# Allosterically Regulated DNA-based Switches: from Design to Bioanalytical Applications

Marianna Rossetti and Alessandro Porchetta*

[1] Chemistry Department, University of Rome, Tor Vergata, Via della Ricerca Scientifica, 00133, Rome, Italy;

*Corresponding author: alessandro.porchetta@uniroma2.it

## 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 a conformational change that alter the function of a connected biomolecule at a distant site. 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.

**Keywords:**

DNA nanotechnology; DNA-based switches; allostery;

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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. We also outline the applications of different classes of allosterically-regulated DNA-based switches (i.e. stem-loop molecular beacons, aptamers, DNAzymes and aptazymes) as functional tools for a broad range of bio-analytical applications.

**BODY TEXT**

## 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 diagnostics, imaging, and biosensing [6–8]. 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 [9,10]. 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 [11], 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 employed for engineering DNA-switches (Figure 1) consists in the generation of a distorted conformation that is not able to bind the target (the *non-binding* "off" state; Figure 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 (Figure 1, top) [12]. 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.

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 dynamic range and detection limit [13,14]. The possibility to finely control the relationship between switch signaling and thermodynamic presents several advantages. The first one is represented by the wide range of inputs that can be used to trigger such switching, including complementary nucleic acid strands [15,16] as well as small molecules or protein targets (i.e. through the use of aptamer sequences or consensus binding sequence) [17–21]. A second advantage is the ease with which secondary chemical and biological effectors (ligands that bind distal sites on the switch) can be used to regulate biomolecular functions via a mechanism called "allostery" (Figure 2).

### 3. *Introducing allostery into DNA-switches.*

Allostery, called "the second secret of life" by Perutz [22], is a ubiquitous strategy employed in nature to regulate the affinities of biomolecules and, through this, to control cellular processes and pathways [23–26]. 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 [27] and G protein-coupled receptors [28] 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 [29]. 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 [30].

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 (Figure 2). Moreover, the unique bio-responsive properties of DNA make it easy the use of complementary nucleic acid and/or DNA-binding biomolecules as allosteric effectors to control the activation/inhibition of DNA-switches. Recently, many efforts to rationally introduce allosteric regulation into nucleic acid based catalytic systems (i.e. ribozymes, DNAzymes) have been done [31–35]. 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 [36].

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 (Figure 1, bottom) or reducing (Figure 1, middle) the overall affinity for its target. 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, DNA-based stem-loop molecular beacons (MBs) are widely employed because of the unique stem–loop structure and fluorophore–quencher pair that make possible the detection of target nucleic acids to be reported in real time with excellent sensitivity and selectivity. MBs are therefore used for a variety of applications [37], such as biosensing platforms for DNA/RNA detection [38], the investigation of enzymatic processes [39], the real time monitoring of living cells processes [40], and the study of protein–DNA interactions [41, 42].

MBs are single-stranded fluorophore-and-quencher-modified DNA sequences that present self-complementary ends. In the absence of the nucleic acid target, MBs adopt a stem−loop "off" configuration that brings the fluorophore/quencher pair into close proximity, 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. It has 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 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) [11].

