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Review

Allosterically regulated DNA-based switches: From design to bioanalytical applications

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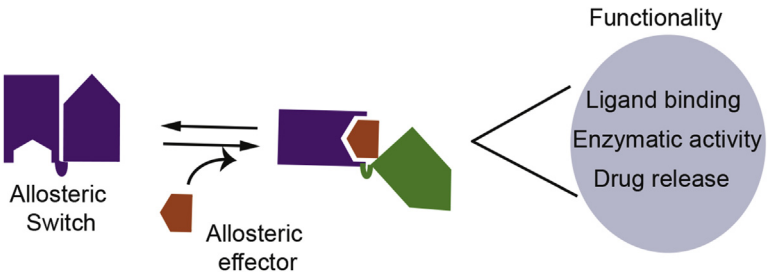
HIGHLIGHTS

- A general strategy for the design of DNA-based switches is described.
- We outlined the usefulness of recreating allostery *in vitro* to rationally control DNA-switches.
- We review bioanalytical applications of DNA-based switches that are allosterically regulated by biological and chemical inputs.

GRAPHICAL ABSTRACT



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ABSTRACT

DNA-based switches are structure-switching biomolecules widely employed in different bioanalytical applications. Of particular interest are DNA–based switches whose activity is regulated through the use of allostery. Allostery is a naturally occurring mechanism in which ligand binding induces the modulation and fine control of a connected biomolecule function as a consequence of changes in concentration of the effector. Through this general mechanism, many different allosteric DNA-based switches able to respond in a highly controlled way at the presence of a specific molecular effector have been engineered. Here, we discuss how to design allosterically regulated DNA-based switches and their applications in the field of molecular sensing, diagnostic and drug release.

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1. Introduction

Over millions of years of evolution, Nature has optimized a complex network of molecular mechanisms for controlling a wide range of cellular activities [1]. In response to different stimuli, for example, cells and organisms have evolved different mechanisms in order to be able to adjust rapidly and precisely to both extracellular and intracellular changes in the physicochemical environment. Most of these naturally occurring mechanisms rely on the use of molecular switches, a class of receptors able to relay information from a cell's exterior to its interior. Structure-switching biomolecules respond to their targets by undergoing specific, binding-induced changes in conformation (i.e. switch) or oligomerization state. These switching events, in turn, trigger specific output signals, such as the opening of an ion channel or the activation of an enzyme [2,3].

Inspired by these mechanisms, which efficiently convert a specific chemical and/or biological input into a desired output, strong efforts to re-create *in vitro* artificial switches using synthetic molecules or re-engineered biomolecules have been done [4,5]. These tools can find broad applications ranging from the measurement of biomolecule concentration to the tuning of biomolecular activity (i.e. enzymatic activity). In particular, DNA-based switches represent an emerging class of structure-switching receptors whose structure and function are regulated (i.e. activated or inhibited) by the presence of specific molecular and chemical inputs. In this Review, we discuss how it is possible to control the activity of DNA-based switches using allostery, a general mechanism widely employed in nature to regulate the functions of a variety of biomolecules. This critical Review differs from previously reported examples because it provides means for the rational design of allosterically-regulated DNA-based switches (i.e. stem-loop molecular beacons, aptamers, DNAzymes and aptazymes), and also outlines their applications for a broad range of bioanalytical applications (see Table 1).

2. Engineering DNA-based switches

The use of DNA-based switches (DNA-switches) presents many advantages so that they have been implemented in a wide range of artificial technologies in the areas of smart materials, diagnostics, imaging, and biosensing [6–11]. This class of DNA-based receptors is not generally affected by non specific adsorption of interfering proteins, so that they work well even in complex matrix samples, such as undiluted blood and serum [12,13]. Recently, DNA-switches have also been employed within living cells in different applications [14,15].

To engineer DNA-switches we can take advantage of the fact that the physic of structure-switching receptors is well described by the population-shift model [16], that provides a route by which we can rationally design and control the affinity of such receptors for a specific target more-or-less at will.

The main strategy for engineering DNA-switches (Fig. 1) consists in the generation of a distorted conformation that is not able to bind the target (the *non-binding* "off" state; Fig. 1, top). The *non-binding* state of DNA-switches can be engineered and finely regulated via the addition of non-native interactions at the molecular level (i.e. through, for example, the introduction of Watson-Crick interactions). In order to couple input recognition to structural motion, which in turn can be coupled to a range of outputs (e.g., fluorescence, electrochemistry, drug release, catalysis), DNA-switches are thus designed to flip from a *non-binding* conformation to a second, *binding-competent* conformation, upon binding to a specific molecular input (Fig. 1, top) [17]. The binding of the target stabilizes the latter state, shifting the pre-existing equilibrium and thus coupling recognition with a large-scale conformational switch and a robust output. Regarding this latter point, alternative label-free strategy based on the use of unmodified DNA [18–20] with chemiluminescence readout [21–23] have been developed to overcome the need of labeled oligonucleotide or bioconjugation steps (i.e. oligonucleotides anchored on metal surfaces) [24].

Because of the physic of the single site binding event, DNA-switches exhibit a general tradeoff: the introduction of non native interactions reduces affinity (binding of the target molecule must overcome a more unfavorable conformational free energy) while ensuring a larger signal change. This, in turn, implies that the thermodynamic of switching mechanism affects the switch's

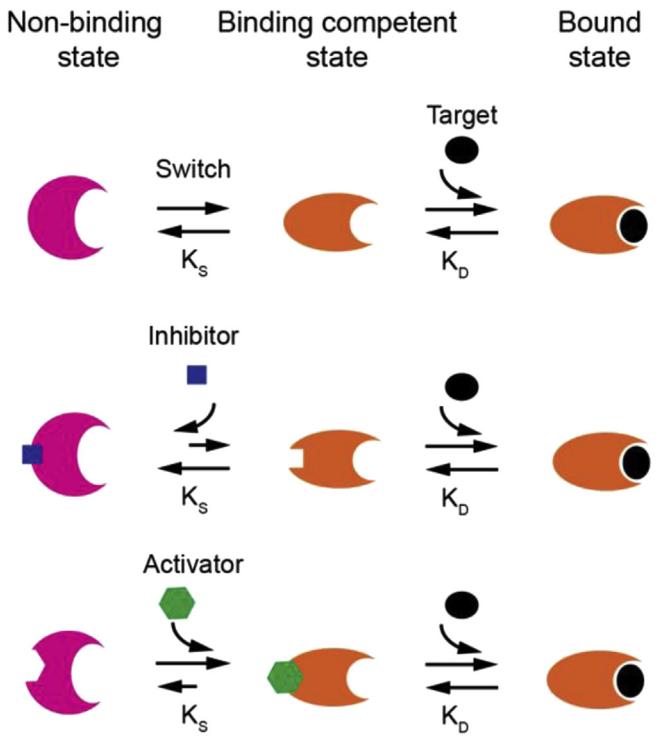

Fig. 1. Many naturally occurring chemo-receptors work via a population-shift mechanism in which target binding pushes a pre-existing equilibrium between the *binding-competent* state and the *non-binding* state to the *bound* state (Top). In allosterically regulated DNA switches, the binding of an external effector (i.e. nucleic acid complementary sequence, small molecule, protein) stabilizes either the non-binding state (Middle) or competent-binding states (Bottom) of the receptor thus reducing or improving the overall affinity for its target.

