

Optimizing the specificity window of biomolecular receptors using structure-switching and allostery

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Abstract:

In order to ensure maximum specificity (i.e., minimize cross-reactivity with structurally similar analogues of the desired target), most bioassays invoke “stringency,” the careful tuning of the conditions employed (e.g., pH, ionic strength or temperature). Willingness to control assay conditions will fall, however, as quantitative, single-step biosensors begin to replace multi-step analytical processes. This is especially true for sensors deployed *in vivo*, where the tuning of such parameters is not just inconvenient, but impossible. In response we describe here the rational adaptation of two strategies employed by nature to tune the affinity of biomolecular receptors so as to optimize the placement of their specificity “windows” without the need to alter measurement conditions: structure-switching and allosteric control. We quantitatively validate these approaches using two distinct, DNA-based receptors: a simple, linear-chain DNA suitable for detecting a complementary DNA strand and a structurally complex DNA aptamer used for the detection of a small molecule drug. Using these models we show that, without altering assay conditions, structure-switching and allostery can tune the concentration range over which a receptor achieves optimal specificity over orders of magnitude, thus optimally matching the specificity window with the range of target concentrations expected to be seen in a given application.

Key words: structure-switching biosensors, molecular beacons, rational design, riboswitches, intrinsically unfolded proteins

Optimizing the specificity of biomolecular receptors, i.e., minimizing cross-reactivity with close structural analogues of the targeted molecule, represents an important challenge in the field of bioengineering¹⁻⁷. A key element of specificity is, obviously, the design of a precise lock-and-key complementarity between the receptor and its targets⁸⁻¹⁴. A receptor's specificity can thus be optimised by increasing the difference in binding energy between the properly matched target and any mismatched molecular analogues via alterations in its binding interface. A second element of specificity, however, is that it is optimal over only a limited range of target concentrations, its "specificity window" (Figure 1A, grey rectangle). That is, at concentrations well below a receptor's dissociation constant (K_D), neither the properly matched target nor mismatched molecular analogues bind avidly enough to generate any significant output; where there is no binding, specificity is "moot." Similarly, even mismatched structural analogues binding less avidly than the properly matched target will, at sufficiently high concentrations, achieve near 100% occupancy, pushing the discriminatory power of the receptor towards zero. Optimal specificity thus depends on both a receptor's lock-and-key complementarity and also on its affinity relative to the range of target/anologue concentrations over which it will be working.

Optimal discriminatory power has historically been achieved in bioassays via careful control over the temperature, pH or ionic strength employed, so as to achieve good "stringency." As biology-based detection moves away from complex, multi-step, bench-top assays (such as PCR and Southern blots) and towards direct, single-step devices (such as the home glucose monitor), willingness to employ these cumbersome methods will fall. This is particularly true when sensors are deployed *in vivo*, where the tuning of assay conditions is not so much inconvenient as it is impossible. How, then, can we tune the affinity of the receptors used in biotechnologies so as to optimize their specificity window without resorting to tuning the

experimental conditions? Alterations of the binding interface are one solution, but given current state of biomolecular design this remains challenging.²⁷ For more facile approaches we look here to evolution, which has also developed methods to tune the specificity windows of biomolecular receptors. One such strategy is to introduce a “conformational switch” that reduces affinity in a “tunable” way by coupling recognition to an unfavorable conformational change (Figure 1B). This mechanism is seen, for example, in the case of intrinsically unfolded proteins, which couple target binding to an unfavorable folding free energy¹⁵, simultaneously altering the receptor’s affinity for both its properly matched target and any mismatched analogues and thus providing a route towards tuning the specificity window. A second strategy used by nature to tune the affinity of a receptor “on-the-fly” is to employ allosteric regulators that, upon binding, likewise alter affinity and thus the placement of the specificity window (Figure 1C)^{16,17}. Here we employ the programmability of synthetic DNA to demonstrate the adaptation of these mechanisms to some of the receptors commonly employed in artificial biotechnologies.