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 (Figure 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 length and concentration [43]. 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)) trigger the conformational change of the stem-loop structure. We demonstrated that the binding of Hg (II) and Ag (I) ions induces a conformational change between two alternative stem-loop states resulting in a change of the fluorescence output in the presence of the heavy metal ions. In this case, we employed complementary single-stranded DNA sequences as allosteric effectors that either stabilize or destabilize the *non-binding* state of the MB, enabling dynamic range tuning over several orders of magnitude of heavy metal ions concentration [44]. Specifically, DNA activator binding destabilizes the *non-binding* state by partially disrupting the duplex stem ($K_S$ increases and affinity improves). Stabilization of the *non-bindin*g 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). Furthermore, allosteric control over DNA-switches also provides a means of broadening the dynamic range of target concentration, over which they respond robustly. This is possible through the use of a mixture of two allosteric effectors in the same tube. As an example, using an activator and an inhibitor in the same tube we have broadened the dynamic range of the sensor 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 [45]. 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, and 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 [46]. 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 [47] . When the specific site in the MBs is methylated by Dam MTase, the probe can be cut by the restriction nuclease DpnI to release 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 bioelectrochemical switches for the detection of complementary DNA sequences [48]. 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 [49]. 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 demonstrated the detection of microRNA (miRNA) let-7a *via* hybridization chain reaction (HCR), where a part of the stem of the hairpin involved is freed by the presence of the miRNA target that acts as initiator sequence strands for the HCR [50]. 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 MBs [51]. In this case, the metal-organic framework can mimetically catalyze the oxidation of *o*-phenylenediamine (*o*-PD) to 2,2′-diaminoazobenzene, which is a good electrochemical indicator for signal readout. The presence of DNA target triggers 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 rationally designed a class of DNA-switches allosterically regulated by biological targets instead of using complementary DNA strands. Such DNA-switches are able to load a molecular cargo (i.e. doxorubicin) and release it only in the presence of antibodies or transcription factors in a very controlled fashion (Figure 3B) [52]. 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 allosteric effector. 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). The binding of the transcription factor pushes this conformational equilibrium towards the "Release" state thus leading to doxorubicin release from the stem of DNA-switch. 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.*

Nucleic acids can not only hybridize to one another, but can also form complex shapes that may act as scaffolds for molecular interactions and support complex formation with protein and small-molecule targets. 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 [53, 54]. Aptamers can be specific as antibodies and can bind their targets with similar affinity, but they are often smaller, easier to generate and more straightforward to modify chemically than their protein-based counterparts [55]. 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 [56]. Most works involve only a few model aptamers to demonstrate the sensing concept with limited biomedical impact. Moreover, other limitations of this technology include the lack of high quality aptamers for clinically important targets and the need to be extensively tested in clinical sample matrices to establish reliability and accuracy. Although recent clinical developments have revived the impetus for this promising class of molecules as targeted therapeutics, different factors (i.e. their inherent physicochemical characteristics, lack of safety data) have delayed their clinical translation as well.

Here we have focused on the last advances in the design of DNA aptamers which present the possibility to be finely regulated through allostery. This mechanism has been widely integrated into

engineered RNA molecules (ribozymes, allosteric RNA aptamers) and remarkable properties of molecular recognition and allosteric function have been demonstrated. Such engineering efforts are made possible by RNA's unique tractability to both rational design and combinatorial selection techniques. Examples of allosterically regulated DNA aptamers, on the contrary, have seen relatively less attention in the DNA-design literature. 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.

Bong and coworkers have used bifacial peptide nucleic acid (bPNA) to allosterically switch-on protein and small-molecule binding in DNA and RNA aptamers [57]. They have functionally crippled a protein-binding DNA aptamer that binds immunoglobulin E (IgE) by replacement of a critical duplex element with poly-thymine tracts (Figure 3C). These tracts fold into triplex stem−loop structures upon binding to a single bPNA strand. Triplex hybridization of the poly-thymine tracts with bPNA mimic, the native duplex and tighten, the overall fold to restore function in an 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 [58] to tune, extend, and narrow the useful dynamic range of target concentration. In particular, we have demonstrated that using a set of easily designed oligonucleotide inhibitors that compete against the three-way junction folding upon cocaine binding, allows rational fine-tuning of the affinity of the DNA aptamer across more than three

orders of magnitude of cocaine concentration [59]. More recently, we have also demonstrated the regulation of the activity of nucleic-acid target-responsive switches with external activators through a general and modular approach based on a modular clamp-like mechanism [60]. 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. This mechanism, commonly referred to as "cooperativity," can thus affect the shape of the binding curve, producing either a more responsive dependence (positive cooperativity) and an improved sensitivity to small changes in target concentration. In order to engineer allosteric cooperativity, Plaxco and coworkers have developed a loop-closure mechanism to the rational engineering of allosterically cooperative receptors [61]. 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) at a position within the single loop and linked tandem repeats of the two resulting half-aptamers via a long, unstructured loop (Figure 3D). The binding-induced association of the first pair of receptor "halves" must pay the entropic cost of loop closure, reducing the affinity of the first binding event relative to that of the second, thus producing a cooperative response. Such allosteric control could be of utility in artificial biotechnologies, such as biosensors, genetic logic gates, and "smart" materials, in which enhanced responsiveness is crucial.

