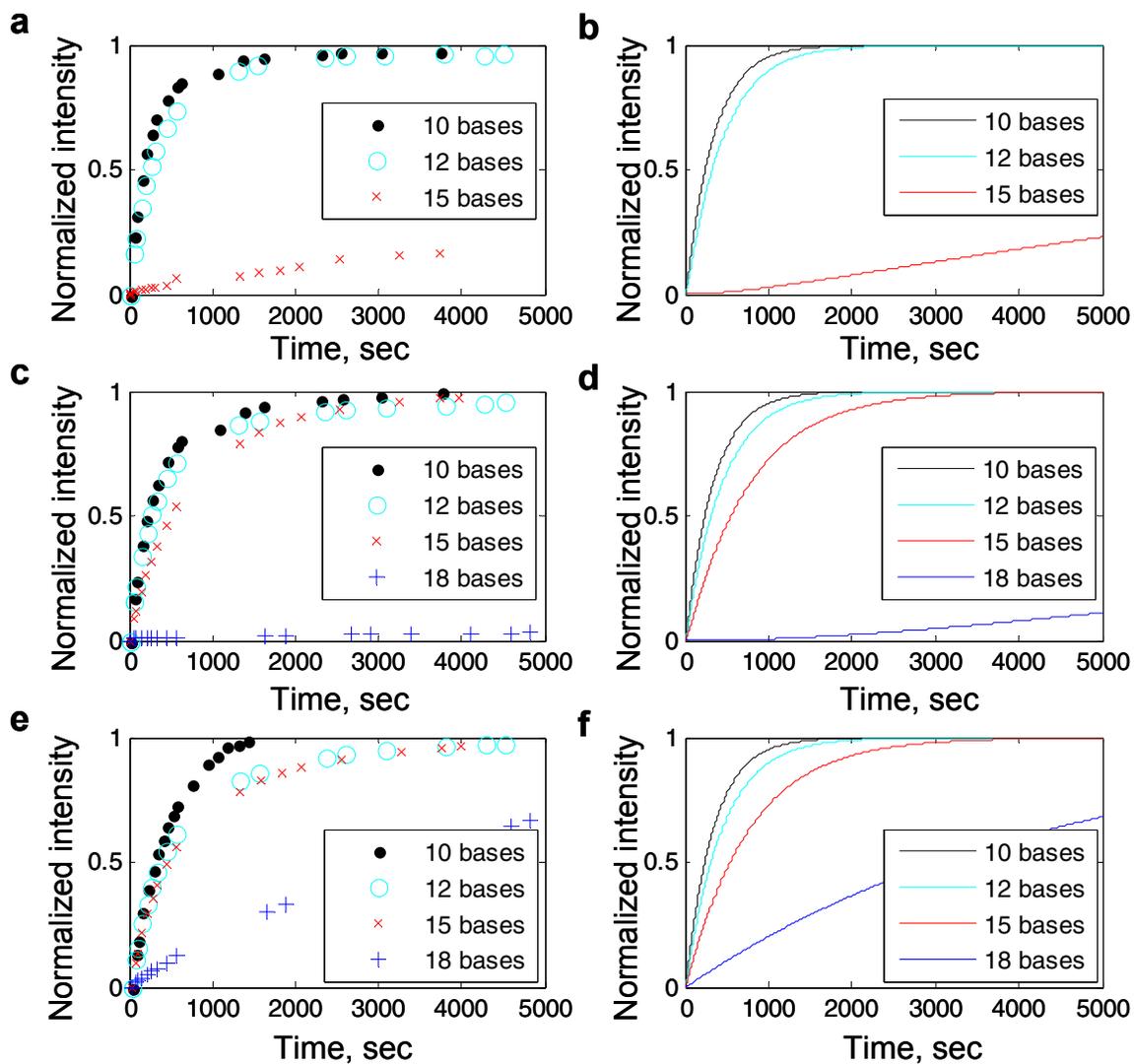


Figure S3. Fitting of experimental data with the two-stage binding model.

S4. Titration curve

Compared to the previous equilibrium analysis, the two-stage binding model introduced two additional reversible reactions to incorporate the intermediate binding step. This could change the steady behavior of the reactions. We studied the equilibrium response of the model to investigate its accuracy in predicting the steady state behavior of the assay. The 24-base donor probe was pre-hybridized with the 10-base quencher probe to determine the titration curve. A serial dilution experiment was performed to prepare the target at different concentrations. The targets were then mixed with the pre-hybridized probe in 384-well plates. Figure S4 shows the comparison of the titration curves determined by the model and experimental data. The result is in good agreement, which demonstrates the applicability of the model for predicting the steady stage response of the assay.