Results

To quantify specificity for our comparisons we employ the following definition. The output signal, S , produced by a receptor is given by:

$$S = \chi(S_F + \Delta S) + (1 - \chi)S_F = S_F + \chi\Delta S \quad (\text{Eq 1})$$

where χ is the fraction of receptor bound to target, S_F is the signal from the unbound (free) receptor and thus represents the background signal, and ΔS is the signal change observed upon

target binding. Using this we define a discrimination factor, Q , such that it reports on the relative signal that would be expected from the properly matched (PM) target versus that that would be seen from a mismatched (MM) analogue of the target at the same concentration. From equation 1 we have thus

$$Q = \frac{S_F + \chi_{PM}\Delta S}{S_F + \chi_{MM}\Delta S} \quad (\text{Eq 2})$$

adding to this the dependence of receptor occupancy on target concentration, we have

$$Q = \frac{S_F + \Delta S \left(\frac{[Target]}{[Target] + K_D^{PM}} \right)}{S_F + \Delta S \left(\frac{[Target]}{[Target] + K_D^{MM}} \right)} \quad (\text{Eq. 3})$$

where K_D^{PM} and K_D^{MM} are the receptor's affinities for the proper target and the mismatched analogue, respectively. For the sake of the work here we arbitrarily define "good specificity" (and the associated specificity window) as concentrations for which $Q > 5$ (i.e., the signal arising from the perfectly matched target is 5-times greater than that arising from the mismatched analogue at the same concentration) (Figure S1). This definition of specificity in hand, we next set out to explore the use of structure-switching and allostery as means of tuning the placement of the specificity window such that maximum Q is, optimally, achieved in the mid-point of the expected target concentration.

To explore the use of structure-switching (Figure 1B) we first employed a linear, 13-base DNA as a receptor for its complementary sequence (black) (Figure 2A). In our constructs, this 13-base recognition element is flanked on both sides by five additional non-complementary nucleotides (red) that, in the studies described below, we later used to introduce structure-switching (Figure 2B). Receptor occupancy is then reported by the fluorophore-quencher pair

attached at the sequences two termini; upon target binding the receptor extends from a random coil configuration, separating its termini and enhancing emission. As with any receptor, the linear DNA binds to both its target and to close structural analogues, albeit its affinity for the latter is poorer. For example, the receptor's dissociation, K_D^{PM} , is 7 ± 1 nM when it is challenged with a perfectly matched (fully complementary) target (Figure 2A, orange). When challenged with a single-base mismatch (blue), in contrast, the receptor's affinity is 26-times poorer ($K_D^{\text{MM}} = 180 \pm 20$ nM). Together these values define a specificity window (again, defined here as the range of concentrations for which $Q > 5$) spans ~ 2 -orders-of-magnitude centered around 4 nM (Figure 2A, right).

Tuning the placement of the specificity window using a structure-switching mechanism

To tune the location of the specificity window of our linear DNA receptor we re-designed it so that it undergoes binding-induced structure-switching (Figure 2B).¹⁸ That is, we modified the 5 bases on each of its termini to render them complementary (Figure 2B), causing the receptor to form a stem-loop structure that must be broken in order for target to bind. This couples binding to an unfavourable free energy that we can alter to tune affinity and thus the specificity window. Under these circumstances the receptor's observed affinity, K_D^{obs} , for both its perfectly matched target and any mismatched analogs is related to the equilibrium constant of the structural switch, K_S , and its affinity in the absence of switching, K_D , by the relationship^{18a}:

$$K_D^{\text{obs}} = K_D \frac{1 + K_S}{K_S} \quad (\text{Eq 4})$$

Following this, a modified receptor containing a fully complementary stem composed of 2GC and 3AT base pairs ("2GC") binds with ~ 40 -fold poorer affinity than that of the "non-switching"

parent receptor ($K_D^{\text{PM}} = 270 \pm 30$ nM versus 7 ± 1 nM; Figure 2B). Moreover, because the change in target affinity is due to alteration of the stem sequence, which is distal to the binding site, the affinity of the mismatched analogue target is likewise shifted ~ 40 -fold ($K_D^{\text{MM}} = 7 \pm 1$ μM versus 180 ± 30 nM; Figure 2B), thus shifting the specificity window of the receptor to 40-fold higher concentrations without altering its two-orders-of-magnitude width (Figure 2B).