### *6. Allosterically regulated DNAzymes and aptazymes.*

DNAzymes are artificial enzymes obtained via in vitro selection [62] 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 that could catalyze reactions such as porphyrin metalation, oxidation of organic molecules, DNA phosphorylation, RNA ligation, carbon- carbon bond formation and hydrolytic cleavage of DNA and RNA [63]. DNAzymes thus represent a promising tool for therapeutic applications (i.e. as antiviral, antibacterial, anti-cancer and anti-inflammatory) [64]. Furthermore, some proofs of concept demonstrate the possible use of DNAzymes for imaging of metal ions in living cells [65, 66]. 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. Indeed, it is well known that allosteric ribozymes control gene expression by cleaving RNA in response to specific metabolites, and riboswitches undergo a conformational change upon ligand binding that enhances or diminishes ribosome binding, thereby influencing the translation efficiency of the mRNA [67].

As one of the most representative example, Willner and coworkers exploited the combined use of tailored molecular beacons and hemin/G-quadruplex DNAzymes [68] to control the peroxidase-mimicking enzymatic activity of a class of DNAzyme employed for sensing, nanobiotechnology and logic gate applications [69]. 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 (Figure 4A) [70]. In the presence of target, it hybridizes with 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 [71], label-free electrochemiluminescent [72] and fluorescence detection [73, 74].

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, the ability of nucleic acids to form branched three-way junctions has been employed to regulate the RNA-cleaving activity of DNAzyme (Figure 4C) [75], [76]. The nucleic acid target molecule acts as a “regulator” module that induces the formation of stable and catalytically competent “three-way” enzyme-substrate-regulator complexes, relative to unstable and catalytically poor enzyme-substrate complexes.

Mokany et al. demonstrated that the catalytic core of a DNAzyme can be splitted 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 (Figure 4D) [77]. 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 [78]. 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 (Figure 4B) [79]. 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 less stable conformation, termed the *on-state*, 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 tested two different peroxidase-like DNAzymes whose activities are triggered upon binding either TATA binding protein or the microphthalmia-associated transcription factor (MITF).

DNAzymes can be further coupled with an aptamer domain to generate aptazymes. In this case, the aptamer–target interaction confers an allosteric control over the coupled catalytic and signaling activity of the DNAzyme. Specifically, aptamer binding site represents the allosteric binding site that enables the enzyme-mimicking activity to be activated and associated with a detectable and measurable output (i.e. fluorescence, colorimetry, chemiluminescence) [80]. The combination of DNA 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 [81]. DNA aptazymes can be rationally designed through the introduction of a communication module which is a short structural DNA sequence that connects the aptamer and the catalytic domain. It 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 [82].

Several strategies have been reported to rationally design allosteric aptazymes. Willner and coworkers have engineered hairpin structures composed by anti-AMP or anti-lysozyme aptamer units linked to a HRP-mimicking DNAzyme sequence [83]. The aptamer unit is part of the loop region whereas DNAzyme sequence is part of the stem region (Figure 5A). In the absence of target, DNAzyme is in its inactive form. When the target is present, instead, it binds the aptamer domain allowing the opening of the hairpin and the self-assembly of the active HRP-mimicking DNAzyme.

The enzymatic activity can be analysed through colorimetric [83, 84, 85] or electrochemical [86, 87, 88] measurements.

So far, however, the available examples of DNA aptazyme sensors are still limited in utilizing only several DNAzymes and DNA aptamers, most likely due to the lack of a general and simple approach for rational design. Zhou et al. showed a general approach for designing fluorescent DNA aptazyme sensors [89]. Specifically, aptamers and DNAzymes are connected at the ends to avoid any change in their original sequences, therefore enabling the general use of different aptamers and DNAzymes in the design. Upon activation of the aptazymes by the targets of interest, the rate of fluorescence enhancement via the cleavage of a dually labeled substrate by the active aptazymes is then monitored for target quantification.