Table 1
Glossary.

Molecular beacon	Single-stranded DNA sequence having self-complementary ends that form a stem-loop structure (hairpin) in its native state.
Aptamer	Single-stranded DNA or RNA sequence that fold into a well-defined three-dimensional structure with high affinity and specificity for its target molecule.
DNAzyme	Catalytically active DNA molecule.
Aptazyme	DNA or RNA sequence composed by an aptamer domain coupled with DNA/RNAzyme unit.

dynamic range and detection limit [25,26]. As an example, if the switching constant (K_s) increases by one order of magnitude thus stabilizing the *nonbinding* state, the observed affinity of the switch (K_D) shifts toward higher target concentrations by the same factor. This relationship thus quantitatively provides a means to finely control the relationship between switch signaling and thermodynamic. This approach presents several advantages if we also consider the unique responsive properties of DNA. The first one is represented by the wide range of inputs that can be used to trigger such switching, including complementary nucleic acid strands [27,28] as well as small molecules or protein targets (i.e. through the use of aptamer sequences or consensus binding sequence) [29–33]. A second advantage is the ease with which secondary chemical and biological effectors (ligands that interact through non-covalent interactions at distal sites on the switch) can be used to regulate biomolecular functions via a mechanism called "allostery" (Fig. 2).

3. Introducing allostery into DNA-switches

Allostery, called "the second secret of life" by Perutz [34], is a ubiquitous strategy employed in nature to regulate the affinities of biomolecules and, through this, to control cellular processes and pathways [35–38]. To achieve this, Nature typically employs allosteric effectors that act on a distal site on the biomolecule to modulate the overall affinity with which a second ligand (i.e. target) binds to the receptor. Key proteins such as myosin [39] and G protein-coupled receptors [40] take advantage of the allostery to control their activity. Similarly, allostery is also strongly involved in processes that allow the transportation of molecular cargoes across the cell [41]. In this regards, one of the best example is the allosteric control of hemoglobin by 2,3-bisphosphoglycerate (BPG). This small molecule binds to hemoglobin and decreases the protein's affinity for oxygen thus enhancing oxygen transport efficiency [42].

Due to the versatility of this mechanism in controlling different biochemical functions, recreation of *in vitro* allosterically-regulated receptors represents one of the main challenges for applications in the field of synthetic biology and biotechnology. Motivated by the above arguments, here we review allosterically-regulated DNA-switches that represent a perfect class of artificial structure-switching receptors that can be programmed through the use of this mechanism. DNA is actually widely used to engineer synthetic switches because its secondary structure is mostly governed by Watson-Crick base-pairing interactions. As a result, the rational design of a structure-switching oligonucleotide in which the ligand binding induces a conformational change, able to alter its function (i.e. binding activity and/or catalytic activity) at a distal site, is relatively simple (Fig. 2). Moreover, the unique bio-responsive properties of DNA make it easy the use of small molecules, complementary nucleic acid and/or DNA-binding biomolecules as allosteric effectors to control the activation/inhibition of DNA-switches [43,44]. Recently, many efforts to rationally introduce allosteric regulation into nucleic acid based catalytic systems (i.e. ribozymes, DNAzymes) have been done [45–49]. Our research team has recently demonstrated the usefulness of recreating allostery *in vitro* to rationally control the dynamic range of DNA-switches for biosensing and drug release purposes [50].

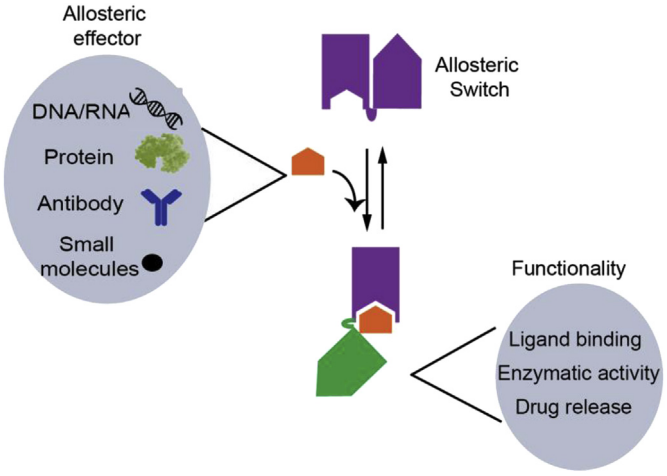

Fig. 2. Allosterically regulated DNA-based switches. Different biological and chemical ligands (DNA, RNA, proteins, antibodies, small molecules) can act as allosteric effectors on DNA-switches. Their binding to the DNA-based receptor generates a conformational change that alters the DNA-switch functionality (i.e. the binding of a second ligand, its enzymatic activity or the drug release activity).

To introduce allosteric control over DNA-switches, the binding of the external effector (i.e. nucleic acid complementary sequence, small molecule, protein) has to stabilize either the *binding-competent* or *non-binding* states (respectively) of the DNA-based receptor, raising or lowering the population of the *binding-competent* state and thus improving (Fig. 1, bottom) or reducing (Fig. 1, middle) the overall affinity for its target (K_D). The main advantage of this approach is represented by the fact that allostery provides a means of tuning the affinity "on the-fly" in a highly predictable way, after the receptor was designed and fabricated. Moreover, it has also been demonstrated that allosteric control does not affect the specificity of the biomolecule for its target because the distal site binding event of the external effector to the receptor does not interact with the target-receptor interface.

4. Allosterically regulated DNA-based molecular beacons (MBs)

Among different classes of DNA-switches, molecular beacons (MBs) present a peculiar stem–loop structure in their native state because of the presence of self-complementary ends. This hairpin structure forces the fluorophore and quencher to be in tight contact thereby suppressing fluorescence emission. Hybridization of a specific target nucleic acid to the loop breaks the stem interactions, thus separating the fluorophore/quencher pair and increasing the emission. MBs have been employed for a variety of applications [51] ranging from biosensing platforms for DNA/RNA detection [52] and the monitoring of enzymatic processes [53], to the real time analysis of living cells pathways [54–56] (see Table 2).