We can rationally and quantitatively control the range of concentration at which optimal specificity is achieved by varying the switching equilibrium constant, K_S . For the stem-loop receptor we do this by varying the stability of the stem via the replacement of A-T base pairs with G-C base pairs, each of which stabilizes the stem by ~ 4 kJ/mol (determined via urea melts; see ref^{18a} for experimental details), shifting both affinity and the specificity window another 4.3-fold (at room temperature) to higher concentrations²². Using this approach, we have thus created a set of structure-switching receptors displaying specificity windows that shift over four orders of magnitude (Figure 3 and Figure S2).

Tuning the placement of the specificity window using allosteric inhibition

Allosteric control provides a second strategy by which we can rationally tune the specificity windows (Figure 1C and Fig 4A). In this the binding of an effector at a site distal from the target binding site alters K_S and thus, in turn, target affinity and the placement of the specificity window.¹⁹⁻²⁰ As a test bed to explore such control we employed a DNA aptamer binding the antimalarial drug quinine (Figure 3A)²⁴⁻²⁶. As an allosteric inhibitor we employed an oligonucleotide complementary to 14 bases in the aptamer's sequence. Hybridization of this to the aptamer creates a switch (between the double stranded state and the native fold) that again alters affinity (Figure 4A). Using this inhibitor we have tuned the specificity window of the

aptamer over many orders of magnitude (Figure 4B). In absence of inhibitor, for example, the aptamer's affinity for quinine (i.e., $K_D^{\text{quinine}} = 0.84 \pm 0.07 \mu\text{M}$) is 19-fold higher than its affinity for the structural analogue cinchonine (i.e., $K_D^{\text{cinchonine}} = 16 \pm 0.8 \mu\text{M}$), with the discrimination factor Q peaking at $0.8 \mu\text{M}$ (Figure 4B, top). Upon addition of the inhibitor (at $10 \mu\text{M}$) the two dissociation constants shift to $63 \pm 6 \mu\text{M}$ and $790 \pm 90 \mu\text{M}$, respectively (Figure 4B, bottom), and optimal discrimination is reached at a 75-fold higher target concentration.

Conclusions

Here we have shown that the placement of the specificity window can be optimized by coupling recognition to an unfavorable conformational switch or to allosteric control, with either approach allowing the precise, rational placement of the specificity window. Moreover, these approaches involve modifications and interactions that are distal from the binding site, thus rendering them easier to introduce than would be the redesigns of the receptor's binding interface itself²⁷.

The findings described here are likely generalizable to other receptors. This is particularly true, obviously, for nucleic acids, as the equilibrium constants of nucleic acid switches, or the binding energy of a DNA or RNA inhibitor can be rationally varied by altering the strength of their base pairing. Indeed, nucleic acid folding and hybridization prediction programs, such as mfold, produce relatively accurate estimates of the thermodynamics of specific RNA or DNA conformations,²² thus providing precision guidance for such rational design efforts. This said, similar approaches can also be employed to tune the specificity of protein-based switches. A single, key residue, for example controls, the switching thermodynamics of the bacterial periplasmic binding protein superfamily²⁸. In a similar context, the ability to introduce

conformational switching into otherwise non-switching proteins has been demonstrated for many proteins in applications ranging from biosensing to smart materials and therapeutics²⁹. Finally, we have shown that binding-induced protein folding, which is perhaps a switching mechanism often employed by proteins^{15,30}, is readily tuned via substitutions distant from the binding interface that stabilize or destabilize the native state³¹. We expect that the strategies we present in this work will be useful to both optimize the specificity of biosensors³² as well as to optimize the specificity of engineered receptors to be implemented in synthetic bioorganisms³³.

In addition to providing a rational framework for optimizing the placement of the specificity windows of structure-switching biosensors, the thermodynamic principles presented here may also improve our understanding of the mechanisms behind the evolution of receptor specificity. A good example is provided by the intrinsically disordered proteins, proteins that only fold upon binding to their specific target^{15,30}. This switching mechanism, which has been employed in several protein based biosensors³¹, has been proposed as an efficient strategy by which nature reduces the affinity of biomolecules without simultaneously reducing their specificity^{15,30,34}. As our knowledge of the thermodynamics of natural biomolecular switches progresses, it will be interesting to uncover if they have evolved switching mechanism or allosteric regulation mechanism to achieve optimal specificity *in vivo*.