Li and coworkers reported a very simple strategy for the rational design of fluorescent aptazyme-based sensor, expanding the methodology developed for the engineering of structure-switching signalling aptamers, i.e. fluorescence-signalling DNA aptamers [90]. The strategy includes three separate oligonucleotides: an ATP aptamer-linked DNAzyme, a regulatory oligonucleotide with a sequence complementary to the nucleotides spanning the aptamer and DNAzyme junction, and a substrate with a fluorophore/quencher pair placed right beside the cleavage site (Figure 5B). In the absence of the aptamer's cognate ligand, the regulatory oligonucleotide inhibits the catalytic ability of the aptazyme by forming a stable DNA–DNA duplex. In the presence of the ligand, the formation of a ligand–aptamer complex reduces the suppressive effect of the regulatory oligonucleotide and restores the catalytic activity of the aptazyme [91].

In the design proposed by Deng and coworkers, on the contrary, DNAzyme is split in two parts to assemble with an aptamer into a functional chimera structure [92]. In the absence of target, the two fragmented enzymatic halves form an intact G-quadruplex that upon hemin binding can

catalyze the oxidation of ABTS into the blue-green colored radical product. 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 combined with molecular beacons modified with a fluorophore/quencher pair. The DNAzyme substrate strand is incorporated in the loop of a molecular beacon that binds to the DNAzyme strand to form a complex structure. In the presence of the target, the substrate is cleaved and the molecular beacon is cut into two pieces, resulting in stem unfolding and enhanced fluorescence signal [93]. 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 [94]. In this strategy, previously employed for the development of label-free aptamer based sensors [95, 96], 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 (Figure 5C) [94].

Many other strategies have been proposed to achieve an enhanced amplification using DNAzyme as a catalytic signal amplifier to construct biosensors [97]. Despite significant efforts in developing allosteric aptazyme-based sensors [98, 99], only few sensors have been tested in biological samples [89, 100, 101]. 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 [102]. 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 [103]. Gold nanoparticles have been modified with fluorophore-labelled substrate strands hybridized to quencher-labelled ATP-specific aptazyme (Figure 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, a process that triggers the formation of the active DNAzyme structure [104].

## *7. Conclusions and outlooks*

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.

## REFERENCES

[1] K. Vermeulen, D.R. Van Bockstaele, Z.N. Berneman, The cell cycle: A review of regulation, deregulation and therapeutic targets in cancer, Cell Prolif. 36 (2003). doi:10.1046/j.1365-2184.2003.00266.x.

[2] J. Cherfils, M. Zeghouf, Chronicles of the GTPase switch, Nat. Chem. Biol. 7 (2011). doi:10.1038/nchembio.608.

[3] J. Soboloff, M. Madesh, D.L. Gill, Sensing cellular stress through STIM proteins, Nat. Chem. Biol. 7 (2011). doi:10.1038/nchembio.619.

[4] A. Vallée-Bélisle, K.W. Plaxco, Structure-switching biosensors: Inspired by Nature, Curr. Opin. Struct. Biol. 20 (2010). doi:10.1016/j.sbi.2010.05.001.

[5] A.A. Lubin, K.W. Plaxco, Folding-based electrochemical biosensors: The case for responsive nucleic acid architectures, Acc. Chem. Res. 43 (2010). doi:10.1021/ar900165x.

[6] K.W. Plaxco, H.T. Soh, Switch-based biosensors: A new approach towards real-time, in vivo molecular detection, Trends Biotechnol. 29 (2011). doi:10.1016/j.tibtech.2010.10.005.

[7] S. Slomovic, K. Pardee, J.J. Collins, Synthetic biology devices for in vitro and in vivo diagnostics, Proc. Natl. Acad. Sci. U. S. A. 112 (2015). doi:10.1073/pnas.1508521112.

[8] H.-M. Meng, H. Liu, H. Kuai, R. Peng, L. Mo, X.-B. Zhang, Aptamer-integrated DNA nanostructures for biosensing, bioimaging and cancer therapy, Chem. Soc. Rev. 45 (2016). doi:10.1039/c5cs00645g.