It has also been demonstrated that, as described by the population-shift model, the affinity of nucleic acid targets to MBs quantitatively depends on both the intrinsic affinity (K_D^{INT}) with which the "open" (linear, stem-broken) state binds its complementary target and on the equilibrium constant for the formation of this state from the "closed" one (*non-binding* stem–loop configuration) [16].

As a proof of concept of the possibility of introducing allostery in MBs, our group designed MBs that present two single-stranded DNA tails, flanking on each of the beacon's two termini. Such single-stranded tails act as distal allosteric binding sites into molecular beacons, so that the binding of inhibitors and/or activators (i.e. complementary single strand DNA sequences) to these sites has enabled the rational modulation of the sensor's affinity to nucleic acid targets (Fig. 3A). Through this strategy, we demonstrated the ability of using allostery to control the placement of the dynamic range of the MBs sensor, spanning over more than three orders of magnitude of target concentration using the same molecular beacon in the presence of allosteric effectors. The extent of the dynamic range modulation can be fine-tuned by changing the activator

Table 2
Allosterically regulated DNA-based molecular beacons (MBs).

Allosteric effector	Binding Target	Output Signal	Detection Range	LOD	Matrix	Reference
ss-DNA	ss-DNA	Fluorescence	nM- μM	n.a.	buffer	[57]
ss-DNA	Heavy metal ions	Fluorescence	nM- μM	n.a.	buffer	[58]
ss-DNA	Hg (II)	Photoluminescence	69–322 nM (buffer); 17–124 nM (tap water); 25–128 nM (river water)	52 nM (buffer)	tap water, river water	[59]
I^-	Hg (II)	Photoluminescence	149–990 nM (buffer); 46–594 nM (tap water); 34–528 nM (river water)	n.a.	tap water, river water	[59]
CN^-	Hg (II)	Photoluminescence	192–853 nM (buffer); 104–456 nM (tap water); 88–553 nM (river water)	n.a.	tap water, river water	[59]
Streptavidin	ss-DNA	Flow cytometry	n.a.	15 pM	cell	[60]
Streptavidin	Thrombin	Flow cytometry	n.a.	0.1 nM	30% serum	[60]
Streptavidin	ATP	Flow cytometry	n.a.	0.03 μM	30% serum	[60]
Streptavidin	DNA Methyltransferase	Flow cytometry	1.1–10 U/mL	1.03 U/mL	20% LB medium	[61]
Streptavidin	Respiratory Syncytial Virus (RSV)	Amperometry	100 pM–100 nM (buffer)	11 pM (buffer)	10% serum samples	[63]
Streptavidin	miRNA let-7a	Amperometry	20 pM–100 nM (buffer)	3.4 pM (buffer)	buffer	[63]
Streptavidin	miRNA let-7a	Chemiluminescence	0.1–1000 fmol	0.1 fmol	Human lung cells	[64]
Streptavidin	ss-DNA	Differential Pulse Voltammetry	10 fM - 10 nM (buffer)	0.48 fM (buffer)	10% human serum	[65]

length and concentration [57]. To extend on this concept, we have rationally designed allosterically controllable, metal ion-triggered MBs. Here MBs switches have been engineered so that the presence of heavy metal ions (i.e. Hg (II) and Ag (I)) triggers the conformational change of the stem-loop structure. Specifically, we demonstrated that the binding of Hg (II) and Ag (I) ions induces a conformational change between two alternative stem-loop states as a function of heavy metal ions concentration resulting in a change of the fluorescence output. This mechanism was finely regulated by the presence of complementary single-stranded DNA sequences which act as allosteric effectors [58]. To activate the *binding* state we used DNA strands of different length that partially open the duplex stem and increase the affinity of the beacon for metal ions. Stabilization of the *non-binding* state, in contrast, is achieved with DNA inhibitor which increases the number of Watson– Crick interactions to be broken to allow the conformational change (K_S and affinity both decrease). In this way the allosteric control over DNA-switches becomes a useful tool also to broaden the dynamic range of heavy metal concentrations over which they respond robustly. As an example, by combining in the same solution two allosteric effectors (i.e. one activator and one inhibitor) we have broadened the dynamic range of the platform to ca. 3 orders of magnitude of Hg (II) concentration. The possibility to control both the width and placement of the useful dynamic ranges, with unprecedented precision, represents a relevant feature that can be of great utility in biosensing applications.

Woo et al. also showed how it is possible to introduce allosteric control over a MBs–based mercury assay to allow the fine modulation of the detection range of the analyte as well [59]. In this study the Hg (II) detection scheme was designed to extend, narrow and shift the range of mercury detection using a variety of inhibitors, functioning as allosteric effectors (i.e. complementary single strand DNA, cationic conjugated polyelectrolyte and I^- or CN^- ions), thus showing the detection range shift in real samples such as Han River and tap water. Although many studies focused on strategies to improve the sensitivity and selectivity of the detection method, the last two examples are interesting because present innovative approaches to finely control the dynamic range of target concentrations. The possibility to arbitrarily extend or narrow the fixed dynamic range of DNA-based receptors would prove advantageous in several biosensing applications (i.e. environmental analysis, biomedical applications) in which the concentration of the target molecule can vary over many orders of magnitude. For example, the ability to extend the dynamic range of biorecognition would likely improve the efficiency of biosensors, whereas the ability to narrow the dynamic range could be of value in the monitoring of drugs with narrow therapeutic windows, as well for point of care medical applications.

Recently, Song et al. extended on this concept showing the usage of allosterically-regulated MBs for highly sensitive detection of nucleic acids, proteins, and small molecules [60]. Specifically, they engineered a platform so that target binding (i.e. allosteric effector) activates the MBs binding affinity to streptavidin magnetic beads through the disruption of the hairpin structure, thereby allowing ultrasensitive simultaneous target detection without any sample treatment, even in complex biological samples. Yang and his group also reported allosteric MBs for sensitive detection of adenine methylation methyltransferase (Dam MTase) activity [61]. The presence of a specific methylation site in the MBs triggers the activation of a restriction nuclease with consequent release of a fluorophore labeled aptamer. This platform appears particularly promising in terms of overall affinity (detection limit of 0.57 U mL^{-1}).

Allosteric MBs can be also immobilized on an electrode (E-MBs) and functionalized with a redox label to operate as bio-electrochemical switches for the detection of complementary DNA sequences [62]. Cai et al. proposed an electrochemical sensor for the detection of target miRNA let-7a, based on label-free functional allosteric MBs, which can form streptavidin aptamers able to bind to streptavidin peroxidase polymer and, by doing so, generate catalytic currents in the presence of the targets [63]. This DNA-based E-sensor showed impressive sensitivity and specificity thanks to the amplification step due to catalytic current and it also performed well in complex biological environments. Moreover, Cai et al. also took advantage of the use of hybridization chain reaction (HCR) to monitor the presence of microRNA (miRNA) let-7a. The binding of the specific miRNA induces the opening of the hairpin stem which in this format acts as initiator strand for the HCR [64].