Material and methods

HPLC purified DNA sequences modified with a FAM (6-carboxyfluorescein) and BHQ-1 (Black Hole Quencher), the quinine-binding aptamer, the 13-nucleotide target (both perfect match and mismatch) and the 15-base inhibitor were all purchased from Sigma-Genosys (all stem-loop constructs possess an additional A -after the FAM- and G nucleotides -before the BHQ-1). The

sequences of these DNA strands are as follows. Linear receptor (non-switching): 5'-(FAM)-A-*TTATT*-GATCGGCGTTTTTA-*AAGAA*-G-(BHQ)-3'; 0GC (stem-loop): 5'-(FAM)-A-*TTATT*-GATCGGCGTTTTTA-*AATAA*-G-(BHQ)-3'; 1GC (stem-loop): 5'-(FAM)-A-*CTATT*-GATCGGCGTTTTTA-*AATAG*-G-(BHQ)-3'; 2GC (stem-loop): 5'-(FAM)-A-*CTCTT*-GATCGGCGTTTTTA-*AAGAG*-G-(BHQ)-3'; 3GC (stem-loop): 5'-(FAM)-A-*CTCTC*-GATCGGCGTTTTTA-*GAGAG*-G-(BHQ)-3'; 4GC (stem-loop): 5'-(FAM)-A-*CTCGC*-GATCGGCGTTTTTA-*GCGAG*-G-(BHQ)-3'; 13-base target: 5'-TAAAACGCCGATC-3'; Quinine-binding aptamer: 5'(FAM)-GGG AGA CAA GGA AAA TCC TTC AAT GAA GTG GGT CGA CA(BHQ)-3'; Inhibitor: 5'-TTT CCT TGT CTC CC-3'.

The length of the DNA target (13 bases) and of the inhibitor (14 bases) were selected to optimize the utilization of the available concentration range for the experiment (between 1 nM and 1000 μ M). For example, the 13-base target provided the smallest K_D that we could measure (around 5 nM) without structure-switching. Mismatch and structure-switching will only reduce affinity^{18a}. The length of the inhibitor was selected using a similar argument and based on our previously knowledge of its effect²⁰.

All reagents (including phosphate monobasic, sodium chloride, quinine and cinchonine) were obtained from Sigma-Aldrich (St. Louis, Missouri) and used without further purification.

All experiments were conducted at pH 7 in 50 mM sodium phosphate buffer, 150 mM NaCl, at 45°C except for the experiments with the quinine aptamers (Figure 4), which were conducted at 37°C. All fluorescence measurements were obtained using a Cary Eclipse Fluorimeter with excitation at 480 (\pm 5) nm and acquisition between 514 and 520 nm using either 5 nm (unfolding curves) or 20 nm (binding curves) bandwidths.

Binding curves were obtained by sequentially increasing the target concentration via the addition of small volumes of solutions with increasing concentration of target with the receptor concentration held constant (at 3 nM for the linear-DNA and stem-loop receptors, and 100 nM for the quinine-binding aptamer). The observed K_D were obtained using the following equation:

$$F([T]) = F(0) + \frac{F_B - F(0)}{1 + \frac{K_D^{obs}}{[T]}} \quad (\text{Eq. 5})$$

Where $F([T])$ is the fluorescence signal obtained at a certain target concentration, F_B is the maximum fluorescence signal and $F(0)$ is the background signal in the absence of target. Since $F(0)$ and F_B vary between the different receptors employed, we simplified data comparison by normalizing the binding curves from 0 to 1 (relative signal). For the linear DNA receptor and the structure-switching bioreceptor (Figure 2) we did this using the equation:

$$Relative\ signal = \left(\frac{F([T]) - F(0)}{F_B - F(0)} \right) \quad (\text{Eq. 6})$$

Because of the signal-off nature of the allosteric-regulated DNA aptamer (Figure 4) in this case we did this using the equation:

$$Relative\ signal = 1 - \left(\frac{F([T]) - F(0)}{F_B - F(0)} \right) \quad (\text{Eq. 7})$$

The stability of the stem-loop (i.e., which defines K_S) was measured using urea unfolding curves (see ref^{18a} for experimental details).

The simulations presented in Figure 3C and 4B (solid lines) were generated using the observed K_D values of each receptor for its target and the selected analogue.

Associated content

Supporting Information available. The following files are available free of charge. File name. Experimental, DNA sequences, supporting figures.