[9] S. Ranallo, M. Rossetti, K.W. Plaxco, A. Vallée-Bélisle, F. Ricci, A Modular, DNA-Based Beacon for Single-Step Fluorescence Detection of Antibodies and Other Proteins, Angew. Chemie - Int. Ed. 54 (2015). doi:10.1002/anie.201505179.

[10] J.S. Swensen, Y. Xiao, B.S. Ferguson, A.A. Lubin, R.Y. Lai, A.J. Heeger, K.W. Plaxco, H.T.

Soh, Continuous, real-time monitoring of cocaine in undiluted blood serum via a microfluidic, electrochemical aptamer-based sensor, J. Am. Chem. Soc. 131 (2009). doi:10.1021/ja806531z.

[11] A. Vallée-Bélisle, F. Ricci, K.W. Plaxco, Thermodynamic basis for the optimization of binding-induced biomolecular switches and structure-switching biosensors, Proc. Natl. Acad. Sci. U. S. A. 106 (2009). doi:10.1073/pnas.0904005106.

[12] Y. Krishnan, F.C. Simmel, Nucleic acid based molecular devices., Angew. Chem. Int. Ed. Engl. 50 (2011) 3124–56. doi:10.1002/anie.200907223.

[13] C.L. Beisel, T.S. Bayer, K.G. Hoff, C.D. Smolke, Model-guided design of ligand-regulated RNAi for programmable control of gene expression, Mol. Syst. Biol. 4 (2008). doi:10.1038/msb.2008.62.

[14] J.S. Marvin, H.W. Hellinga, Manipulation of ligand binding affinity by exploitation of conformational coupling, Nat. Struct. Biol. 8 (2001). doi:10.1038/nsb0901-795.

[15] R.R.F. Machinek, T.E. Ouldridge, N.E.C. Haley, J. Bath, A.J. Turberfield, Programmable energy landscapes for kinetic control of DNA strand displacement, Nat. Commun. 5 (2014). doi:10.1038/ncomms6324.

[16] D.Y. Zhang, E. Winfree, Control of DNA strand displacement kinetics using toehold exchange, J. Am. Chem. Soc. 131 (2009). doi:10.1021/ja906987s.

[17] L. Chen, J. Chao, X. Qu, H. Zhang, D. Zhu, S. Su, A. Aldalbahi, L. Wang, H. Pei, Probing Cellular Molecules with PolyA-Based Engineered Aptamer Nanobeacon, ACS Appl. Mater. Interfaces. 9 (2017). doi:10.1021/acsami.6b16764.

[18] N. Wu, I. Willner, Programmed dissociation of dimer and trimer origami structures by aptamer-ligand complexes, Nanoscale. 9 (2017). doi:10.1039/c6nr08209b.

[19] S. Perrier, P. Bouilloud, G. de Oliveira Coelho, M. Henry, E. Peyrin, Small molecule aptamer assays based on fluorescence anisotropy signal-enhancer oligonucleotides, Biosens.

Bioelectron. 82 (2016). doi:10.1016/j.bios.2016.04.010.

[20] A. Desrosiers, A. Vallée-Bélisle, Nature-inspired DNA switches: Applications in medicine, Nanomedicine. 12 (2017). doi:10.2217/nnm-2016-0349.

[21] Y. Tang, F. Long, C. Gu, C. Wang, S. Han, M. He, Reusable split-aptamer-based biosensor for rapid detection of cocaine in serum by using an all-fiber evanescent wave optical biosensing platform, Anal. Chim. Acta. 933 (2016). doi:10.1016/j.aca.2016.05.021.

[22] M.F. Perutz, Mechanisms of cooperativity and allosteric regulation in proteins, Q. Rev. Biophys. 22 (1989). doi:10.1017/S0033583500003826.

[23] M.V. Levine, M.A. Cuendet, G. Khelashvili, H. Weinstein, Allosteric Mechanisms of Molecular Machines at the Membrane: Transport by Sodium-Coupled Symporters, Chem. Rev. 116 (2016). doi:10.1021/acs.chemrev.5b00627.

