A general strategy to introduce pH-induced allostery in DNA-based receptors to achieve controlled release of ligands

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Supporting information

Reagents:

Reagent-grade chemicals, including tris-(2-carboxyethyl) phosphine hydrochloride, sodium chloride, magnesium chloride (all from Sigma-Aldrich, St Louis, Missouri) were used without further purifications. HPLC purified oligonucleotides modified with a pH-insensitive fluorophore (AlexaFluor488, Alexa488) and a quencher (Black Hole Quencher 1, BHQ1) were purchased from IBA (Gottingen, Germany) and employed without further purification. HPLC purified oligonucleotides modified with a pH-insensitive FRET couple, Quasar 570 and Quasar 670 were purchased from Biosearch Technologies (Risskov, Denmark) and employed without further purification.
The following oligos modified and non-modified were used:

Re-engineered triplex-based molecular beacon:

5’- TTCCCT TTTTT (Alexa488) TTCCTT GATTTGGCTA GAGAG AAGGAA T (BHQ1)- 3’

Re-engineered triplex-based molecular beacon (used for pH titration curves, Figure 2c):

5’- (Alexa488) TTCCCT TTTTT TTCCTT GATTTGGCTAGAGAG A(BHQ1)AGGAA- 3’

Original molecular beacon (used as a control without the triplex forming tail):

5’- (Alexa488) T TTCCCT GATTTGGCTAGAGAG AAGGAA T(BHQ1)- 3’

pH-independent molecular beacon (used as a control with a tail of random sequence that cannot form a triplex):

5’-TCAATG T(Alexa488)GTA TTCCCT TATTTGGCTAGAGAG AAGGAA T(BHQ1)- 3’

Re-engineered pH-dependent molecular beacon (used for target-release applications):

5’-TTCCCT TTTTT TTCCTT T(Quasar570)TTGGCTAGAG AAGGAA- 3’

In all the sequences above the underlined bases form the duplex stem of the molecular beacon and the italic bases represent the triplex-forming portion.

Complementary used for binding curves experiments:

5’-CTCTCTAGCCAAA-3’
Fluorophore-labeled target used for load/release experiments:

5’-CTCTAGCCAAA (Quasar 670)- 3’

Re-engineered triplex-based cocaine-binding aptamer (labeled at the triplex-forming stem, see Figure 4a):

5’- (Alexa488) \textbf{CCCTCT} ATTTT TCTCCC TTT(BHQ1) GGGAGA CAAG GAAAA TCCTT CAATG AAGTG GGTCG ACA -3’

Re-engineered triplex-based cocaine-binding aptamer (labeled at the three-way junction closure, see Figure S9a):

5’- \textbf{CCCTCT} ATTTT TCTCCC TTT(BHQ1) GGGAGA CAAG GAAAA TCCTT CAATG AAGTG GGTCG ACA (Alexa488) -3’

In the sequences above the italic bases represent the triplex-forming portion and the underlined bases represent the Watson-Crick portion of the triplex-forming stem.

Original cocaine-binding aptamer:

5’- (Alexa488) GGGAG ACAAG GAAAA TCCTT CAATG AAGTG GGTCG ACA (BHQ1)-3’

**Buffer conditions.** DNA oligonucleotides were suspended to a final concentration of 100 μM and stored in 0.01 M Tris + 1 mM MgCl₂, pH 7, at -20 °C. Fluorescence experiments (binding curves) with the cocaine-binding aptamer were performed by adding increasing concentrations of cocaine to a 1 mL
solution of a 10 mM Tris buffer + 10 mM MgCl₂ in the presence of 100 nM of cocaine-binding aptamer. Fluorescence experiments (binding curves) with the molecular beacon were performed by adding increasing concentrations of the complementary target in a 1 mL solution of a 10 mM Tris buffer + 150 mM NaCl + 10 mM MgCl₂ in the presence of 10 nM of molecular beacon. The pH of the above buffers was adjusted with the addition of 1 M HCl or 1 M NaOH.

Load/release experiments using the molecular beacon were performed in a 1 mL solution using an equimolar concentration of molecular beacon and target (30 nM). Initially, a buffer solution at a pH of 5.0 was used. The pH was then increased to 8.0 by adding a small aliquot of 1 M NaOH. Similarly, to decrease the pH from 8.0 to 5.0 we added a small aliquot of a 1 M HCl solution. In this case the molecular beacon and the target are each labeled with a single pH-insensitive fluorophore (Quasar570 and Quasar 670 respectively). The fluorescence signal of the Quasar570 was followed as described below (see fluorescence measurements).