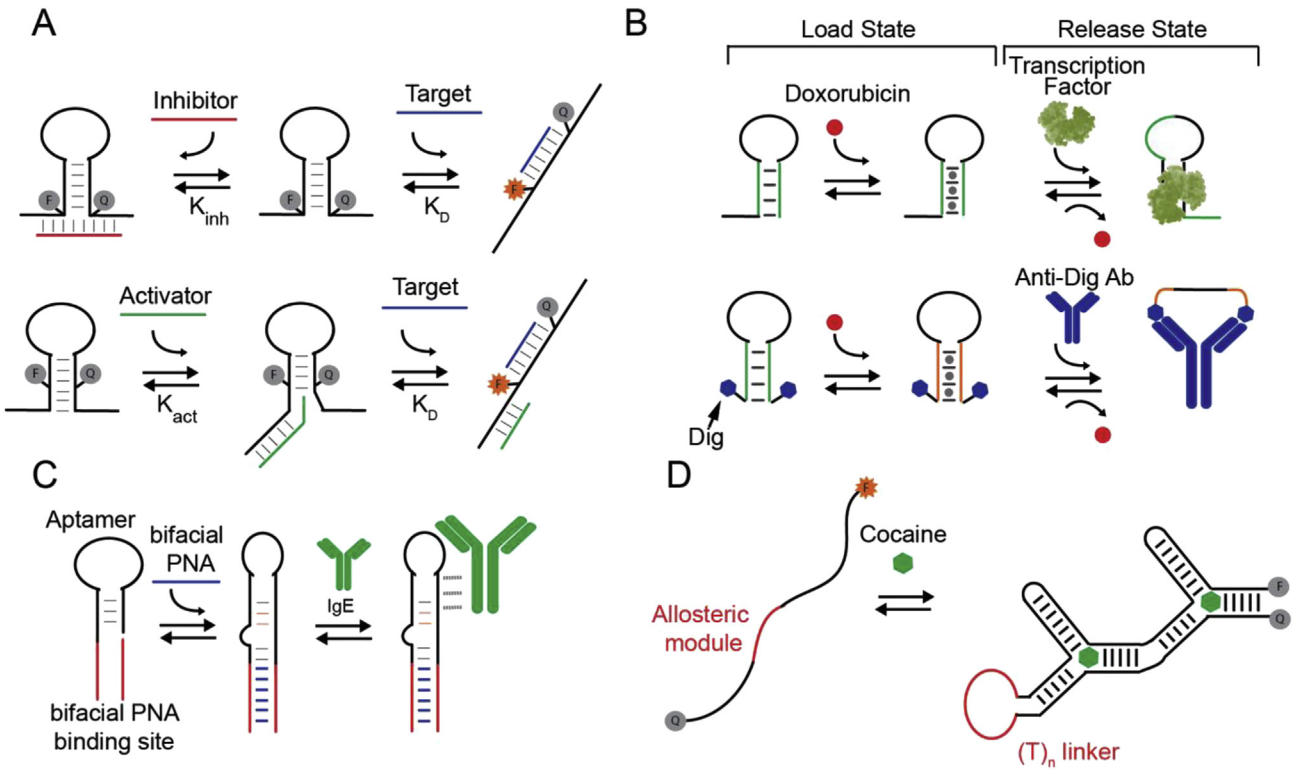

Fig. 3. Examples of allosterically regulated DNA-based molecular beacons and DNA-aptamers. A) Molecular beacons are allosterically regulated via the introduction of single-stranded tails on each of the beacon's two termini. Two single-stranded "tails" added to a molecular beacon serve as allosteric binding site. The binding of the inhibitor (red) to the two tails bridges the junction between them, stabilizing the *non-binding* state of the beacon. In the case of activator (green), instead, only one single-stranded "tail" serves as allosteric binding site. The activator partially invades the stem, destabilizing the *non-binding* state and thus improving the target affinity. Adapted with permission from F. Ricci, A. Vallée-Bélisle, A. Porchetta, K.W. Plaxco, J. Am. Chem. Soc. 134 (2012). https://doi.org/10.1021/ja304672h. Copyright (2012) American Chemical Society. B) DNA-based switches allosterically regulated by biological targets (i.e transcription factors, antibodies) are able to load and release doxorubicin as a function of their concentration. Adapted from Ref. 66 with permission from the Royal Society of Chemistry. C) IgE-binding aptamers can be allosterically regulated by bifacial PNA (bPNA). The addition of bPNA stabilizes the terminal T-tracts into a triplex hybrid stem thus restoring the IgE recognition interface. Adapted with permission from X. Xia, X. Piao, D. Bong, J. Am. Chem. Soc. 136 (2014). https://doi.org/10.1021/ja5032584. Copyright (2014) American Chemical Society. D) Allosterically regulated aptamer via intrinsic-disorder–based mechanism. The parent aptamer has been cut and tandem repeats of the two halves of the aptamer are linked *via* an unstructured, 50-base polythymine sequence (in red). The first binding event requires the energetically unfavorable closing of this loop, reducing its affinity relative to that of the second binding event. Adapted with permission from A.J. Simon, A. Vallée-Bélisle, F. Ricci, K.W. Plaxco, Proc. Natl. Acad. Sci. 111 (2014) 15048–15053. https://doi.org/10.1073/pnas.1410796111. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Recently, a sensitive electrochemical sensor for DNA detection has been also designed by Ju and co-workers, based on mimetic catalysis of metal–organic framework and an allosteric MB [65]. In this case a metal-organic framework is employed to catalyze the oxidation of *o*-phenylenediamine (*o*-PD) to 2,2′-diaminoazobenzene, the electrochemical indicator for signal readout. The presence of DNA target pushes the metal-organic framework into close proximity on the electrode surface resulting in the enhancement of electrochemical signal. The "signal-on" electrochemical sensor can detect target DNA down to 0.48 fM with extended linear range from 10 fM to 10 nM.

Finally, our group have also rationally designed a class of DNA-switches allosterically regulated by biological targets instead of using complementary DNA strands. These DNA-switches loaded with a molecular cargo (i.e. doxorubicin) are able to release it only in the presence of a biological input acting as allosteric effector (i.e. antibodies or transcription factors, Fig. 3B) [66]. The idea was to activate the release of the molecular cargo as a function of the change in the affinity of the MBs in the presence of the biological input. Specifically, in our first proof of concept demonstration we rationally designed a MB able to adopt two mutually exclusive conformations: a "Load" conformation containing a doxorubicin-intercalating domain (the stem of the MB) and a "Release" conformation containing a double stranded consensus sequence, which is recognized by the specific transcription-factor (i.e. Tata Binding Protein). As expected, the presence of the transcription factor shifts the equilibrium towards the "Release" state thus inducing doxorubicin release in a highly controlled fashion. In our second model system we designed a similar DNA-switch, whose conformational equilibrium and subsequent doxorubicin release is regulated through the binding of a specific antibody (i.e. anti-DIG antibody) working as allosteric effector. To allow the binding of the antibody to the DNA-switch which ultimately trigger the doxorubicin release, we employed terminally modified DNA-switch with a specific recognition element (digoxigenin).