[24] S.S. Grimm, E.Y. Isacoff, Allosteric substrate switching in a voltage-sensing lipid phosphatase, Nat. Chem. Biol. 12 (2016). doi:10.1038/nchembio.2022.

[25] N.D. Taylor, A.S. Garruss, R. Moretti, S. Chan, M.A. Arbing, D. Cascio, J.K. Rogers, F.J. Isaacs, S. Kosuri, D. Baker, S. Fields, G.M. Church, S. Raman, Engineering an allosteric transcription factor to respond to new ligands, Nat. Methods. 13 (2016). doi:10.1038/nmeth.3696.

[26] P. Tompa, The principle of conformational signaling, Chem. Soc. Rev. 45 (2016). doi:10.1039/c6cs00011h.

[27] M. Cecchini, A. Houdusse, M. Karplus, Allosteric communication in myosin V: From small conformational changes to large directed movements, PLoS Comput. Biol. 4 (2008). doi:10.1371/journal.pcbi.1000129.

[28] N.J. Smith, G. Milligan, Allostery at G protein-coupled receptor homo- and heteromers: uncharted pharmacological landscapes., Pharmacol. Rev. 62 (2010) 701–25. doi:10.1124/pr.110.002667.

[29] R. Nussinov, C.-J. Tsai, B. Ma, The Underappreciated Role of Allostery in the Cellular Network, Annu. Rev. Biophys. 42 (2013) 169–189. doi:10.1146/annurev-biophys-083012-130257.

[30] Y. Yuan, M.F. Tam, V. Simplaceanu, C. Ho, New look at hemoglobin allostery, Chem. Rev. 115 (2015) 1702–1724. doi:10.1021/cr500495x.

[31] B. Strobel, B. Klauser, J.S. Hartig, T. Lamla, F. Gantner, S. Kreuz, Riboswitch-mediated Attenuation of Transgene Cytotoxicity Increases Adeno-associated Virus Vector Yields in HEK-293 Cells, Mol. Ther. 23 (2015). doi:10.1038/mt.2015.123.

[32] B. Klauser, J. Atanasov, L.K. Siewert, J.S. Hartig, Ribozyme-Based Aminoglycoside Switches of Gene Expression Engineered by Genetic Selection in S. cerevisiae, ACS Synth. Biol. 4 (2015). doi:10.1021/sb500062p.

[33] M.A. Aleman-Garcia, R. Orbach, I. Willner, Ion-responsive hemin-G-quadruplexes for switchable DNAzyme and enzyme functions, Chem. - A Eur. J. 20 (2014). doi:10.1002/chem.201304702.

[34] M. Kirchner, S. Schneider, Gene expression control by Bacillus anthracis purine riboswitches, RNA. 23 (2017). doi:10.1261/rna.058792.116.

[35] P. Ketzer, J.K. Kaufmann, S. Engelhardt, S. Bossow, C. Von Kalle, J.S. Hartig, G. Ungerechts, D.M. Nettelbeck, Artificial riboswitches for gene expression and replication control of DNA and RNA viruses, Proc. Natl. Acad. Sci. U. S. A. 111 (2014). doi:10.1073/pnas.1318563111.

[36] F. Ricci, A. Vallée-Bélisle, A.J. Simon, A. Porchetta, K.W. Plaxco, Using Nature's "tricks" to Rationally Tune the Binding Properties of Biomolecular Receptors, Acc. Chem. Res. 49 (2016). doi:10.1021/acs.accounts.6b00276.

[37] W. Tan, K. Wang, T.J. Drake, Molecular beacons, Curr. Opin. Chem. Biol. 8 (2004). doi:10.1016/j.cbpa.2004.08.010.

[38] A.A. Martí, S. Jockusch, N. Stevens, J. Ju, N.J. Turro, Fluorescent hybridization probes for sensitive and selective DNA and RNA detection, Acc. Chem. Res. 40 (2007). doi:10.1021/ar600013q.

