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General Strategy to Introduce pH-Induced Allostery in DNA-Based Receptors to Achieve Controlled Release of Ligands

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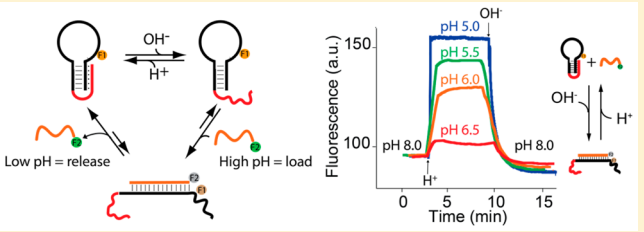
Supporting Information

ABSTRACT: Inspired by naturally occurring pH-regulated receptors, here we propose a rational approach to introduce pH-induced allostery into a wide range of DNA-based receptors. To demonstrate this we re-engineered two model DNA-based probes, a molecular beacon and a cocaine-binding aptamer, by introducing in their sequence a pH-dependent domain. We demonstrate here that we can finely tune the affinity of these model receptors and control the load/release of their specific target molecule by a simple pH change.



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The regulation of the pH inside or outside the cell and in different tissues of our body represents one of the most efficient strategies that Nature has optimized during evolution to control biological pathways.[1−3] Nature, for example, uses pH changes to control the load and release of important species. A classic example is represented by hemoglobin, whose affinity toward oxygen gets poorer as the pH decreases.[4] This allows hemoglobin to load oxygen in the lung (where pH is higher) and release it into the muscle tissues (where pH is lower). This pH-induced allostery is often achieved through the exploitation of hydrogen bonds or other pH-dependent interactions in specific domains of the receptor[2] that can either activate or inhibit its binding capacity (Figure 1).

Because it is well-known that pH varies significantly in different disease states including tumorogenesis, several attempts have been made recently to develop in vitro systems able to respond to pH changes[5] that could be applied for smart drug-delivery approaches. In this context pH-sensitive hydrogels, polymers, and nanocarriers have been widely studied.[5−8] Recently, several DNA-based nanodevices have been also engineered to undergo pH-triggered conformational changes.[9−26] Since Watson−Crick interactions are largely insensitive to pH variations, the majority of such DNA-based nanodevices rely on the use of pH-dependent alternative secondary structures such as *i-motif*,[16,17,21−24,27−29] A-motif,[25,26] and triplex DNA.[11,30−35] Despite this, only a limited fraction of such pH-dependent DNA-based nanomachines has been applied to the pH-induced release of specific ligands.[22,23,27] These examples, despite being very interesting, present some limitations. First, the pH-induced release of a ligand is usually achieved by modifying the recognition sequence of the DNA-based receptor so that it contains a pH-dependent motif that can fold/unfold at different pHs. This limits the possible generalization of similar approaches to aptamers or other DNA receptors whose recognition site is not pH-dependent. The second limitation is associated with the fact that these approaches do not appear easily tunable. To the best of our knowledge, in fact, the fine-tune control of affinity and thus the possibility to gradually control the ligand's loading/release with pH has not been yet demonstrated with DNA-based receptors.

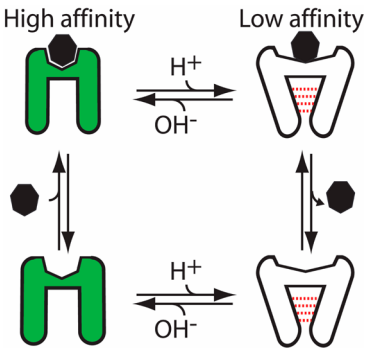

Figure 1. Nature often employs finely pH-regulated biomolecules to modulate a number of biological activities including target recognition and molecular transport. Many of these naturally occurring pH-regulated receptors switch between a *high-affinity* state and *low-affinity* state upon pH changes. Here we mimic such systems and propose a general strategy to engineer a pH-regulated switching element into DNA-based receptors such that they can transport and release a ligand in response to pH changes.

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In response to these limitations and inspired by naturally occurring pH-regulated receptors, we demonstrate here a general strategy to introduce pH-induced allostery into a wide range of DNA-based receptors. More specifically, we re-engineered two different model DNA-based receptors by introducing in their original sequence a pH-dependent domain. In contrast to previously reported examples[16,17,21−35] of pH-regulated DNA-based receptors, in our approach the pH-dependent domain is introduced in a location *distal* from the recognition site. This allows to finely regulate the binding affinity of these DNA-based receptors with pH without affecting and modifying their recognition sequence. Such re-engineered receptors can thus act as pH-responsive nano-machines that load or release a specific target in a controlled and gradual fashion via simple pH changes.