5. Allosterically regulated DNA aptamers

Short, single-stranded DNA or RNA molecules (20–100 nucleotides in length) that can specifically bind via three-dimensional structure to a molecular target, ranging from small molecules and proteins complexes and even entire cells, are called aptamers [67,68]. Aptamers can be specific as antibodies and can bind their targets with similar affinity, but they are often smaller, easier to

Table 3
Allosterically regulated DNA-aptamers.

Allosteric effector	Binding Target	Output Signal	Detection Range	LOD	Matrix	Reference
Bifacial peptide nucleic acid	IgE	Electrophoretic mobility shift assays and filter-binding assays	n.a.	5 nM	buffer	[73]
ss-DNA	Cocaine	Fluorescence	nM- mM	n.a.	buffer	[75]
ATP	ss-DNA	Fluorescence	nM- μM	n.a.	buffer	[76]
ss-DNA	ATP	Fluorescence	μM- mM	n.a.	buffer	[76]
Cocaine	ss-DNA	Fluorescence	nM- mM	n.a.	buffer	[76]
Gentamicin	ss-DNA	Fluorescence	nM- μM	n.a.	buffer	[76]
ss-DNA	ss-DNA	Fluorescence	nM- μM	n.a.	buffer	[76]
Hg (II)	Hg (II)	Fluorescence	μM	n.a.	buffer	[77]
Cocaine	Cocaine	Fluorescence	μM- mM	n.a.	buffer	[77]
Doxorubicin	Doxorubicin	Fluorescence	nM	n.a.	buffer	[77]

generate and more straightforward to modify chemically than their protein-based counterparts [69]. It is possible to find a plethora of applications in which aptamers have been tested as a functional responsive biomaterial. Concerning their use as recognition elements in biosensing and bio-imaging platforms, as well as in biomedical diagnosis, the application of aptamer-based technology is still in a quite preliminary stage [70]. To date, most works involved the use of few model aptamers operating as sensing elements with limited biomedical impact. The high quality aptamers needed for clinical applications and the obligation to be extensively tested in clinical samples in order to establish their reliability and accuracy represent crucial issues. Nevertheless, recent clinical developments have revived the impetus for this promising class of molecules as targeted therapeutics.

Here we have focused on the last advances in the design of allosteric DNA aptamers. Allostery has been widely integrated into engineered RNA molecules (ribozymes, allosteric RNA aptamers) and remarkable properties of molecular recognition and allosteric function have been demonstrated. Examples of allosterically regulated DNA aptamers, on the contrary, have seen relatively less attention in the DNA-design literature [71,72]. Nevertheless, we believe that it would be particularly advantageous to find new ways to modulate and control the functional activity of DNA aptamers with external inputs and stimuli at will. For example, the rational design of responsive DNA-based molecular machines that, like naturally occurring proteins, can perform specific and highly optimized functions in response to a given molecular input represents a forefront field of investigation with unexplored bio-analytical applications (see Table 3).

Bong and coworkers have used bifacial peptide nucleic acid (bPNA) to cripple a protein-binding DNA aptamer that binds immunoglobulin E (IgE) by replacement of a critical duplex element with poly-thymine tracts (Fig. 3C) [73]. These poly-thymine portion folds into triplex stem–loop structures upon binding to a bPNA strand. Triplex hybridization in the presence of bPNA mimic restores binding function in the allosterically coupled domain. The so redesigned aptamer exhibits low nanomolar affinity (5 nM) to IgE, similar to that reported for the native aptamer (7 nM), indicating full recovery of molecular recognition via bPNA triplex stem replacement.

Our research unit also demonstrated the use of allostery within the original cocaine–binding DNA aptamer developed by Stojanovic [74] to tune, extend, and narrow the useful dynamic range of target concentration. In particular, we have used a set of DNA inhibitors that compete against the folding upon cocaine binding. This allows rational fine-tuning of the observed affinity of the cocaine-binding aptamer across more than three orders of magnitude of cocaine concentration [75]. More recently, we have also extended on the concept of activity regulation of nucleic-acid target-responsive switches through a general approach based on a modular clamp-like mechanism [76]. We did so by splitting the two recognition module (the ATP-binding aptamer and a triplex forming unit) and then joined them together through a random 20-base linker loop. In the presence of one triplex forming oligonucleotide target, the ATP-binding aptamer ability to bind ATP is restored. With this approach we finely modulated the affinity of ATP aptamer using an external actuator working as an allosteric effector.

Allostery can occur also when the effectors and the target ligands are exactly the same molecules (i.e heterotropic allostery); that is, when the binding of one copy of a ligand changes the affinity with which subsequent copies of the same molecule bind the receptor. In order to engineer allosteric cooperativity, Plaxco and coworkers have developed a loop-closure mechanism to the rational engineering of allosterically cooperative receptors [77]. Indeed, this approach is so simple that it can be performed even in the absence of detailed knowledge of the parent receptor's structure. They cut the wild type DNA aptamer sequence (cocaine-binding aptamer, Hg (II) – binding aptamer, doxorubicin-binding aptamer) at a position within the single loop and linked tandem repeats of the two resulting half-aptamers via a long, unstructured loop (Fig. 3D). The possibility of engineering cooperative DNA aptamers could be of utility in applications that require enhanced responsiveness such as genetic logic gates and “smart” materials.

6. Allosterically regulated DNAzymes and aptazymes

DNAzymes are artificial enzymes obtained via *in vitro* selection [78] and consist of single strand of DNA organized into domains required for enzymatic activity (catalytic core domains) and for substrate recognition (substrate binding domains). To date, many classes of DNAzymes have been selected from fully randomized libraries and they are able to catalyze reactions such as oxidation of organic molecules, ligation, phosphorylation, and hydrolytic cleavage of DNA and RNA [79], thus representing promising tools for therapeutic applications [80] (see Table 4). Furthermore, some proofs of concept demonstrate the possible use of DNAzymes for imaging of metal ions in living cells [81,82]. The use of catalytic nucleic acids has been widely employed to achieve amplified biosensing by designing aptamer–DNAzyme conjugates (i.e. aptazyme) that combine recognition units and amplifying readout units. The mechanisms of action of such responsive functional molecules has been inspired by naturally-occurring regulatory RNA elements [83].

