Title: Allosteric Regulation of Aptamer Affinity through Mechano-Chemical Coupling

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Allosteric Regulation of Aptamer Affinity through Mechano-Chemical Coupling


Abstract: The capacity to precisely modulate aptamer affinity is important for a wide variety of applications. However, most such engineering strategies entail laborious trial-and-error testing or require prior knowledge of an aptamer’s structure and ligand-binding domain. We describe here a simple and generalizable strategy for allosteric modulation of aptamer affinity by employing a double-stranded molecular clamp that destabilizes aptamer secondary structure through mechanical tension. We demonstrate the effectiveness of the approach with a thrombin-binding aptamer and show that we can alter its affinity by as much as 65-fold. We also show that this modulation can be rendered reversible by introducing a restriction enzyme cleavage site into the molecular clamp domain and describe a design strategy for achieving even more finely-tuned affinity modulation. This strategy requires no prior knowledge of the aptamer’s structure and binding mechanism and should thus be generalizable across aptamers.

Introduction

Aptamer reagents such as antibodies and aptamers are essential tools in both basic and applied research for a wide variety of functional materials and molecular diagnostics systems.[1] The operating parameters of such applications include biorecognition,[2] affinity,[3] selectivity,[4] stability,[5] and specificity.[6] The binding properties of affinity reagents are inherent to the nucleic acid or amino acid sequence and structure generated during the screening and selection process, and further engineering is therefore required to manipulate that structure and thereby achieve the desired effect on ligand affinity. In general, this is a much more straightforward process for aptamers relative to antibodies, because their structure can be readily engineered based on predictable base-pairing principles. Furthermore, researchers have identified a number of well-known and -defined structural motifs, such as hairpins and G-quadruplexes, which can be incorporated into an aptamer-based system in order to alter its folding and binding properties in a semi-predictable fashion.

Affinity modulation is usually achieved through direct mutations in aptamer sequences, followed by extensive screening and validation procedures to locate variants with appropriately altered affinity.[6–9] However, this mutagenesis-based approach is time-consuming and inefficient, due to its trial-and-error nature and because it is difficult to ensure that a given mutation will not only meaningfully alter affinity, but also achieve this by modifying the conformational equilibrium rather than the chemical interaction between the reagent and the analyte.[6] As an alternative, one can rationally design aptamer-based molecular switch constructs with modulated affinity, which transition between a binding-ready state and a binding-inactive stem-loop structure.[9,10] Effective affinity can also be controlled through the use of split aptamer constructs, in which affinity is modulated by the purely entropic change associated with the length of the intramolecular linker joining the two fragments.[11] Alternatively, aptamer affinity can be modulated by adding a triple helix structure to the two termini of an aptamer, thereby trapping them in a fixed position and limiting the aptamer’s flexibility.[12] However, these design strategies typically require precise knowledge of both the structure of the folded aptamer as well as the basis of its interaction with the ligand, limiting their general applicability. Several groups have described strategies in which an aptamer is reversibly stabilized in a fixed position and limiting the aptamer’s flexibility.[12] One important limitation of this allosteric approach is that the inhibitor strands typically interact with the active binding domain in direct binding competition with the ligand. Unfortunately, this approach is challenging to implement for applications in which the goal is to partially down-
tune aptamer affinity by a defined amount rather than inhibit
aptamer binding entirely.

In this work, we describe a simple and effective strategy for
reversibly fine-tuning aptamer affinity by adding an allosteric
‘tuning clamp domain’ that acts at a distance from the binding site.
Specifically, we use a molecular clamp design that perturbs
aptamer folding through mechanical extension, forcing it into a
state in which its binding competency is considerably reduced.
This molecular clamp structure self-assembles through the
hybridization of an allosteric inhibitor DNA strand with two clamp
domain sequences flanking the aptamer. We also describe simple
design strategies that make it possible to precisely and
predictably tune the affinity of a given aptamer by a defined
amount, along with a restriction enzyme-based strategy for
releasing the tension of the molecular clamp and restoring normal
aptamer affinity. Previous work has demonstrated the
nanomechanical control of enzymatic activity,\textsuperscript{[13]} but to our
knowledge, this represents the first attempt to modulate aptamer
affinity in such a fashion. As a proof of concept, we have
modulated the affinity of HD22, an aptamer for human α-
thrombin,\textsuperscript{[14]} by ~65-fold, and show that the mechanically-inhibited
HD22 aptamer can be reactivated and restored to its initial affinity
via endonuclease digestion. We have also demonstrated this
approach with a second aptamer that binds to the small-molecule
target Ochratoxin A (OTA).\textsuperscript{[15]} and we believe this strategy should
generally applicable across a range of aptamers without any
prior knowledge of the aptamer structure or binding domain.