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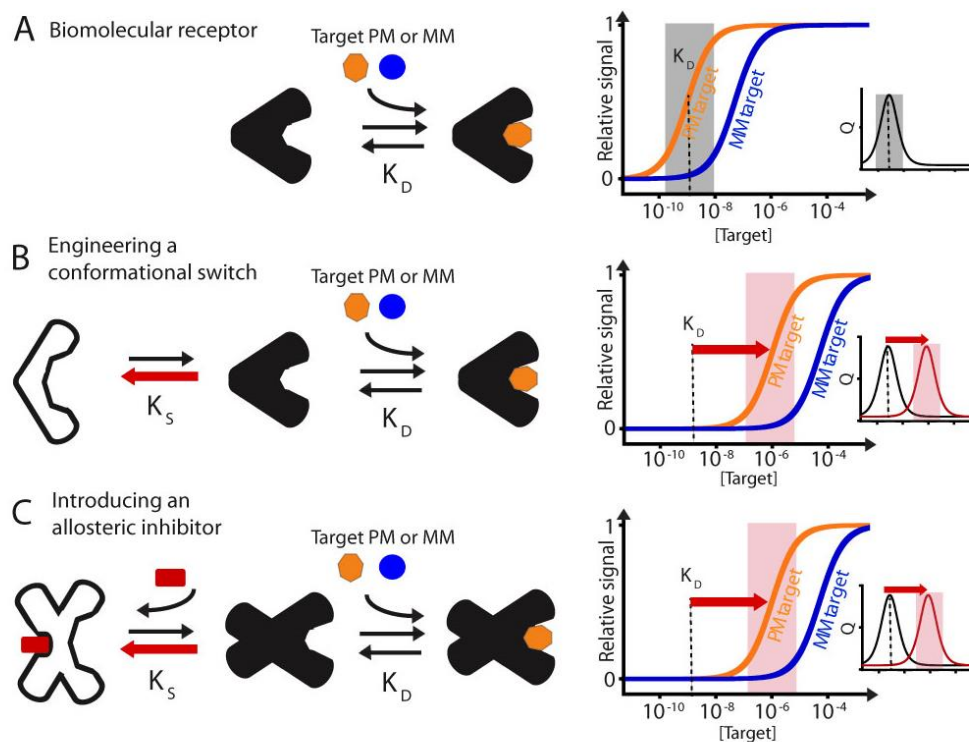


Figure 1. A) Biomolecular receptors achieve high specificity over only a limited range of target concentrations (the “specificity window” –see grey rectangle). Here we define this specificity window using the discrimination factor, Q , which is the ratio of the output signal seen for the properly matched (PM) target to that produced by a structurally analogous mismatched (MM) target at the same concentration²¹. In this work we have explored two strategies to tune the specificity window so as to achieve maximal specificity over a given range of target concentrations. The first is by engineering a structure-switching system (conformational switch) (B) and the second by introducing allosteric inhibition (C).

