Nanoscale

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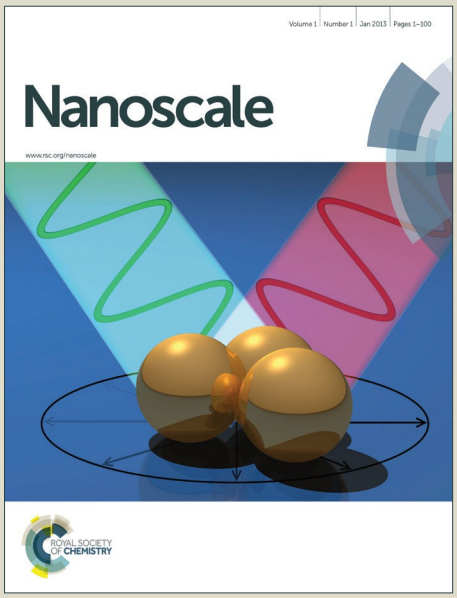

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View Article Online
DOI: 10.1039/C6NR06026A

Journal Name

COMMUNICATION

A modular clamp-like mechanism to regulate the activity of nucleic-acid target-responsive nanoswitches with external activators

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Here we demonstrate a general and modular approach to regulate the activity of target-responsive DNA-based nanoswitches. We do so by coupling together two DNA-based responsive elements: a triplex-forming clamp-like probe able to bind a specific DNA sequence and a split aptamer selected to bind a small molecule. In the presence of the specific target of one of the above responsive elements, the nanoswitch partially folds and its ability to bind the second target is restored. With this approach we can finely modulate the affinity of both DNA-recognition elements and aptamers using an external ligand. The modular nature of our strategy makes it easily generalizable to different DNA based recognition elements. As a demonstration of this we successfully designed five different DNA nanoswitches whose responsiveness can be regulated by different molecular effectors and targets. The convenience with which this mechanism is designed suggests that it may prove a useful tool by which sensors, genetic networks and other biotechnology devices employing nucleic-acid based receptors can be controlled with an external input.

The high programmability of its basic interactions has made DNA one of the most used biomaterials to create complex molecular nanodevices.[1-6] For example, DNA has been used to build 2d and 3d nanostructures[7-13] with a precision that would be inconceivable with other biomolecular materials. By using small nucleic-acid responsive elements in specific locations of these nanostructures it is also possible to obtain complex functional nanodevices that can respond to specific molecular cues.[14-18] To this end in recent years several target-responsive DNA nanodevices have been rationally designed to recognize a wide variety of molecular inputs and, in response to the binding, perform a specific function.[12,14,15,19-25] This has been usually achieved by designing DNA-based nanoswitches that, upon the binding of a specific target, can undergo a conformational change that triggers their activity. For example, DNA-based nanoswitches have been largely employed as sensing nanodevices for diagnostic or imaging purposes to detect specific DNA sequences, proteins, antibodies and other relevant targets.[6,16,26-29] In the emerging field of synthetic biology, rationally designed target-responsive DNA nanoswitches have been also employed to control biological pathways and cellular functions including transcription and gene expression.[30-37] Finally, the possibility to couple a target recognition with a conformational change has been used to develop DNA-based nanomachines for drug-release applications that are able to load and release a cargo upon the binding of a specific target.[7,24,25,38-43]

In order to improve the efficacy and performances of the above described target-responsive DNA-based nanodevices, however, novel strategies would be needed to control their activity in a more flexible way. For example, it would be particularly advantageous to find new ways that can allow to modulate and control the activity of such DNA-based nanodevices with multiple inputs and stimuli. As an example, the platform recently reported by Collins, Yin and Silver using DNA toehold switches to signal the presence of specific nucleic acid sequences could be further implemented with a modular approach so that it can be triggered by multiple inputs.[33]

Motivated by the above arguments, here we demonstrate a general straightforward and highly modular strategy to introduce an external control in the functionality of nucleic acid target-responsive nanoswitches. We did so by designing a DNA-based nanoswitch achieved through the coupling of two recognition modules: a triplex-forming recognition element that binds a sequence-specific DNA target through a clamp-like mechanism involving both Watson-Crick and Hoogsteen interactions[44,45] (control module, Fig. 1A) and a split aptamer acting as the second functional responsive unit (target-responsive module, Fig. 1B).