As a first model DNA receptor we have used a molecular beacon, a stem-loop fluorescent probe widely used for the detection of specific DNA or RNA sequences.[36−38] The observed affinity of a molecular beacon for its target depends quantitatively on the equilibrium constant between the *high-affinity* (open) and *low-affinity* (closed) conformation.[39] That is, upon increasing the stability of the stem-loop conformation (*low-affinity* state) (simply achieved by increasing the stability of the stem), the observed affinity of the molecular beacon for its target becomes poorer. Because Watson−Crick interactions are equally stable over a wide pH window, a conventional molecular beacon shows no change in the affinity for its specific target at different pHs (Figure 2a). Here, we introduced a pH-induced allostery in a molecular beacon by designing a pH-sensitive stem. We did so by adding at one end of the molecular beacon a short tail that is able to form an intramolecular triplex with the stem through parallel Hoogsteen interactions (Figure 2b).[35] Because protonation of the N3 of cytosine in the third strand is required to form stable triplets (CGC^+)[40] the formation of a triplex stem will preferentially occur at acid pHs.[41,42] The additional Hoogsteen interactions in the triplex will increase the stability of the stem compared to a duplex-only stem and will thus affect the affinity of the molecular beacon for its target in a pH-dependent manner.

We first demonstrated the pH-dependent conformational change of the DNA receptor by monitoring the folding/unfolding of the triplex stem structure at different pHs. We did so by labeling the DNA molecular beacon sequence with a pH-insensitive[43] fluorophore (AlexaFluor488) and a quencher (BHQ1) at locations that could signal the folding/unfolding of the triplex structure (see cartoons in Figure 2c). As expected, at higher pHs the unfolding of the triplex DNA separates the fluorophore away from the quencher thus producing an increase in the fluorescence signal (Figure 2c). The pH-window of the duplex-to-triplex transition is consistent with triplexes of similar sequences,[35] and we observe a pH of semiprotonation (defined here as the average pK_a due to several interacting protonation sites) of 6.5. Melting curves performed at different pHs also demonstrate the additional stabilization provided by the Hoogsteen interactions under more acid conditions (Figure S1).

Because the affinity of a molecular beacon is strongly dependent on the stability of its stem,[39] we can finely modulate the affinity of our molecular beacon over more than 2 orders of magnitude by varying the pH of the solution from pH 4.5 to pH 7.0 ($K_{D\_pH4.5} = 8.9 \pm 0.9 \times 10^{-7}$ M; $K_{D\_pH7} = 5.7 \pm 0.7 \times 10^{-9}$ M; Figure 2d). Of note, a control molecular beacon (i.e., with the same recognition sequence and stem but with a random tail that cannot form a triplex) does not show any significant variation in affinity over the entire pH range we have investigated (Figure S2). We also note that the affinity of the re-engineered pH-dependent molecular-beacon at pH 7.0 ($K_{D\_pH7} = 5.7 \pm 0.7 \times 10^{-9}$ M) is within error from the affinity of the original molecular-beacon at the same pH ($K_{D\_pH7} = 6.3 \pm 0.5 \times 10^{-9}$ M) thus confirming that the triplex-forming tail does not affect affinity under conditions at which triplex does not form.