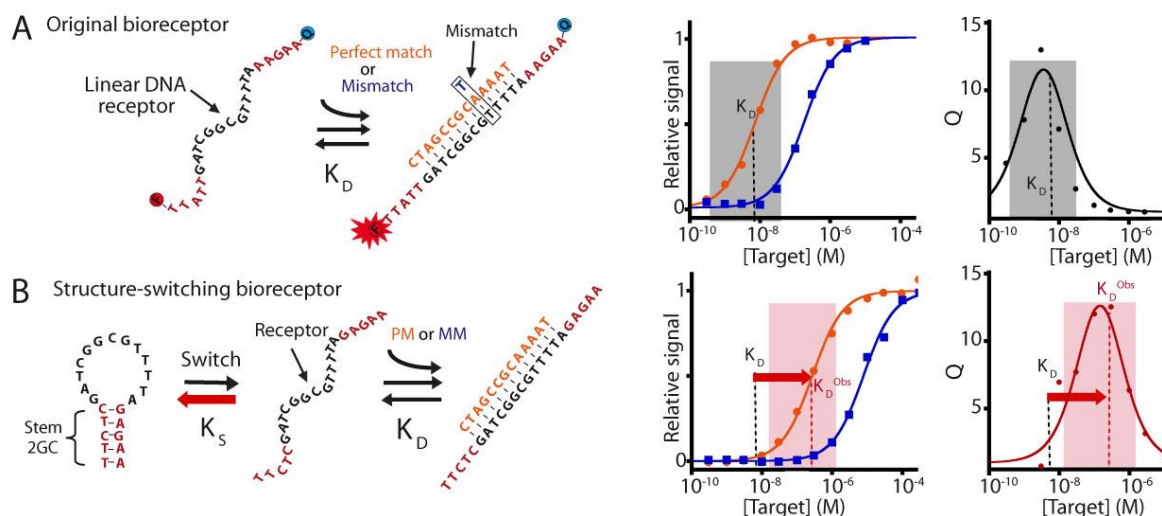


Figure 2: Coupling recognition to an unfavorable conformational change (a “switch”) provides a convenient means of tuning the specificity window of many receptors. A) To demonstrate this, we have used a DNA receptor (labeled with a fluorophore and a quencher) containing a 13-base target recognition element (black) and five additional bases at either end (red). If the two end sequences lack complementarity there is no switch and the receptor recognizes its perfectly matched (PM) complement with high affinity ($K_D^{PM} = 7 \pm 1$ nM). It also recognizes, however, a sequence containing a single mismatch displaying, albeit with lower affinity ($K_D^{MM} = 180 \pm 20$ nM). The concentration window over which we can observe good specificity (here defined as the concentration range at which we achieve a discrimination factor, Q , of greater than 5, see grey box) is fixed and located close to K_D^{MM} . B) By altering the tail sequences such that they self-hybridize, causing the receptor to form a stem-loop structure, we introduce a structure-switching mechanism that provides a means of tuning its specificity window (see red box). Experimental values represent the average of three independent measurements. To simplify data comparison we

have presented relative fluorescence (see Material and Methods), which corrects for variations in the background fluorescence caused by the differing conformations of the original receptor and its structure-switching variant.

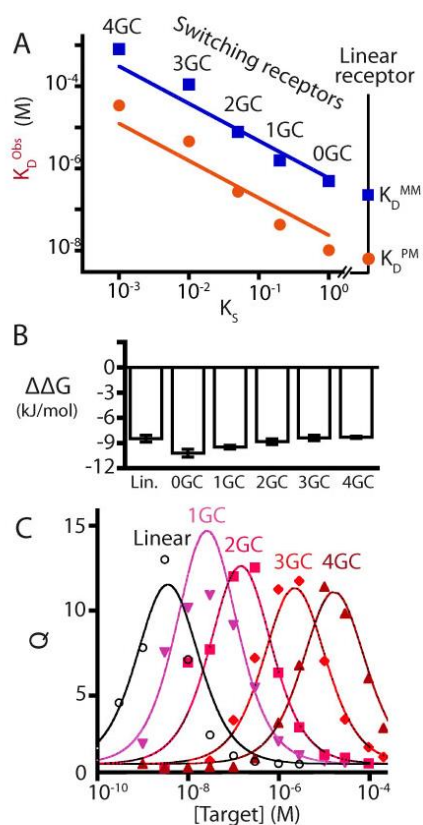


Figure 3: (A) The placement of the specificity window can be tuned by altering the equilibrium constant, K_S , of the receptor's conformational switch (ref²³). In the case of our model DNA receptor, this can be achieved by increasing the G-C content of the stem (i.e., stabilizing the non-binding conformation). B) Introducing the switching mechanism does not perturb the overall specificity of the receptor ($\Delta\Delta G_{PM-MM}$) nor its discrimination factor (Q); it only changes the range of concentration over which the receptor achieves its maximum specificity (C).