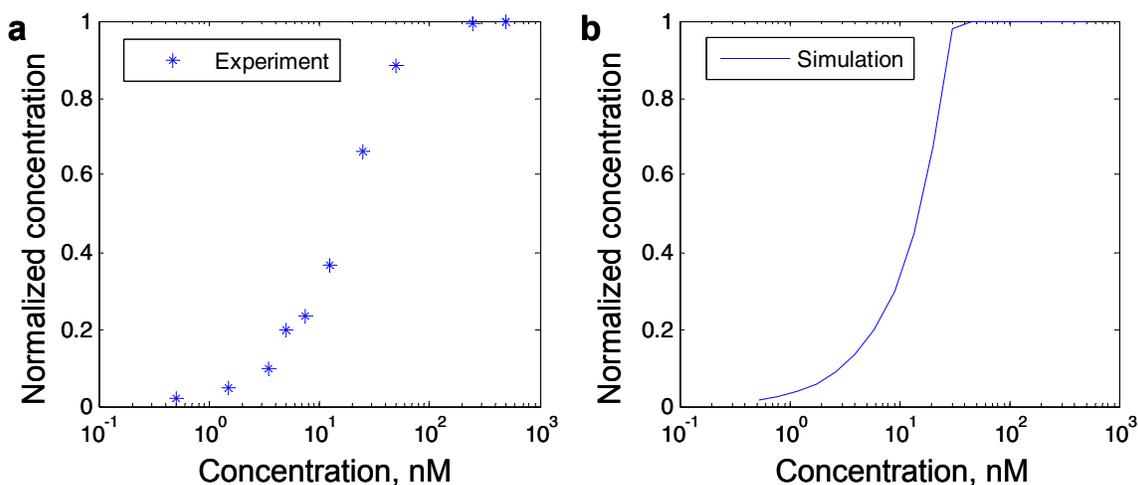


Figure S4. Comparison of the titration curves determined by (a) the two-stage binding model and (b) experiment investigation.

S5. Model fitting for ionic strengths

The dependence of the reaction rate constants on the ionic strength was determined by fitting the experimental data with the two-stage binding model. Since the reaction kinetics was dominated by the displacement reactions, the kinetics was only sensitive to the displacement rate constant, k_{4-} , in the fitting procedure.

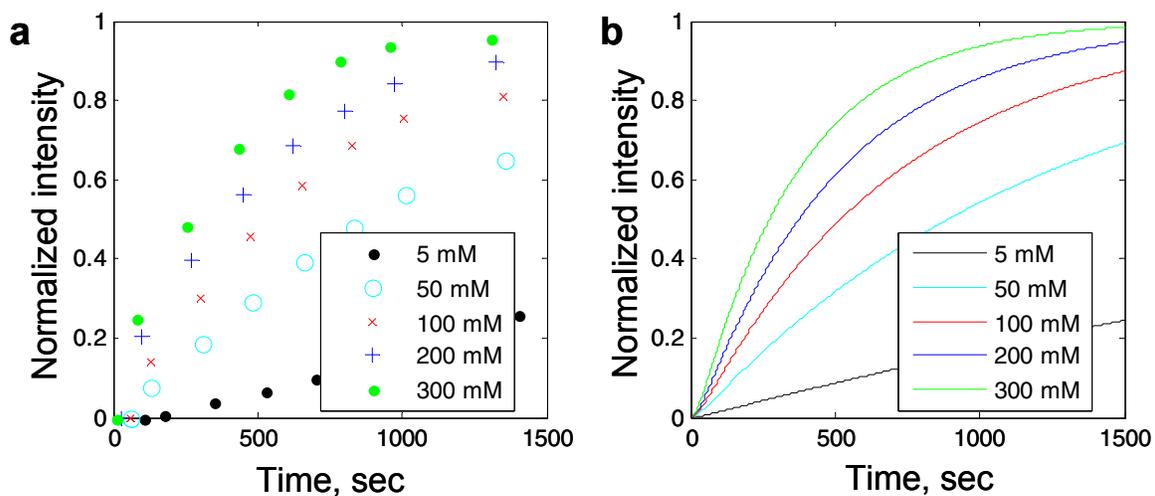


Figure S5. Fitting of kinetic responses at different NaCl concentrations. Kinetics determined by (a) the two-stage binding model and (b) experiment.

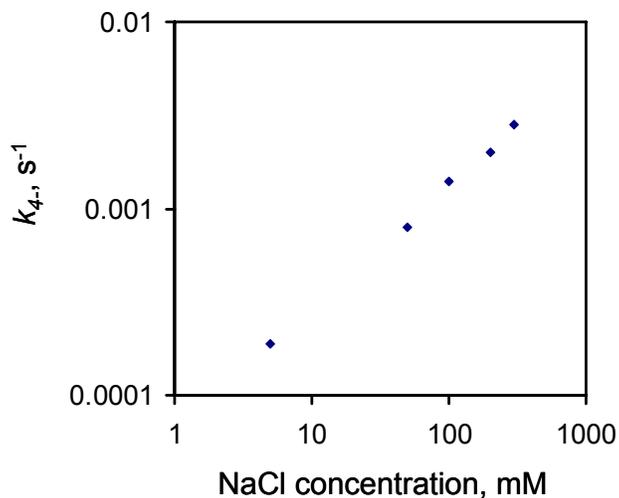


Figure S6. Dependences of the disassociation rate constant, k_{4-} , as a function of the salt concentration.