As one of the most representative example, Willner and coworkers exploited the combined use of tailored molecular beacons and hemin/G-quadruplex DNAzymes [84] to control the peroxidase-mimicking enzymatic activity of a class of DNAzymes [85]. They designed a molecular beacon consisting of a loop complementary to the nucleic acid target and a stem including half of the DNAzyme-forming sequence, locked by a complementary sequence (Fig. 4A) [86]. In the presence of target, it hybridizes with

Table 4
Allosterically regulated DNAzymes.

Allosteric effector	Output Signal	Detection Range	LOD	Matrix	References
ss-DNA	Absorbance	0.2–4.3 μM	n.a.	buffer	[86]
dNTP telomerase	Absorbance	0-10000 cells	500 cells	HeLa cells	[86]
ss-DNA	Amperometry	1-1000 nM	0.6 nM	buffer	[87]
ss-DNA	Electrochemiluminescence	5.0 fM-0.1 nM	0.9 fM	buffer	[88]
ss-DNA	Fluorescence	1-20 nM	1 nM	buffer	[89]
ss-DNA	Fluorescence	1-10 nM	1 nM	buffer	[90]
Thrombin	Fluorescence	10-100 nM	10 nM	buffer	[90]
ss-DNA	Polyacrylamide gel electrophoresis (PAGE)	n.a.	n.a	buffer	[91]
Small molecules (Adenosine, FMN)	Polyacrylamide gel electrophoresis (PAGE)	n.a.	n.a	buffer	[92]
ss-DNA	Fluorescence	n.a.	5 pMM	buffer	[93]
Transcription Factor (TBP, MITF)	Absorbance	nM- μM	n.a.	buffer	[93]

the loop domain, opening the hairpin structure and releasing the DNAzyme-forming sequence. At this stage, the formed G-quadruplex in the presence of hemin can exhibit its peroxidase-like activity and an optical signal output is measured. Such strategy has been adapted to create sensors based on electrochemical [87], label-free electrochemiluminescent [88] and fluorescence detection [89,90].

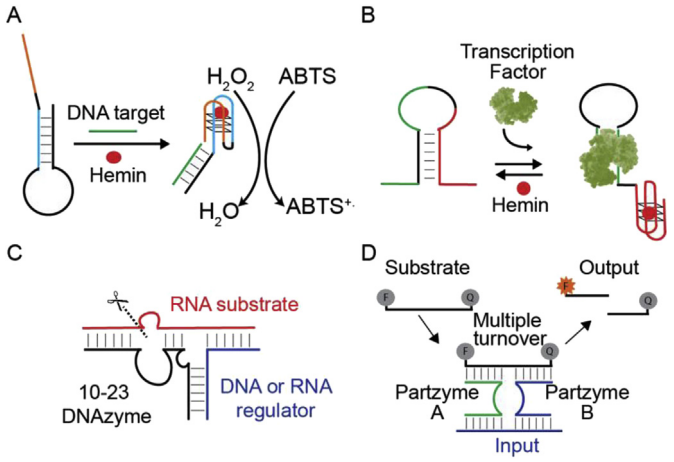

Fig. 4. Examples of DNAzymes whose enzymatic activity is regulated through allostery.
A) Peroxidase-mimicking enzymatic activity of DNAzyme allosterically controlled by combining tailored molecular beacons and hemin/G-quadruplex DNAzymes. When the blue segment is hybridized in the hairpin conformation, the activity of the catalytic DNAzyme is inhibited. The binding of the DNA target (in green) to the loop opens the beacon, and the two segments (light blue and orange segments) fold with hemin thus activating the peroxidase-like activity of the DNAzyme. Adapted with permission from Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler, I. Willner, J. Am. Chem. Soc. 126 (2004). https://doi.org/10.1021/ja031875r. Copyright (2004) American Chemical Society. B) DNAzyme allosterically activated by transcription factors. A catalytic DNAzyme domain (red sequence) is coupled whit a double-stranded transcription factor-binding domain (green). A sequence element complementary to the sequence of the DNAzyme stabilizes an alternative conformation (left) that "sequesters" both domains in an inactive state. This off-state is in equilibrium with a second conformation, the on-state, in which both domains are functional. The binding of the transcription factor pushes this conformational equilibrium towards the on-state, activating catalysis. In the presence of hemin and H_2O_2, this domain catalyzes the oxidation of the HRP substrate TMB to give a coloured product. Adapted from Ref. 95 with permission from the Royal Society of Chemistry. C) RNA-cleaving activity of 10–23 DNAzyme can be allosterically regulated *via* the use of oligonucleotide effectors. The regulator oligonucleotide is complementary to both the RNA substrate and the DNAzyme. The dashed line identifies the site of RNA cleavage. Adapted from J. Mol. Biol. 318, D.Y. Wang, B·H.Y. Lai, D. Sen, 33–43, Copyright (2002), with permission from Elsevier. D) The DNAzyme is split into two inactive halves, each one containing a partial catalytic core, a substrate arm and a sensor portion that generate a multicomponent complex (MNAzyme) only at the presence of the DNA oligonucleotide working as an allosteric effector (input, blue). Adapted with permission from E. Mokany, S.M. Bone, P.E. Young, T.B. Doan, A. V. Todd, J. Am. Chem. Soc. 132 (2009) 1051–1059. https://doi.org/10.1021/ja9076777. Copyright (2010) American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Allosteric modulation of DNAzyme activity has been also demonstrated through the introduction of a DNA strand that works as an allosteric effector. In this case, nucleic acids form branched three-way junctions to control the RNA-cleaving activity of DNAzymes (Fig. 4C) [91,92]. The "regulator" element induces the formation of stable and catalytically competent "three-way" enzyme-substrate-regulator complexes, which operate on unstable and catalytically poor enzyme-substrate complexes.

Mokany et al. demonstrated instead that the catalytic core of a DNAzyme can be split into two inactive halves, each one containing a partial catalytic core, a substrate arm and a sensor arm that generate a multicomponent complex (MNAzyme) only in the presence of the nucleic acid "assembly facilitator" that acts as an allosteric effector (Fig. 4D) [93]. Using this platform, they demonstrated several applications including sensitive, isothermal target detection (detection limit down to 10 copies of the target gene), discrimination of polymorphisms, and highly specific monitoring of real-time polymerase chain reaction (PCR). Recently, a programmable nanodevice based on MNazyme has been proposed for intracellular imaging of miRNA and logic gated-drug delivery [94]. The nanodevice presents high specificity and sensitivity, suggesting promising applications in accurate classification of cancer subtypes, dynamic monitoring of therapy response, and prognostic evaluation.