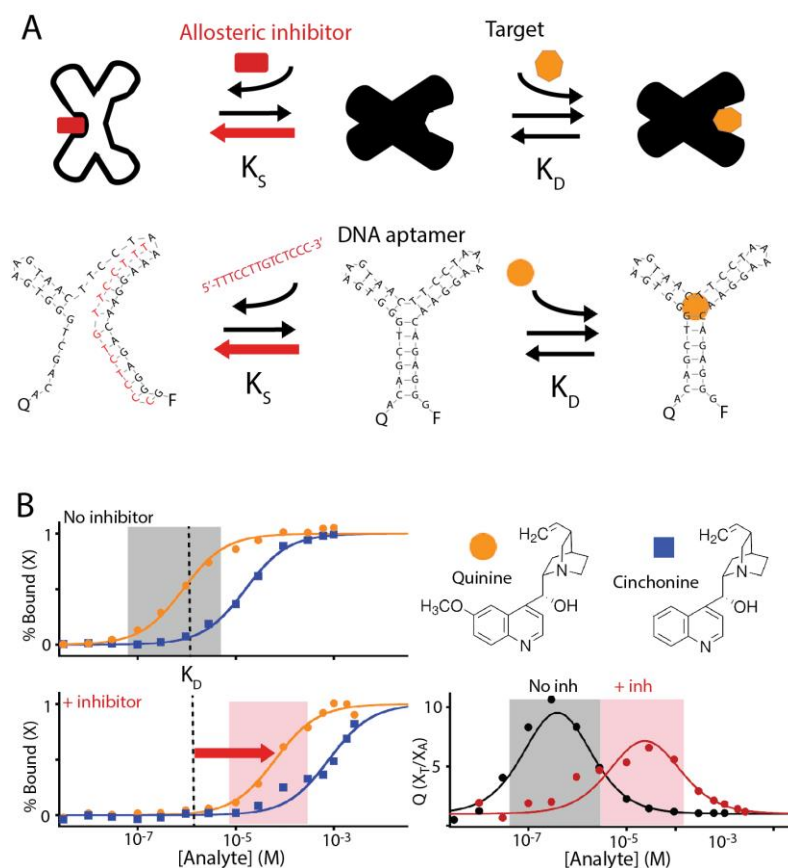
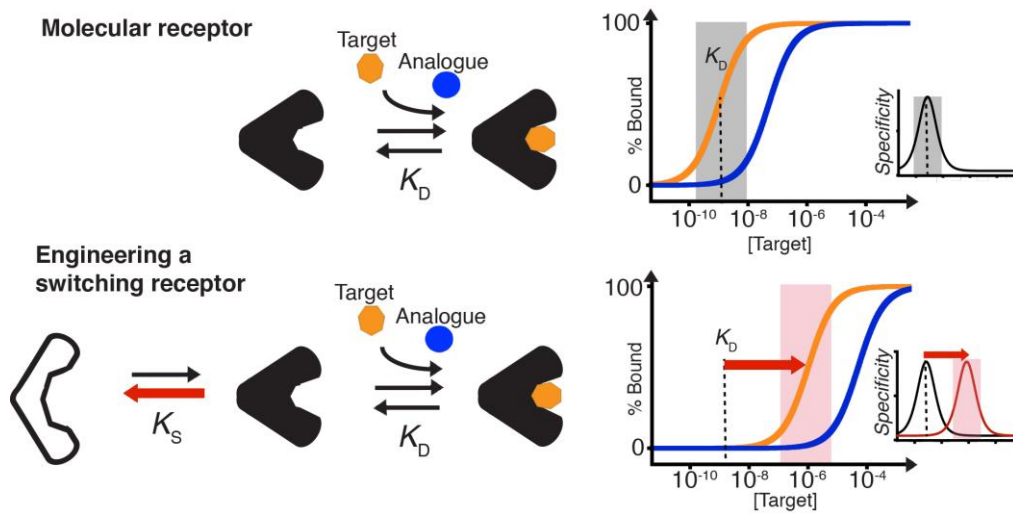


Figure 4: Allosteric regulation provides a second approach to tuning the placement of the specificity window. A) To demonstrate this we employed a quinine-binding aptamer²⁴ labeled at its two termini with an optically reporting fluorophore-quencher pair. The aptamer folds upon binding to its molecular target²⁴, causing emission to fall. B) We can reduce the aptamer's affinity and thus shift its specificity window to higher target concentrations using a complementary DNA strand that, upon hybridization to the aptamer, stabilizes a non-binding, double-stranded conformation²⁰. The introduction of this inhibitor changes the range of concentration at which the receptor binds to its perfectly matched target (quinine) and a mismatched analog (cinchonine), thus shifting its specificity window to 75-fold higher target concentrations. Also in this case to simplify data comparison we have presented relative

fluorescence (see Material and Methods), which corrects for variations in the background fluorescence caused by the allosteric inhibitor (see Figure S3).



TOC Figure