Results and Discussion

Design Principles for the Tuning Clamp Domain System

Our clamp design consists of two strands (Fig. 1a). The first
strand is composed of the aptamer flanked by ‘clamp domain’
sequences at both ends. These flanking sequences are designed
to hybridize with a complementary allosteric inhibitor strand (red).
When fully assembled, the two strands form a stressed
aptamer molecule (SAM). This forces the aptamer into an unfolded state (U),
shifting the equilibrium away from the folded, binding-competent (F) and target-
bound (B) states. The influence of the unfolded state on overall aptamer affinity can
be analytically determined based on the population shift model\textsuperscript{[24]}
Briefly, the dissociation constant ($K_D$) of SAMs for the aptamer
target can be described by the following equation:

$$K_D = rac{[A][T]}{[B]} = \frac{([F] + [U])[T]}{[B]} = \frac{(1 + K_s)[F][T]}{[B]} = (1 + K_s)K_D^0$$

where $[A]$, $[T]$, $[B]$ are the respective concentrations of aptamer,
target, and aptamer-target complex, $[F]$ and $[U]$ are the
concentration of the folded and unfolded states, $K_D^0$ is the initial
dissociation constant of the parent aptamer, and $K_s$ is the interconversion rate between the folded and unfolded states. This
final term is described by:

$$K_s = \frac{[U]}{[F]} = \exp\left(\frac{\Delta G}{k_B T}\right)$$

Thus, the $K_D$ of the SAM can be described as follows:

$$K_D = 1 + \exp\left(\frac{\Delta G}{k_B T}\right)K_D^0$$

in which $k_B$ is the Boltzmann constant, $T$ is the temperature, and
$\Delta G$ is the free energy difference between the folded and unfolded
states. Based on Eq. (2), it is clear that one can control aptamer
affinity by tuning the $\Delta G$ value.

To determine $\Delta G$ quantitatively for the SAM, one can
calculate the total energy profile of the SAM using the bending
energy model for the molecular clamp and the stretching energy
model for the aptamer (see Methods). We demonstrated this with
a series of modeling experiments based on SAMs incorporating
the thrombin-binding aptamer HD22.\textsuperscript{[14]} When we plotted the total
elastic energy ($E_{tot}$) of the system as a function of EED (Fig. 2a),
we identified two local minima that correspond to the folded and
unfolded states of the aptamer. The difference between these two
minima yields the $\Delta G$ value. A stiffer molecular clamp will exert
greater force on the aptamer and will therefore make the unfolded state more energetically favorable. We therefore expect that the
balance between the folded and unfolded states can be shifted by
changing the stiffness of the molecular clamp, which can in turn

\begin{figure}
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\caption{Schematic of allosteric control with the tuning clamp domain. (a) An aptamer sequence (yellow) is flanked by two clamp domain sequences (cyan). These domains are designed to hybridize with a complementary allosteric inhibitor strand (red). When fully assembled, the two strands form a stressed aptamer molecule (SAM). This forces the aptamer into an unfolded state (U), shifting the equilibrium away from the folded, binding-competent (F) and target-bound (B) states.}
\end{figure}
be adjusted by altering its length ($N_0$), as shown in our previous work.\textsuperscript{[16]} To analyze the influence of the clamp length, we plotted the total energy profiles of SAMs with molecular clamps ranging in length from 16 to 24 bp (Fig. 2b). The simulated constructs are denoted as SAM16-18, SAM18-20, SAM20-22, and SAM22-24, where the first number refers to the length of the tuning clamp domain and the latter number refers to the length of the allosteric inhibitor strand. The energy minimum for the folded state was barely changed, while that for the unfolded state was dramatically shifted, suggesting that $\Delta G$ can be readily regulated by varying $N_0$.