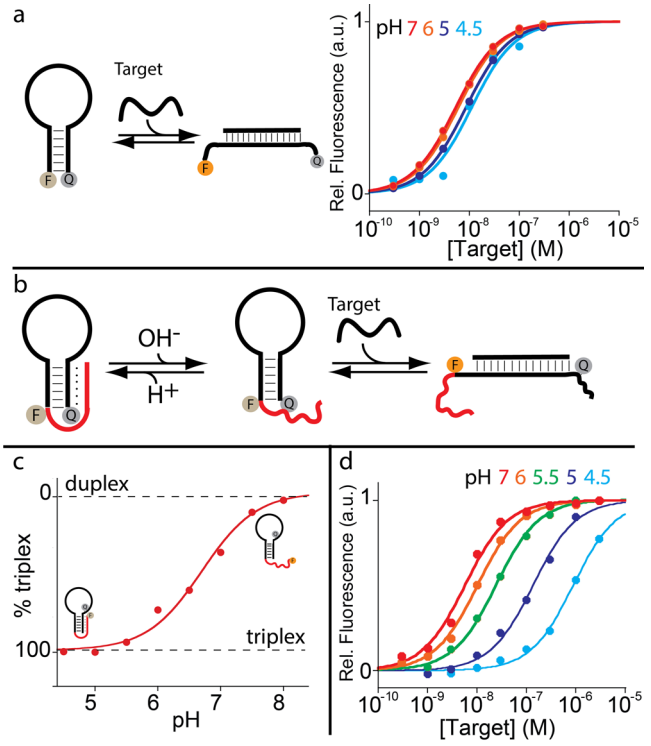

Figure 2. (a) As a first proof-of-principle of this strategy we used the classic stem-loop molecular beacon, an optical labeled DNA-based biosensor whose affinity for its specific target is normally independent to pH over a wide range. (b) We re-engineer pH-induced allostery in this molecular beacon by designing a pH-dependent triplex-forming stem, which is *distal* from the recognition sequence (loop). (c) While at acid pHs this triplex stem is highly stable, it completely unfolds to a simple duplex stem at pHs higher than 7.5. (d) Because the additional interactions of the triplex structure makes the stem (and thus the *nonbinding* state) more stable and difficult to be opened by the target, we observe a poorer affinity for the target at acid pHs. Here normalized curves are shown for a matter of clarity (curves with absolute signals are reported in Figure S3). Both triplex folding/unfolding and target binding were followed here by labeling the molecular beacon with a pH-insensitive fluorophore (AlexaFluor488) at the 5′-end and a quencher (BHQ1) in an internal position. See SI for experimental details.

The ability to modulate the affinity of DNA-based receptors through pH changes may be used to trigger the pH-dependent loading or release of a specific target in a controlled way. Here we demonstrate this by using our re-engineered molecular beacon and showing that it can reversibly load and release its specific target through various cycles of pH changes. We did so by labeling the target strand and the molecular beacon with a pH-insensitive FRET pair (Figure 3a). The binding of the target (load) is thus associated with a decrease of fluorescence signal, while its release results in an increase in fluorescence signal. By sequentially changing the pH of the solution we were

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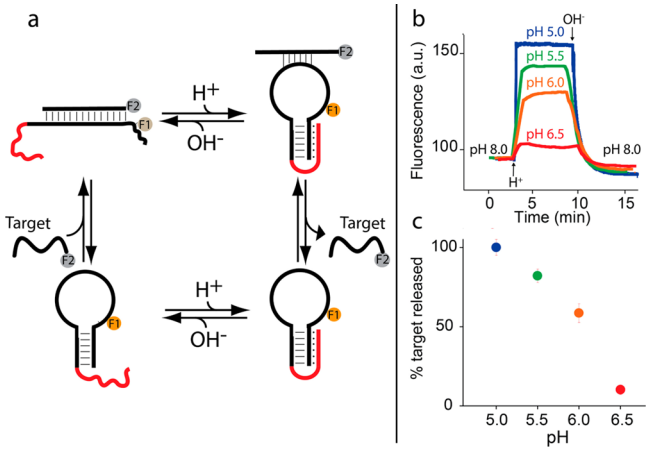

Figure 3. (a) The pH-controlled DNA receptor we have engineered in this work can act as a DNA-based nanomachine that, through pH changes, can reversibly load and release its target in a controlled fashion. (b) By gradually decreasing the pH of the solution from pH 8.0 to pH 6.5, 6.0, 5.5, and 5.0 we can observe a reversible and gradual loading and release of the target from the molecular beacon. (c) Shown are also the percentages of target released calculated from the increase in fluorescence signal. Of note, the fluorescence signal observed at pH 5.0 is indistinguishable from the signal of the molecular beacon in the absence of the target under the same experimental conditions. See SI for experimental details.