Load/release experiments using the cocaine-binding aptamer were performed in a 1 mL solution using a concentration of cocaine-binding aptamer of 100 nM and a concentration of cocaine target of 300 μM. We started with a buffer solution at a pH of 5.0. The pH was then increased to 6.0 by adding a small aliquot of 1 M NaOH. Similarly, to decrease the pH from 6.0 to 5.0 we added a small aliquot of a 1 M HCl solution.

To know the exact volume of the aliquots of NaOH and HCl to add for each pH cycle we have performed preliminary experiments in the same volume (1 mL) and same solution measuring the pH with a micro-pH electrode. During the load/release experiments we also intermittently measured the pH to make sure the pH value was the one expected. Moreover, in order to have a better control of pH during the pH cycles we have used an universal buffer prepared as reported elsewhere1.
**Fluorescence measurements.** All fluorescence measurements were obtained using a Cary Eclipse Fluorometer (Varian) at 25° C. When using Alexa488-labeled DNA sequences an excitation at 488 nM (± 5 nm) and acquisition between 510 and 520 nm with 5 nm bandwidths were used. When using Quasar570-labeled DNA sequences an excitation at 558 nM (± 5 nm) and acquisition between 565 and 580 nm with 5 nm bandwidths were used. Binding curves were obtained by sequentially adding increasing concentration of the specific target in a solution containing the relevant receptor (i.e. 10 nM of molecular beacon or 100 nM of cocaine-binding aptamer). All the signals have been recorded at the equilibrium.

The observed $K_D$ were obtained using Eq.1:

$$F(T) = F(0) + \left[ \frac{[T][F_B - F_0]}{K_D^{obs} + [T]} \right]$$

where $[T]$ = target concentration; $F_B$= fluorescence in the presence of saturating concentration of target and $F_0$ = background fluorescence.

Melting curves analysis. Fluorescence melting curves were obtained in solution of 100 nM molecular beacon in 10 mM phosphate buffer + 150 mM NaCl + 10 mM MgCl₂. The experiments were performed by heating from 25 °C to 90 °C at a rate of 1 °C s⁻¹. The reported melting curves have been normalized.
through the use of the interpolation model\(^2\). Melting temperatures (T\(_m\)) have been obtained using the same model from the intersection of the calculated median and the experimental melting curve.

References:

(1) Östling, S.; Virtama, P. *Acta Phys. Scandinav.* **1946**, *11*, 289-293.