![Figure 2](image)

**Figure 2.** Regulating aptamer affinity by tuning the mechanical properties of the molecular clamp. (a) Predicted total energy ($E_{tot}$) profile vs EED for various SAM constructs, using the bending energy model for the molecular clamp and the stretching energy model for the aptamer. The two energy minima correspond to the folded (left) and unfolded (right) states of the aptamer. (b) Predicted total energy profiles for SAMs with molecular clamp length ($N_0$) ranging from 16 to 24 bp. (c) Binding curve modulation using molecular clamps of varying lengths. (d) The aptamer $K_D$ values extracted from the binding curves in (c). The black crosses are the predicted $K_D$ value based on Eq. (2), with the free energy difference $\Delta G$ extracted from the model shown in (b). The sequences used in this figure are listed at the bottom.

**Experimental Validation of Molecular Clamp-Based Tuning of Aptamer Affinity**

To verify these modeling-based predictions, we synthesized a series of HD22-based aptamer strands with tuning clamp domains of varying length (16, 18, 20, 22, and 24 nt). These were then hybridized with fully complementary allosteric inhibitor strands to produce SAMs with molecular clamps of $N_0$ ranging from 16–24 bp. We measured the apparent $K_D$ using a standard fluorescence binding assay, in which we incubated thrombin-coated beads with various concentrations of fluorescently labeled SAMs and determined the target-bound fraction with a microplate reader (see Methods). $K_D$ values for all SAM constructs studied in this work are shown in Table S2. The results of this assay showed that SAMs with lower-stiffness molecular clamps ($N_0 = 24, 22, and 20$) did not meaningfully alter the aptamer binding curve, whereas stiffer molecular clamps ($N_0 = 18 and 16$) dramatically shifted the curve to the right (Fig. 2c). We derived the $K_D$ values for these various constructs by fitting the binding curve to the Langmuir isotherm (Fig. 2d),\textsuperscript{[10, 17]} and determined that the affinity of the SAM16-16 construct (2.010.88 $\pm$ 87.33 nM) was reduced by nearly 65-fold relative to the SAM24-24 construct (30.95 $\pm$ 5.67 nM). Further statistical analysis showed a significant change in $K_D$ for SAM20 relative to that of SAM22-22 or SAM24-24 (Table S3). These results confirmed that one can achieve effective modulation of aptamer affinity through this molecular clamp design. It is worth noting that the $K_D$ values of SAM22-22 and SAM24-24 were significantly higher than that of the original HD22 aptamer ($K_D = 16.32$ $\pm$ 0.84 nM; Fig. S2). This is possibly due to the slight mechanical perturbation induced by these long molecular clamps, which is > 0 even for these fully-folded SAMs (Fig. S3), as well as steric hindrance from the clamp domain.

We then calculated the total elastic energy profiles for these SAMs with an identical set of parameters: bending modulus $B = 50$ k_BT or 200 pN $\times$ nm$^{-1}$, critical torque $\tau_c = 28$ pN $\times$ nm for the dsDNA bending energy, persistence length $\lambda_p = 11.2$ nm, and aptamer folding energy $\Delta G_{fold} = 4.34$ k_BT (as determined previously).\textsuperscript{[18]} The $\Delta G$ values were extracted from Fig. 2b, and the predicted $K_D$ values were calculated using Eq. (2) as plotted in Fig. 2d. Remarkably, this single set of parameters showed an excellent fit for the experimental $K_D$ values of all the tested SAMs.