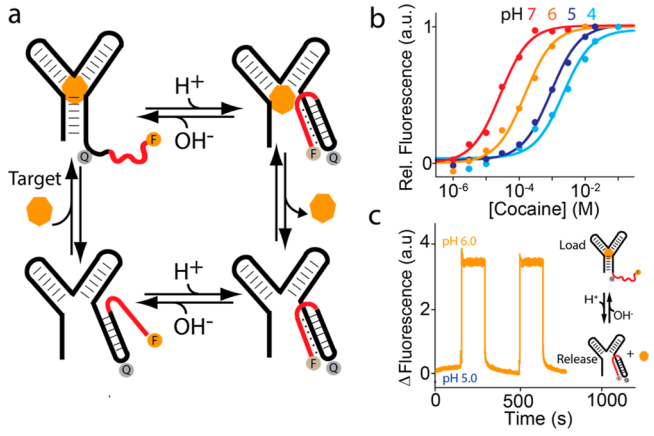

Figure 4. Rational design of pH-induced allostery in a more complex DNA-based aptamer. (a) We have re-engineered the classic cocaine-binding aptamer,[44] which is thought to fold into a three-way junction upon target binding, by introducing at its 3′-end a triplex-DNA forming tail that, when folded, inhibits folding of the aptamer and thus its ability to bind its target. Triplex-to-duplex transition of this tail 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 (Figure S6). (b) Because triplex formation stabilizes an alternative *nonactive* conformation of the aptamer we can modulate the affinity of this engineered cocaine-binding aptamer by changing the pH of the solution. (c) We also demonstrate the pH-induced load/release of cocaine using this re-engineered aptamer. In the presence of cocaine (i.e., 300 μM) at pH 5.0 no detectable signal increase is observed thus suggesting that no binding occurs. A pH change from 5.0 to 6.0 triggers the aptamer's ability to bind cocaine, and this results in a fluorescence signal increase. Shown are the fluorescence signals subtracted from the background signals at both pH 5.0 and 6.0. See SI for experimental details.

able to observe a reversible loading and release of the target strand from the molecular beacon (Figures 3 and S4). Moreover, because the affinity of the molecular beacon (and thus its loading capacity) is gradually modulated at different pHs (see Figure 2d) we can achieve a gradual and controlled release of the target DNA sequence at different pHs (Figure 3b,c).

To demonstrate the generality of our strategy we also re-engineered the more complex cocaine-binding aptamer[44,45] so that its binding activity can be modulated by pH changes. The original cocaine-binding aptamer is thought to fold into a three-way junction upon binding to its target analyte. As previously reported,[46] the affinity of this aptamer for its target is virtually the same over a wide pH range (Figure S5). For example, the dissociation constant achieved at pH 5.0 (K_{D_pH5} = 12.9 ± 0.9 × 10^{-6} M) is only slightly different from that observed at pH 7.0 (K_{D_pH7} = 8.1 ± 0.8 × 10^{-6} M) (Figure S5). We observe that, because the reported pK_a of cocaine is 8.60,[47] such small difference in affinity is unlikely due to the difference in protonation of the cocaine itself but could be ascribed to other experimental reasons such as, for example, the effect of fluorophore/quencher interaction which, at different pHs, might slightly affect the aptamer's folding event.[39]

Similarly to what we have done with the molecular beacon, we re-engineered the cocaine-binding aptamer sequence by introducing at the 3′-end a tail that is able to form an intramolecular triplex DNA. Upon triplex formation at low pH, the folding of the aptamer, and thus target binding, is inhibited (Figure 4a). At higher pHs the triplex structure unfolds leading to an active aptamer that is able to bind its specific target (Figure 4a). The pK_a of this duplex-to-triplex transition (pK_a = 6.3) remains similar to that observed in the re-engineered molecular beacon (Figure S6). We also note that this triplex DNA *opening/closing* is highly reversible and shows fast kinetics[35] (Figure S7). Because the triplex-DNA motif allosterically regulates the cocaine-induced folding of the aptamer, the affinity of this re-engineered cocaine-binding aptamer is modulated by pH (Figures 4b and S8, right). By varying the pH of the solution from pH 4.0 to pH 7.0 we were able to gradually modulate the affinity of the aptamer for its target over more than 2 orders of magnitude (K_{D_pH4} = 2.1 ± 0.5 × 10^{-3} M; K_{D_pH7} = 2.7 ± 0.6 × 10^{-5} M; Figure 4b). Consistent with the proposed mechanism, the affinity of the aptamer for its target shows a pH-dependence that is almost indistinguishable from that observed for the opening/closing transition of the DNA triplex motif (Figure S8, right). We also note that, as expected,[48] the Watson−Crick duplex portion of the triplex-forming stem even in the absence of Hoogsteen interactions slightly affects the aptamer's affinity. At pH 8.0 (where triplex formation does not occur (Figure S6)) we observe in fact an affinity (K_{D_pH8} = 2.3 ± 0.5 × 10^{-5} M) that is slightly poorer than that observed with the classic cocaine-binding aptamer under the same conditions (K_D = 1.1 ± 0.5 × 10^{-5} M). To further demonstrate the pH-dependent tuning of the aptamer's affinity toward its target we have also labeled the same triplex-forming aptamer at different locations that allow to directly measure the three-way junction aptamer's folding upon cocaine binding (Figure S9a).[44] This new aptamer shows a pH-dependency toward cocaine affinity that is almost indistinguishable from the aptamer labeled on the triplex-forming stem (Figure S9b). Difference in the absolute affinity values might be explained by the different effect that the fluorophore/quencher pair interaction may play in the overall stability of the nonbinding and binding states.[39]