Our research unit have also engineered DNAzymes allosterically activated by specific nucleic acid binding proteins (Fig. 4B) [95]. In this case, we designed structure-switching catalytic DNAzyme sequences which present consensus sequences recognized by specific transcription factors (TF). Specifically, a construct exhibiting two low-energy conformational states has been engineered: in the more stable of these, termed the *off-state*, the catalytic domain and the double-stranded, TF-binding region are "sequestered" and thus inactive. In the *on-state* conformation, both the domains are in their active forms. The presence of the TF target pushes the equilibrium between these states towards the latter conformation, concomitantly activating peroxidise catalysis. We demonstrated the versatility of this approach on two different peroxidase-like DNAzymes whose activities are triggered upon binding either TATA binding protein or the microphthalmia-associated transcription factor (MITF).

DNAzymes joined with an aptamer unit are generally called aptazymes. In this case, the aptamer allows for an allosteric control over the catalytic DNAzyme action as a function of the target binding. Thus an aptamer binding site represents the allosteric binding site that enables the enzyme-mimicking activity to be activated and associated with a detectable output (i.e. fluorescence, colorimetry, chemiluminescence) [96]. The combination of DNA

Table 5
Allosterically regulated aptazymes.

Allosteric effector	Output Signal	Detection Range	LOD	Matrix	References
AMP	Absorbance	n.a.	50 μM	buffer	[99]
Lysozyme	Absorbance	n.a.	0.5 pM	buffer	[99]
AMP	Fluorescence	18-130 μM	18 μM	10% serum	[105]
IFN-γ interferon-gamma	Fluorescence	4.6–900 nM	4.6 nM	buffer	[105]
ATP	Fluorescence	n.a.	n.a.	buffer	[107]
Adenosine	Absorbance	6-50 μM	6 μM	buffer	[108]
Adenosine	Fluorescence	500 nM-40 μM	500 nM	buffer	[109]
Adenosine	Fluorescence	n.a.	6.7 μM	10% human serum	[110]
Adenosine	Fluorescence	n.a.	n.a.	75% human serum	[118]
ATP	Fluorescence	200 nM-20 μM	200 nM	Hela cells	[119]

aptamer unit within re-engineered DNAzyme that act as catalytic signal amplifier, have opened the doors to the development of sensors for a wide range of analytes, besides cofactors [97]. DNA aptazymes have been rationally engineered through the introduction of a *communication* module which is a short structural DNA sequence that connects the aptamer and the catalytic domain. This element has a critical role because it is responsible for translating the binding event occurring in the aptamer domain into an activity-associated conformational change within the catalytic domain [98].

Several strategies have been reported to rationally design allosteric aptazymes (see Table 5). Willner and coworkers have engineered hairpin structures composed by anti-AMP or anti-lysozyme aptamer units linked to a HRP-mimicking DNAzyme sequence [99]. The anti-AMP aptamer is a portion of the loop region whereas the DNAzyme sequence is part of the stem region (Fig. 5A). In the absence of target, DNAzyme is not active. When the target is