(2) Mergny, J.; Lacroix, L. *Oligonucleotides* **2003**, *13*, 515-537.
Figure S1. Fluorescence melting curves of the triplex-based molecular beacon were used to characterize the molecular beacon folding/unfolding at different pHs. **(Left)** Fluorescence melting curves of the triplex-based molecular beacon internally labelled shows that triplex folding increases the stability of the non-binding state at acid pH (pH = 4.5, $T_m = 58.6 \degree C$) compared to neutral pH (pH = 7, $T_m = 56.2 \degree C$) because of the additional stabilization provided by the Hoogsteen interactions. **(Right)** Fluorescence melting curves of a molecular beacon with the same exact sequence as that used before but with a random tail not able to form a triplex stem shows no significant change in melting temperature at the pHs investigated. For experimental details see the methods section.
Figure S2. A molecular beacon with the same stem/loop sequence of that used in Figure 2d but with a random tail that cannot fold into a triplex structure shows no change in affinity over the entire pH range we have investigated. Experimental conditions are the same used for pH-controlled molecular beacon (see methods section).
Figure S3. The re-engineered molecular beacon used in Figure 2d is labelled with a fluorophore/quencher pair at the end of the stem/loop (see cartoon, top). Because of this, no significant change of signal gain can be observed over the entire pH range we have investigated. Experimental conditions are the same used for pH-controlled molecular beacon (see methods section).
Figure S4. The pH-controlled molecular beacon we have re-engineered can act as a DNA-based nanomachine that, through pH changes, can reversibly load and release its target (Figure 3). Here we demonstrate that the load/release is fast and reversible through different pH cycles. Experimental conditions are the same used for pH-controlled molecular beacon (see Figure 3 and methods section). The signal drift observed after each addition of HCl and NaOH is likely due to the change in the overall ionic strength of the solution (see Figure S12).
Figure S5. The original cocaine-binding aptamer, which is thought to fold into a three-way junction upon target binding, shows similar affinity for cocaine over a wide pH window. Experimental conditions are the same used for pH-controlled cocaine binding aptamer (see Figure 4 and methods section).
Figure S6. Triplex-to-duplex transition of the re-engineered cocaine aptamer was studied by measuring the fluorescence signal at different pHs. As expected, at increasing pHs the triplex-structure unfolds thus increasing the relative fluorescence signal. The $pK_a$ of this duplex-to-triplex transition ($pK_a = 6.3$) is similar to that observed in the re-engineered molecular beacon ($pK_a = 6.5$, Figure 2c).
Figure S7. Folding/unfolding of the pH-allosterically regulated cocaine-binding aptamer (at a concentration of 100 nM) upon pH changes is highly reversible and fast. We demonstrate this by sequentially cycling the pH of the solution from 5.0 to 8.0 using different aliquots of 1 M HCl and 1 M NaOH solution. Experimental conditions are the same used for binding curve experiments (see Figure 4 and methods section).
Figure S8. The affinity of the triplex-based cocaine-binding aptamer for its target strongly depends on pH. Here we show that the pH-dependence of the measured constant affinities ($K_A=1/K_D$, taken from the binding curves of Figure 4) is practically indistinguishable from the pH-dependence observed for the opening/closing transition of the DNA triplex motif (Figure S6).
Figure S9. a) To further demonstrate the possibility to tune the aptamer’s affinity with pH we have labeled the pH-dependent cocaine-binding aptamer shown in Figure 4 at positions that signal the aptamer’s folding upon cocaine binding. In this case, contrarily to what we have observed in Figure 4, the binding of the target leads to the aptamer’s folding causing a signal decrease. b) As expected, also with this aptamer we observe a strong pH-dependence of the aptamer’s affinity and because triplex formation stabilizes an alternative non-active conformation of the aptamer we can modulate the affinity of this engineered cocaine-binding aptamer by changing the pH of the solution.
**Figure S10.** Raw fluorescence binding curves at pH 5.0 and pH 6.0 of the triplex-based cocaine-binding aptamer. The difference of fluorescent background signal ($\Delta F_{\text{pH}}$) achieved at pH 5.0 and pH 6.0 is due to the partial opening of the DNA triplex motif (see Figure 4b). The signal increase ($\Delta F_{\text{coc}}$) at pH 6.0 in presence of 300 µM cocaine is comparable to the observed signal change we measured by performing cyclic pH changes in the presence of the same amount of target (Figure 4c).
Figure S11. As an additional demonstration of the possibility to load/release cocaine using the pH-dependent cocaine-binding aptamer we have performed a pH-jump experiment (similar to that shown in Figure 4c) where the pH-dependent aptamer labeled at the 3-way junction closure was employed. This aptamer gives a more direct measurement of the aptamer’s folding upon target binding. As expected, in the presence of cocaine (i.e. 50 µM) at pH 5.0 no detectable signal increase is observed thus suggesting that no binding occurs. A pH change from 5.0 to 7.0 triggers the aptamer’s ability to bind cocaine and this results in a fluorescence signal decrease (in this case the folding of the aptamer leads to a signal decrease). Shown are the fluorescence signals subtracted from the background signals at both pH 5.0 and 7.0. Here an aptamer concentration of 300 nM (maximum concentration allowed for fluorescence measurements) was used.
Figure S12. The signal drift observed during pH-jump experiments using the pH-controlled molecular beacon (see Figure S4) is related to differences in ionic strength (following acid/base addition) that causes a change of fluorescence signal or a partial rearrangement of the molecular beacon structure. We have demonstrated this by following the fluorescence signal of the molecular beacon under the same experimental conditions shown in Figure S4 (but at a fixed pH of 8.0) and by sequentially injecting NaCl aliquots to obtain the same ionic strength change obtained in Figure S4 after each addition of HCl and NaOH. As expected, we observe a very similar signal drift after each NaCl addition (red curve). The signal observed under the same conditions but without the addition of NaCl is very stable (blue curve).