**Releasing the Mechanical Tension in SAMs to Restore Normal Aptamer Binding**

The above experiments strongly indicate that aptamer affinity can be directly modulated by introducing changes in mechanical tension of the molecular clamp, and we subsequently validated this in series of control experiments. Every SAM features a gap in the middle of the molecular clamp structure, where the flanking arms of the aptamer strand meet (i.e., the space between the two cyan domains in Fig. 1b). In principle, widening this gap should release the mechanical tension applied to the aptamer (Fig. 3a), since most of the elastic energy in the molecular clamp is stored in the middle of the double-stranded DNA domain.\textsuperscript{[19]} We tested this by combining an aptamer strand with a 16-nt tuning clamp domain with various allosteric inhibitor strands that were designed to extend the width of this gap. Our experiments confirmed that as the gap increases in length from 0 to 3 nt, the aptamer $K_D$ dropped back to $K_D^0$ ($22.38$ $\pm$ 3.48 nM; Fig. 3b, c), clearly indicating that allosteric control of aptamer affinity in the context of the SAM was being achieved through the strain exerted by the molecular clamp. We also note that the $K_D$ values for constructs with a gap of 2 or 3 nt were significantly lower than that of the control SAM16 sequence (Table S4). This effect might be induced by restricting aptamer flexibility through terminal fixation\textsuperscript{[21]} by the molecular clamp with zero tension.
Mechanical tension is the key to molecular clamp-mediated regulation of aptamer binding. (a) Schematic showing how the size of single-stranded gaps in the molecular clamp affect mechanical tension. (b) The thrombin binding curve can be modulated by increasing the single-stranded gap at the center of the molecular clamp from 0 to 3 nt. Control is the aptamer strand alone, without the allosteric inhibitor strand. (c) K_D values extracted from the binding curves (b), ranging from 2.010.88 ± 87.33 nM for a 0 nt gap to 11.58 ± 2.87 nM for a 3 nt gap. The sequences used in this figure are listed at the bottom.

We also identified a mechanism to efficiently alleviate this mechanically-induced allosteric regulation, thereby restoring the baseline affinity of the aptamer. To achieve this, we incorporated an EcoRI restriction endonuclease site into the molecular clamp region of construct SAM16-16. After hybridization, we measured a K_D value of 1.692.55 ± 184.03 nM, demonstrating strong inhibition of the aptamer. But after digestion with EcoRI, the aptamer was restored to near-basline affinity, with a K_D of 56.33 ± 5.48 nM (Fig. 4), indicating successful disassembly of the molecular clamp. If the restriction endonuclease works properly, the position of the cut site will not affect the restoration of aptamer affinity, since the molecular clamp will always break into two pieces and completely release the mechanical tension after digestion. In this work, the EcoRI cut site was specifically positioned to make use of the nick at the center of the molecular clamp, so that the original clamp domain was not damaged in a manner that might impede subsequent re-hybridization with the allosteric inhibitor.

Fine-Tuning of Aptamer Affinity Using Single-Stranded ‘Hinges’

It is worth noting that according to Eq. (2), when ΔG < 0, K_D = K_D^0, but when ΔG > 0, K_D increases exponentially with ΔG, and we also observed this in our experimental results (Fig. 2d). These results indicate that this simple molecular clamp system is not necessarily suitable for precisely fine-tuning an aptamer’s target-binding affinity on its own. We subsequently devised a mechanism for achieving such fine calibration, wherein we introduced single-stranded ‘joints’ between the molecular clamp and the aptamer that partially release the internal energy of the SAM. We achieved this by using shorter allosteric inhibitor strands that are only complementary to a partial stretch of the aptamer strand’s tuning clamp domain. For example, an aptamer strand with a clamp domain of 22 nt might be hybridized with a 16-nt allosteric inhibitor strand (SAM22-16), yielding a SAM with a molecular clamp of N_c = 16 bp flanked by a 3-nt single-stranded joint on either side. We expected that for any given length of the clamp domain, the existence of flanking single-stranded joints would act as a hinge that releases some of the mechanical tension applied to the aptamer and thereby reduces the effect of the molecular clamp on the K_D value. As these single-stranded joints increase in length from 1 to 3 nt, we would expect this to further soften the flanking hinge and thereby enhance this dampening effect on the molecular clamp (Fig. 5a).