As a first model system of such modular control strategy we have employed the well characterized ATP-binding aptamer.[46-47] The two recognition modules (ATP-binding aptamer and triplex forming module) are split and then joined together through a random 20-base linker loop and the resulting nanoswitch has been labeled with a fluorophore/quencher pair to follow target-induced folding (Fig. 1C). As expected, the nanoswitch has a poor binding activity

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†.Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

towards ATP (Fig. 1D, black curve) due to the high energetic cost associated with the closure of the 40-base unfolded sequence (linker strand + triplex forming module) upon target binding. In the presence of the specific DNA sequence recognized by the triplex forming control module (Activator), the partial folding of the nanoswitch

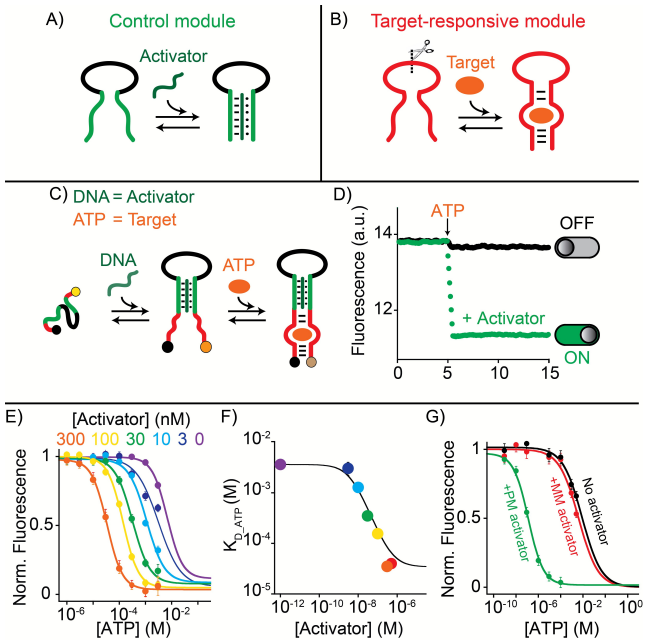

Fig. 1 Clamp-like nucleic acid nanoswitches by using two different target-responsive recognition elements (A) as the control module (green portion) and (B) as a target-responsive module (red portion). (C) As the first test-bed of this strategy we employed the ATP-binding split aptamer as the target-responsive module. (D) Only in the presence of the activator the nanoswitch partially folds and it is able to bind the target (ATP) (green curve). (E) The effect of the activator on the ATP affinity (K_D_ATP) is concentration-dependent and (F) is well described by a classic allosteric model (Eq. 1, solid curve). (G) Moreover, the effect is sequence-specific and no activation is observed in the presence of the single-base mismatch activator (MM activator), but only in the presence of the perfect match activator (PM activator). Here and in the following figures binding curves experiments have been obtained by adding increasing concentrations of the target to a 100 mM Tris, 10 mM $MgCl_2$ solution, pH 7.4 at 25 °C, containing a concentration of clamp-like nanoswitch of 10^{-8} M and the indicated concentration of activator and target.

activates the ATP binding module and we observe a strong binding activity (Fig. 1D, green curve). By varying the concentration of the DNA activator from 3 x 10^{-9} to 3 x 10^{-7} M we can finely regulate the efficiency with which the nanoswitch binds to ATP (K_D_ATP obtained between 9 (± 2) x 10^{-3} M and 3.5 (± 0.6) x 10^{-5} M, respectively) (Fig. 1E). The observed change in affinity at different activator concentrations is well modeled (Fig. 1F) by the equation describing a classic allosteric activation mechanism[48]:

Eq. 1 $$K_{D\_ATP} = K_D^{No\ act}\left(\frac{1+\frac{[Activator]}{K_{Act}}}{1+\frac{[Activator]}{\alpha K_{Act}}}\right)$$

where K_{Act} is the dissociation constant between the activator and the nanoswitch[49] (Fig. S1) and α is the ratio between the target dissociation constants in the presence of saturating activator concentration and in the absence of activator. We can thus simulate quantitatively the activation of our nanoswitch over a range of activator concentrations without the use of any fitted parameters (solid line, Fig. 1F) and we find that the experimental results perfectly fit such simulation (Fig. 1F). Because the binding of the activator strand to the clamp-like nanoswitch is sequence-specific the effect is also highly specific. For example, even in the presence of saturating concentrations of a single-base mismatch activator strand (i.e. 3 X 10^{-7} M), the clamp-like nanoswitch remains in the low-affinity state towards ATP (Fig. 1G).