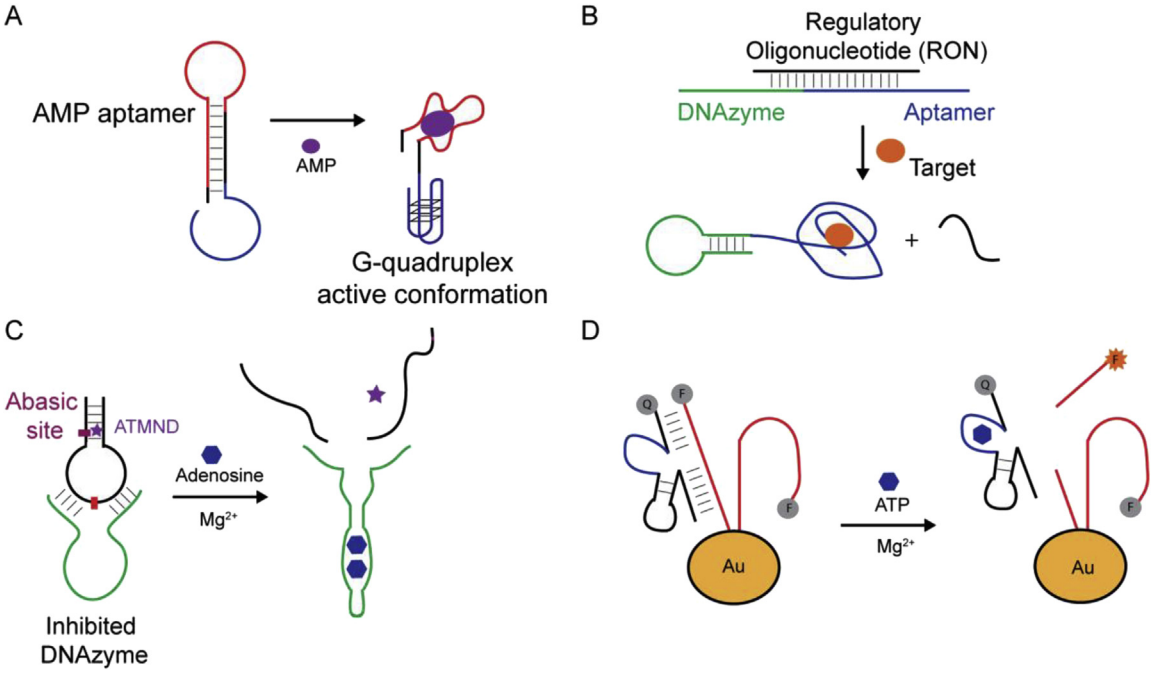

Fig. 5. Examples of allosterically regulated DNA-aptazymes.
A) The anti-AMP aptamer (red sequence) is linked to the horseradish peroxidase (HRP)-mimicking DNAzyme sequence (blue sequence) to form a hairpin structure. The HRP-mimicking DNAzyme sequence is protected in an inactive structure in the stem regions of the hairpin, whereas the loop regions includes a part of the aptamer sequence. The presence of target AMP opens the hairpin resulting in the active HRP-mimicking DNAzyme. Adapted with permission from C. Teller, S. Shimron, I. Willner, Anal. Chem. 81 (2009). https://doi.org/10.1021/ac901773b. Copyright (2009) American Chemical Society. B) A DNA aptamer (blue sequence) is linked to a DNAzyme (green sequence). In the absence of ligand, a complementary oligonucleotide effector (grey portion, RON) hybridizes to a portion of both aptamer and DNAzyme, thus inhibiting the DNAzyme function. The binding of the target to the aptamer significantly weakens the duplex between the DNAzyme sequence and the effector. As a result, the inhibitory effect of the regulatory oligonucleotide is much reduced and the enzymatic activity is largely restored. Adapted with permission from J.C. Achenbach, R. Nutiu, Y. Li, Anal. Chim. Acta. 534 (2005) 41–51. Elsevier. https://doi.org/10.1016/j.aca.2004.03.080. C) The DNAzyme substrate is incorporated in the loop of a molecular beacon having an abasic site in the hybridized stem region. The sequence of the Mg^{2+}-dependent 10-23 DNAzyme, instead, is conjugated with the nucleic acid sequence of the anti-adenosine aptamer. In the absence of target adenosine, the DNAzyme is inhibited, even in the presence of Mg^{2+} ions. Upon addition of adenosine, instead, the DNAzyme is activated by restoring the stem-loop structure and its function. Adapted with permission from P. Song, Y. Xiang, H. Xing, Z. Zhou, A. Tong, Y. Lu, Anal. Chem. 84 (2012). https://doi.org/10.1021/ac203488p. Copyright (2012) American Chemical Society. D) The ATP-specific aptazyme strand and the relative substrate are immobilized on gold nanoparticles (AuNP). In absence of ATP, the aptazyme cannot form a stable and active structure, and the fluorescence of the fluorophore is quenched by both AuNP and molecular quencher. In presence of ATP, instead, the aptamer domain binds to ATP forming a compact structure, and activates the aptazyme. The activated aptazyme cleaves the fluorophore-labeled substrate strand, releasing the shorter fluorophore-labeled DNA fragment. The active aptazyme can catalitically bind to another substrate strand on the AuNP, causing the cleavage of another substrate strand, providing an amplified fluorescent signal for the target ATP. Adapted with permission from Y. Yang, J. Huang, X. Yang, K. Quan, H. Wang, L. Ying, N. Xie, M. Ou, K. Wang, Anal. Chem. 88 (2016) 5981–5987. https://doi.org/10.1021/acs.analchem.6b00999. Copyright (2016) American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

present, instead, it binds the aptamer domain allowing the self-assembly of the active HRP-mimicking DNAzyme. The enzymatic activity has been analysed through colorimetric [99–101] and electrochemical [102–104] readout.

Thus far, however, the available examples of DNA aptazyme sensors are still limited, especially because of the lack of a universal bioengineering approach that allows for a rational molecular design. In this regards, Zhou et al. showed an innovative strategy for the engineering of optical DNA aptazyme [105]. In this case a DNA aptamer and DNAzymes are joined together without any change in their original sequences. The active aptazyme results in the increase of the fluorescence emission via cleavage of a dually-labeled substrate upon target binding and its real-time monitoring allows for target quantification.

Li and coworkers also reported a very simple strategy for the design of fluorescent aptazyme-based sensor, expanding the methodology developed for the engineering of switching DNA aptamers [106]. They employed three different oligonucleotides: an ATP aptamer-linked DNAzyme, a *regulatory* oligonucleotide, and a substrate with a fluorophore/quencher pair close to the cleavage site (Fig. 5B). In the absence of the aptamer's target, the *regulatory* oligonucleotide inhibits the catalytic activity of the aptazyme by forming a stable DNA–DNA duplex. In the presence of the ligand, instead, the formation of a ligand–aptamer complex restores the catalytic efficacy of the aptazyme [107].

In the design proposed by Deng and coworkers, on the contrary, DNAzyme is split in two portions to assemble with an aptamer into an active chimera structure [108]. In the absence of the target, the two split enzymatic parts generate a G-quadruplex that can catalyze the oxidation of ABTS which generates a colorimetric output. In the presence of target, instead, the two fragmented enzymatic halves remain at a distance from each other and the DNAzyme activity is inhibited. To obtain a significant signal amplification, aptamer-tailored DNAzyme can be also combined with molecular beacons. In this case, the DNAzyme substrate strand is incorporated in the loop of a molecular beacon that binds to the DNAzyme strand to form a complex structure. The presence of the target induces the substrate cleavage and the molecular beacon cut into two portions, resulting in enhanced fluorescence signal [109]. The functionalization of the molecular beacon can be overcome through the introduction of an abasic site containing a tetrahydrofuranyl residue (named dSpacer) into the functional DNA duplexes [110]. In this strategy, previously employed for the development of label-free aptamer based sensors [111,112], an external fluorophore binds to the abasic site in DNA duplex, resulting in its quenched fluorescence. In the presence of the specific target, the aptamer-target interactions induce the cleavage of the substrate and the subsequent release of fluorophore from the stem region into the solution, and the recovery of its fluorescence. By using the aptazyme of adenosine based on 10-23 DNAzyme, Lu and coworkers have detected adenosine with high sensitivity and selectivity in diluted serum samples (Fig. 5C) [110].

Many other strategies have been proposed to achieve an enhanced amplification using DNAzyme as a catalytic signal amplifier to construct biosensors [113]. Despite significant efforts in developing allosteric aptazyme-based sensors [114,115], only few sensors have been tested in biological samples [105,116,117]. This is probably due to the difficulty to transfer aptazymes developed for test tube to work in complex biological matrices. For example, regarding the DNA aptazymes that cleave RNA substrates, a reason is that natural RNases present in biological samples cleave the RNA substrate, producing false-positive signals. Because the substrate of natural RNases is RNA present in the natural D-configuration (D-RNA), the development of RNase-resistant RNA-cleaving DNAzymes could be a solution to overcome this issue. With this aim, Li and coworkers have *in vitro* selected DNAzymes that cleave L-RNA, the enantiomer of D-RNA, which is completely resistant to RNases. The most active L-RNA-cleaving DNAzyme isolated has been employed to build an aptazyme that detect ATP in biological samples containing RNases [118]. Nevertheless, the use of DNA aptazymes for intracellular biological molecules detection and imaging is still limited. Only recently, an aptazyme sensor for amplified molecular probing in living cells has been reported [119]. Gold nanoparticles have been modified with fluorophore-labeled substrate strands hybridized to quencher-labeled ATP-specific aptazyme (Fig. 5D). The amplification of the fluorescence signal has been obtained by cycling and regeneration of the aptazyme. Furthermore, the sensor can readily enter living cells and works at physiological Mg^{2+} concentration.

Allosteric aptazymes can also be useful tools for other applications, i.e. drug release. Willner and coworkers have demonstrated the triggered release of the anticancer drug (doxorubicin) from the pores of mesoporous silica by using aptazymes as functional triggers for opening the pores. The opening of the pores proceeds only in the presence of target following the cooperative formation of an aptamer–substrate complex [120].

7. Conclusions and outlook

This review article sheds useful insights into the design and functions of different classes of DNA-based switches. We outlined relevant examples of DNA-based switches whose functional activities are allosterically regulated in response to specific binding molecules, including proteins, nucleic acids and small molecules, acting as allosteric effector. By doing so, we demonstrated that it is possible to rationally introduce Nature's solutions, such as allostery, into a wide range of DNA-based switches. Given the efforts currently devoted to engineering allosteric nucleic acids for various applications in cellular and molecular biology, and the growing success of research groups working on nucleic acid biomolecular design, we are confident that these and similar approaches, will offer viable solutions to a wide range of bioanalytical problems.

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