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As its molecular beacon counterpart, the pH-dependent cocaine-binding aptamer can be also used to achieve pH-triggered load/release of the ligand (Figure 4c). Because cocaine is not optically active, its load/release can be followed only through indirect measurements. To do so, we first monitored the signal of the pH-dependent aptamer in the absence of cocaine by sequentially changing the pH from 5.0 to 6.0. The observed fluorescence signal change was consistent with partial opening of the DNA-triplex motif (Figure S6). At pH 5.0, upon addition of cocaine (i.e., 300 μM, a concentration chosen in order to have the highest change in affinity), no change in fluorescence was observed because under this pH the binding property of the aptamer is inactive (Figures 4c and S10). By increasing the pH of the solution (from 5.0 to 6.0) the affinity of the aptamer is restored and cocaine binding to the aptamer can be inferred from the higher fluorescence signal observed (cocaine binding shifts the equilibrium toward the active conformation of the aptamer) (Figure 4c). By decreasing again the pH of the solution (from 6.0 to 5.0) we observe a signal comparable to that in absence of cocaine thus suggesting that the formation of the alternative triplex-structure leads to the complete release of the cocaine target from the aptamer (Figure 4c). Of note, such behavior is reversible and seems to demonstrate that we can load/release the aptamer's target by changing the pH of the solution by a single unit. A similar pH-jump experiment using the pH-dependent aptamer labeled to signal the three-way junction aptamer's closing was also performed as an additional demonstration of the possibility to load/release the cocaine-binding aptamer's target at different pHs (Figure S11).

We have demonstrated here a general strategy to re-engineer DNA-based receptors so that their binding affinity can be finely regulated by changes of pH. We have demonstrated the versatility of this strategy by re-engineering two different DNA-based receptors: the classic molecular beacon and the cocaine-binding aptamer. We have rationally inserted, in these two receptors, a pH-sensitive triplex-DNA domain (*distal* from the recognition site) that upon forming at low pHs, stabilizes an inactive form of the receptor thus reducing its affinity for the target. The strategy proposed here appears suitable to generate DNA-based switching receptors that can be activated/inhibited through pH-changes.

Compared to other previously reported approaches where pH-dependent motifs have been used to modulate the affinity of a DNA-based receptor toward a target and to release a specific ligand,[16,17,21−35] our strategy appears more versatile and more easily tunable. In fact, inspired by naturally occurring allosterically regulated receptors, we have rationally engineered our DNA-based receptors so that the pH-dependent domain is *distal* from the recognition site. This strategy thus overcomes sequence-specific limitations of previously reported pH-dependent DNA probes and nanomachines where the sequence of the recognition element needs to be modified to be pH-dependent. Our strategy can thus be adapted to potentially any nucleic acid receptor without sequence-specific limitations. Finally, to the best of our knowledge, this is the first time that a fine-tuned load/release of a ligand through pH changes is reported using DNA-based receptors. In fact, by gradually changing the solution's pH we were able to finely modulate the stability of the distal pH-dependent motif, thus finely controlling the amount of ligand released. The possibility to control the amount of ligand released upon pH changes and the adaptability to potentially any DNA-based recognition sequence make our strategy of possible value for the development of novel nucleotide-based methods for the controlled release of drugs[49−52] or to site-specifically release and thus activate nucleotide-based therapeutic agents (i.e., aptamers) against diseases characterized by pH disregulation.[53−55]

■ ASSOCIATED CONTENT

Supporting Information

Sequences, materials and methods, and additional experiments. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.5b00852.

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Author Contributions

[§]These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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