The activation mecahnism of the clamp-like nanoswitch is highly reversible. To demonstrate this we have initially designed a new activator strand flanking a 10-base sequence at the 5' end that can be displaced from the nanoswitch using a competitor strand and a strand-displacement reaction (see Fig. 2A). In this way it is possible to activate/inhibit the nanoswitch in a reversible way so that the binding activity towards ATP can be regulated on-the-fly (Fig. 2B). And because the binding of the activator to the clamp-like nanoswitch involves pH-dependent Hoogsteen interactions, we can also regulate the activation of the nanoswitch by changing the solution's pH. For example, by varying the pH of a solution containing the activator (10^{-7} M) and ATP (5 x 10^{-5} M) it is possible to bind (low pH) or release (high pH) the activator from the nanoswitch. As a consequence, the binding ability of the nanoswitch towards ATP can be activated or inhibited, respectively and ATP can be loaded and released to the nanoswitch in a reversible way (Fig. S2).

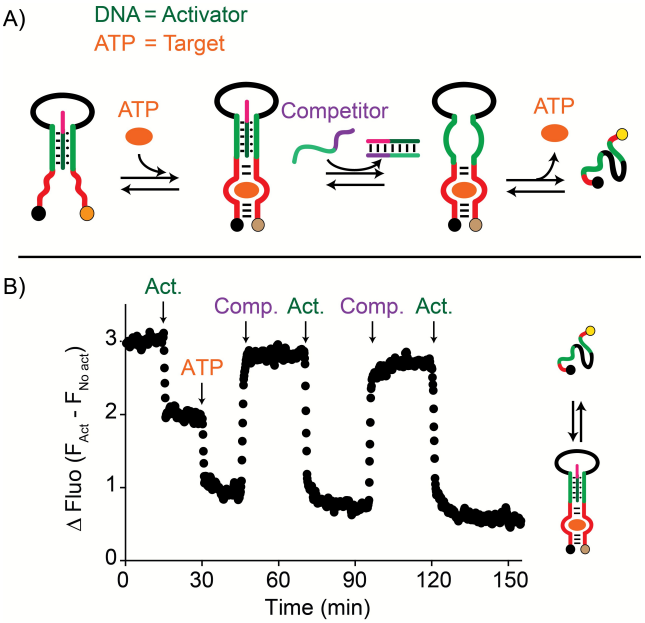

Fig. 2 (A) Our clamp-like nanoswitches are highly reversible. (B) In the presence of the activator the nanoswitch partially folds restoring ATP binding activity. By adding the competitor it is possible to selectively displace the activator. This, in turn, leads to the release of the ATP from the nanoswitch. Here the ATP loading/release to the clamp-like nanoswitch is followed by time-course fluorescence measurements obtained in a solution

of nanoswitch (10^{-8} M) in a 100 mM Tris buffer + 10 mM $MgCl_2$, pH 7.4, 25°C with the injection of the activator (10^{-7} M), ATP (5 x 10^{-5} M) and competitor (2 x 10^{-7} M).

Our strategy is highly modular and flexible. For example, the roles of the two recognition elements used above can be easily exchanged. To do this we have simply switched the position of the two recognition modules in a newly designed nanoswitch so that the ATP-binding aptamer now acts as the control module and the triplex-forming probe as the target-responsive module (Fig. 3A). In this case, binding of the DNA target to the triplex-forming module is energetically less favourable due to the intrinsic disorder of the long unfolded loop (30-base of random loop + 25-base of the split ATP-binding aptamer) (Fig. 3B, black curve). Only upon the binding of ATP to the control module, the binding activity towards the DNA target can be restored (Fig. 3B, green curve and Fig. S3). Also in this case, the effect of the activator (here ATP) on the activity of the nanoswitch is concentration-dependent (Fig. 3C) and well described by the allosteric model (Fig. 3D, solid line). We note here that the length of the random loop represents an important parameter in the control behavior of our nanoswitches. We demonstrate this by showing that a nanoswitch with a shorter loop (i.e. 5 bases) will not provide enough intrinsic disorder to observe an efficient regulation (Fig. S5). Our approach is easily generalizable to other DNA-based recognition elements. To demonstrate this, we have designed two different aptamer-based nanoswitches using the cocaine-binding[50] and the gentamicin-binding aptamer[51]. In both cases we used the split aptamer as the control module and we were able to modulate in a fine way the affinity of our nanoswitch towards a specific DNA sequence by using different concentrations of cocaine (Fig. 3E, S6) and gentamicin (Fig. 3F, S7).