[39] Z. Tang, K. Wang, W. Tan, C. Ma, J. Li, L. Liu, Q. Guo, X. Meng, Real-time investigation of nucleic acids phosphorylation process using molecular beacons, Nucleic Acids Res. 33 (2005) 1–6. doi:10.1093/nar/gni096.

[40] W. Pan, Y. Li, M. Wang, H. Yang, N. Li, B. Tang, FRET-based nanoprobes for simultaneous monitoring of multiple mRNAs in living cells using single wavelength excitation., Chem. Commun. (Camb). 52 (2016) 4569–72. doi:10.1039/c5cc10147f.

[41] A.J. Bonham, K. Hsieh, B.S. Ferguson, A. Vallée-Bélisle, F. Ricci, H.T. Soh, K.W. Plaxco, Quantification of transcription factor binding in cell extracts using an electrochemical, structure-switching biosensor, J. Am. Chem. Soc. 134 (2012) 3346–3348. doi:10.1021/ja2115663.

[42] A. Vallée-Bélisle, A.J. Bonham, N.O. Reich, F. Ricci, K.W. Plaxco, Transcription factor beacons for the quantitative detection of DNA binding activity, J. Am. Chem. Soc. 133 (2011) 13836–13839. doi:10.1021/ja204775k.

[43] F. Ricci, A. Vallée-Bélisle, A. Porchetta, K.W. Plaxco, Rational design of allosteric inhibitors and activators using the population-shift model: In vitro validation and application to an artificial biosensor, J. Am. Chem. Soc. 134 (2012). doi:10.1021/ja304672h.

[44] A. Porchetta, A. Vallée-Bélisle, K.W. Plaxco, F. Ricci, Allosterically tunable, DNA-based switches triggered by heavy metals, J. Am. Chem. Soc. 135 (2013). doi:10.1021/ja404653q.

[45] V.S. Le, J.-E. Jeong, B. Kim, J. Lee, K. Kyhm, H.Y. Woo, Inhibitor effects on molecular beacon-based mercury assays for tuning of detection range, Sensors Actuators, B Chem. 240 (2017). doi:10.1016/j.snb.2016.09.034.

[46] Y. Song, L. Cui, J. Wu, W. Zhang, W.Y. Zhang, H. Kang, C.J. Yang, Allosteric molecular

beacons for sensitive detection of nucleic acids, proteins, and small molecules in complex biological samples, Chem. - A Eur. J. 17 (2011). doi:10.1002/chem.201101353.

[47] W. Zhang, X. Zu, Y. Song, Z. Zhu, C.J. Yang, Detection of DNA methyltransferase activity using allosteric molecular beacons, Analyst. 141 (2016). doi:10.1039/c5an01763g.

[48] D. Kang, A. Vallée-Bélisle, A. Porchetta, K.W. Plaxco, F. Ricci, Re-engineering electrochemical biosensors to narrow or extend their useful dynamic range, Angew. Chemie - Int. Ed. 51 (2012). doi:10.1002/anie.201202204.

[49] Z. Cai, Y. Song, Y. Wu, Z. Zhu, C. James Yang, X. Chen, An electrochemical sensor based on label-free functional allosteric molecular beacons for detection target DNA/miRNA, Biosens. Bioelectron. 41 (2013). doi:10.1016/j.bios.2012.10.002.

[50] S. Cai, Z. Cao, C. Lau, J. Lu, Label-free technology for the amplified detection of microRNA based on the allosteric hairpin DNA switch and hybridization chain reaction, Analyst. 139 (2014). doi:10.1039/c4an01178c.

[51] P. Ling, J. Lei, L. Zhang, H. Ju, Porphyrin-Encapsulated Metal-Organic Frameworks as Mimetic Catalysts for Electrochemical DNA Sensing via Allosteric Switch of Hairpin DNA, Anal. Chem. 87 (2015). doi:10.1021/acs.analchem.5b00001.

[52] M. Rossetti, S. Ranallo, A. Idili, G. Palleschi, A. Porchetta, F. Ricci, Allosteric DNA nanoswitches for controlled release of a molecular cargo triggered by biological inputs, Chem. Sci. 8 (2017). doi:10.1039/c6sc03404g.