In order to test this directly, we constructed and generated binding curves for a number of SAMs with varying lengths of molecular clamp and single-stranded joint regions: SAM18-16, SAM20-16, SAM20-18, SAM22-16, SAM22-18, and SAM22-20 (Fig. 5b). The affinity measurements for these various SAMs (blue bars in Fig. 5c) confirmed our initial predictions, and we consistently observed K_D values that were lower than those of the equivalent SAMs containing no single-stranded joints (red bars in Fig. 5c). The affinity achieved with these SAMs with single-stranded joints effectively filled the gaps in Fig. 2d, clearly demonstrating that this fine-tuning strategy allows us to better access the full range of possible affinities that can be achieved with our SAMs.
This approach is not without limitations. The affinity tuning range is relatively small due to the intrinsic gap in the molecular clamp,\(^\text{[19]}\) although repair of this gap by cyclizing the aptamer strand should further broaden this range. Since circular aptamers have been reported to possess improved biostability and affinity,\(^\text{[26]}\) such constructs could be useful for controlled cargo release.\(^\text{[27]}\) Another limitation is that aptamer affinity can only be down-tuned using this molecular clamp system. It is generally difficult to further increase aptamer affinity through rational design beyond the target affinity that has been achieved over the course of the selection process. However, one could envision a directed selection process that incorporates the molecular clamp mechanism into the screening library as a means for directly isolating aptamers with allosterically-enhanced affinity, and this will be an interesting avenue for future investigations.

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**Keywords:** Affinity • Allosteryism • Aptamers • Mechanochemical Coupling • Molecular Clamp

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**Figure 5.** Fine-tuning aptamer affinity by adding ‘hinges’ adjacent to the molecular clamp domain. (a) Schematic showing how single-stranded hinges of increasing length affect SAM mechanical tension. (b) Binding curves for a variety of SAMs containing single-stranded hinges at either end of the tuning clamp domain. (c) Affinity measurements for fully complementary SAMs (red) and constructs that contain flanking hinges (blue). The sequences used in this figure are listed at the bottom.

**Conclusion**

We present a strategy for aptamer affinity regulation based on an intuitive design that requires only the addition of tuning clamp domain sequences at both ends of the aptamer and the synthesis of a complementary allosteric inhibitor strand. The allosteric inhibitor strand forms a physical module that is physically distinct from the binding domain of the aptamer, avoiding direct competition with the ligand, thereby achieving affinity regulation in a straightforward and predictable fashion. The allosteric inhibition of aptamer binding can be rapidly alleviated in a controlled fashion by incorporating a restriction enzyme cleavage site within the tuning clamp domain sequence. Finally, we have demonstrated that we can achieve even finer control over the final affinity of the aptamer by introducing single-stranded ‘hinge’ domains that partially alleviate the mechanical tension from the molecular clamp. Importantly, this approach does not require any a priori knowledge of the aptamer’s structure or thermodynamic parameters, and we believe it should be broadly generalizable across aptamers. Indeed, we have further validated this allosteric inhibition strategy with another aptamer that binds to the small-molecule mycotoxin OTA (see Supporting Information for details).\(^\text{[15]}\) The ability to readily convert existing aptamers into allosterically-regulated systems could prove valuable in many applications, including as tools for evaluating the affinity of molecular detection reagents,\(^\text{[22]}\) adaptive biosensors,\(^\text{[23]}\) reagents for super-resolution imaging,\(^\text{[24]}\) components of programmable nanodevices or networks,\(^\text{[25]}\) systems for precisely controlled drug release,\(^\text{[26]}\) and even as tools for rational gene regulation.\(^\text{[1b, 24]}\)
RESEARCH ARTICLE


We describe a simple and effective strategy for reversibly fine-tuning aptamer affinity by an allosteric molecular clamp that perturbs aptamer folding through mechanical extension, forcing it into a state in which its binding competency is considerably reduced. Aptamer affinity is regulated by tuning the mechanical properties of the molecular clamp.