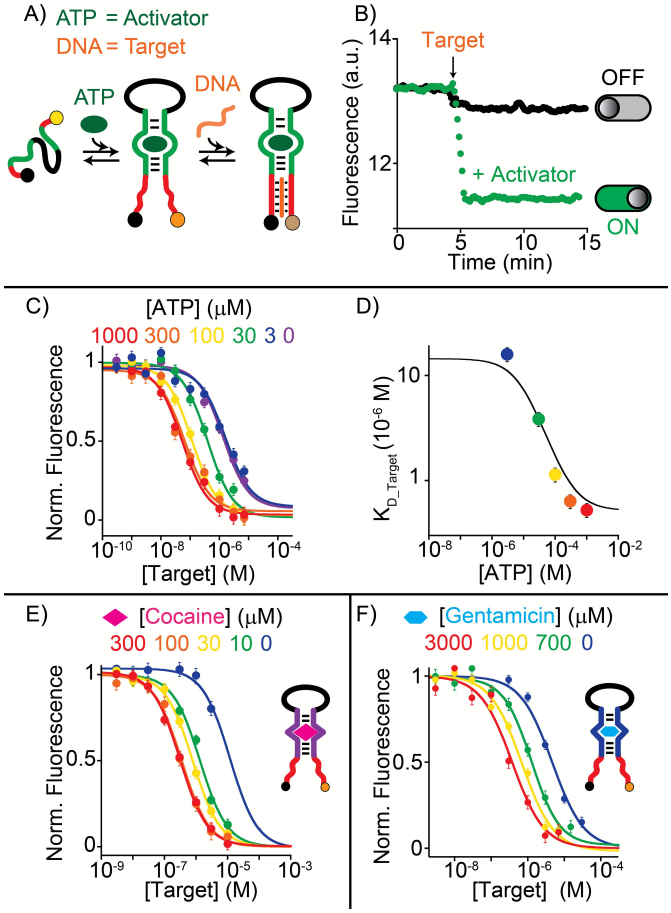

Fig. 3 (A) Clamp-like regulated nucleic acid nanoswitch using a split ATP-binding aptamer as the control module. (B) Efficient DNA target binding to this nanoswitch is restored only in the presence of ATP. (C) As expected we observe a concentration-dependent effect and (D) a behavior well described by the allosteric model (solid curve, see also Fig. S4). We also used the cocaine-binding aptamer (E) and the gentamicin-binding aptamer (F) as the control module.

As a final example of the generality and versatility of our approach we have also designed a clamp-like nanoswitch composed of two different triplex-forming recognition modules (Fig. 4A). The two modules are designed to recognize in a sequence-specific way two different DNA strands. As expected, the quite long (40-base) unfolded sequence comprising the loop linker strand (20-base) and the two halves of the control module (10-base each) contributes to the high entropic cost associated with target binding thus resulting in a poor target binding affinity (Fig. 4B, black curve). Binding activity of the nanoswitch can be restored in the presence of the activator (DNA sequence binding to the control module) (Fig. 4B, green curve). Of note, also in this case we can finely modulate the target affinity over ca. 2 orders of magnitude of target concentration (dissociation constants ranging from 2.5 (± 0.3) x 10^{-7} M to 6 (± 1) x 10^{-9} M) by varying the concentration of the control effector from 5 x 10^{-9} to 3 x 10^{-7} M, respectively (Fig. 4C). Again, the observed behavior is well described by the allosteric model (Fig. 4D, solid line, Fig. S8), is highly specific (Fig. 4E) and reversible (Fig. S9).

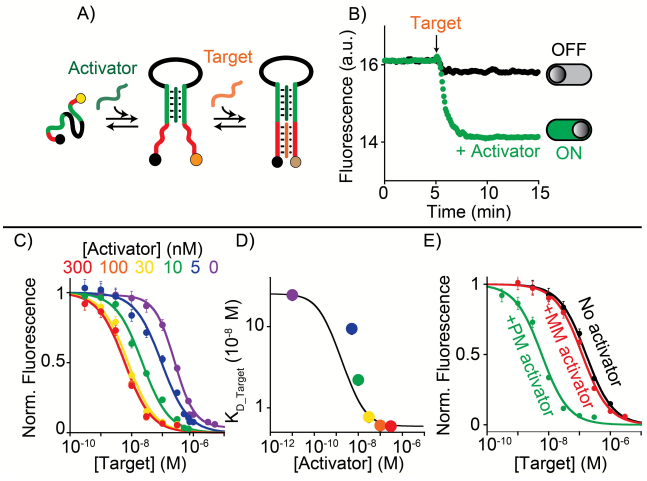

Fig. 4 (A) Clamp-like regulated nucleic acid nanoswitch based on two triplex-forming recognition modules. (B) Only in the presence of the activator, the partial folding caused by the activator restores target binding activity. (C) The concentration-dependent regulatory effect on the observed target affinity (K_D) is well described by the allosteric model (Eq. 1) (D, solid curve). (E) Moreover, the effect is sequence-specific and no activation is observed in the presence of the single-base mismatch activator (MM activator), but only in the presence of the perfect match activator (PM activator). See Supporting Info for experimental details.

Conclusions

Nature has evolved many different strategies to finely control and regulate cellular processes and pathways. The most used and intriguing of these strategies is *allostery*. This mechanism allows proteins and other biorecognition elements to modulate their affinity through the binding of a molecular input (effector) to a site different (i.e. in greek *allos*) from the target binding-site. The binding of the effector to this topographically distinct site can thus improve (positive allostery) or worsen (negative allostery) the affinity of the recognition element so that its functionality can be triggered or silenced in a very precise concentration-dependent fashion. Pioneer works from the groups of Breaker and Famulok have recreated in-vitro allosteric regulation to control ribozymes[52] and nucleic acid target-responsive elements.[53] Other applications of allosteric regulation include sensing[53-57] and those in the emerging field of synthetic biology.[58-59] Here we have expanded on this theme and have rationally designed novel clamp-like target-responsive DNA-based nanoswitches that, by using a strategy similar to that of allosterically controlled intrinsically disordered proteins, can be activated by a partial folding induced by the binding of an external activator. We did so by joining together, through a random loop, two responsive elements: a triplex-forming clamp-like probe able to bind a specific sequence and a split aptamer selected to bind a small molecule target. We demonstrate that, by doing so, we can finely tune the affinity of both DNA-recognition elements and aptamers over different orders of magnitude through the binding of an external ligand.

Compared to other methods where modulation of the activity of DNA-based receptors[54-57] has been achieved with allosteric approaches our strategy appears particularly advantageous for several reasons. Because it takes advantage of a triplex-forming nucleic acid recognition module that can be triggered in a highly specific and sensitive way by natural DNA strands,[44] our approach appears far more suitable for sensing and synthetic biology applications than those based on triplex-forming portions triggered by non-natural targets (i.e. non-native PNAs).[56] The high specificity of triplex recognition through the clamp like mechanism[44] would also allow in principle to activate different nanoswitches using different specific sequences of DNA activators. The mechanism we propose could thus be parallelized with different DNA activators that can trigger the binding activity of different aptamers in an orthogonal way. Moreoever, we have demonstrated that the allosteric effect is robust and reversible making it suitable as a way to load and release a molecular cargo in a reversible way upon the binding of an activator molecule. Such allosterically controlled DNA nanomachine can be particularly advantageous for, for example, drug-delivery applications.

Given the above attributes, the use of clamp-switch, triplex-based DNA switching mechanism holds great promise also as a new way to control and regulate target-responsive structural switching DNA-based sensors.[16] As these sensors have been largely employed for the detection of several targets we envision that our strategy can also be applied to control the activity of surface-confined DNA structures labeled for sensing applications.[16]

Finally, our strategy is so versatile and flexible that we can easily apply it to a number of different aptamers and we can invariably use both the aptamer or the triplex-forming module as either the control module or the target binding module. This flexibility could prove useful in other biotechnology-based applications. For example, a similar strategy could be applied in the field of synthetic biology to design genetic circuits that, through different modulators, can finely control biological pathways and cellular functions including transcription and gene expression.[30-37,58-59]

Acknowledgements

This work was supported by the European Research Council, ERC (project n.336493) (FR), by Associazione Italiana per la Ricerca sul Cancro, AIRC (project n. 14420) (FR), by the Italian Ministry of Health (project n. GR-2010-2317212) and by the Italian Ministry of University and Research (Project of National Interest, PRIN, 2012CTAYSY).

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