[53] F. Pfeiffer, M. Rosenthal, J. Siegl, J. Ewers, G. Mayer, Customised nucleic acid libraries for enhanced aptamer selection and performance, Curr. Opin. Biotechnol. 48 (2017). doi:10.1016/j.copbio.2017.03.026.

[54] X. Fang, W. Tan, Aptamers generated from cell-SELEX for molecular medicine: A chemical biology approach, Acc. Chem. Res. 43 (2010). doi:10.1021/ar900101s.

[55] T.-H. Ku, T. Zhang, H. Luo, T.M. Yen, P.-W. Chen, Y. Han, Y.-H. Lo, Nucleic acid

aptamers: An emerging tool for biotechnology and biomedical sensing, Sensors (Switzerland). 15 (2015). doi:10.3390/s150716281.

[56] W. Zhou, P.-J.J. Huang, J. Ding, J. Liu, Aptamer-based biosensors for biomedical diagnostics., Analyst. 139 (2014) 2627–40. doi:10.1039/c4an00132j.

[57] X. Xia, X. Piao, D. Bong, Bifacial peptide nucleic acid as an allosteric switch for aptamer and ribozyme function, J. Am. Chem. Soc. 136 (2014). doi:10.1021/ja5032584.

[58] M.N. Stojanovic, P. de Prada, D.W. Landry, Aptamer-based folding fluorescent sensor for cocaine, J. Am. Chem. Soc. 123 (2001) 4928–4931. doi:10.1021/ja0038171.

[59] A. Porchetta, A. Vallée-Bélisle, K.W. Plaxco, F. Ricci, Using distal-site mutations and allosteric inhibition to tune, extend, and narrow the useful dynamic range of aptamer-based sensors, J. Am. Chem. Soc. 134 (2012). doi:10.1021/ja310585e.

[60] E. Del Grosso, A. Idili, A. Porchetta, F. Ricci, A modular clamp-like mechanism to regulate the activity of nucleic-acid target-responsive nanoswitches with external activators, Nanoscale. 8 (2016). doi:10.1039/c6nr06026a.

[61] A.J. Simon, A. Vallée-Bélisle, F. Ricci, K.W. Plaxco, Intrinsic disorder as a generalizable strategy for the rational design of highly responsive, allosterically cooperative receptors, Proc. Natl. Acad. Sci. 111 (2014) 15048–15053. doi:10.1073/pnas.1410796111.

[62] R. Fiammengo, A. Jäschke, Nucleic acid enzymes, Curr. Opin. Biotechnol. 16 (2005) 614–621. doi:10.1016/j.copbio.2005.10.006.

[63] M. Hollenstein, DNA catalysis: The chemical repertoire of DNAzymes, Molecules. 20 (2015) 20777–20804. doi:10.3390/molecules201119730.

[64] A. a Fokina, D. a Stetsenko, J.-C. François, DNA enzymes as potential therapeutics: towards clinical application of 10-23 DNAzymes., Expert Opin. Biol. Ther. 15 (2015) 689–711. doi:10.1517/14712598.2015.1025048.

[65] Z. Wu, H. Fan, N.S.R. Satyavolu, W. Wang, R. Lake, J.-H. Jiang, Y. Lu, Imaging

Endogenous Metal Ions in Living Cells Using a DNAzyme–Catalytic Hairpin Assembly Probe, Angew. Chemie - Int. Ed. 56 (2017). doi:10.1002/anie.201703540.

[66] C.E. McGhee, K.Y. Loh, Y. Lu, DNAzyme sensors for detection of metal ions in the environment and imaging them in living cells, Curr. Opin. Biotechnol. 45 (2017). doi:10.1016/j.copbio.2017.03.002.

[67] J.L. Vinkenborg, N. Karnowski, M. Famulok, Aptamers for allosteric regulation, Nat. Chem. Biol. 7 (2011). doi:10.1038/nchembio.609.

[68] P. Travascio, Y. Li, D. Sen, DNA-enhanced peroxidase activity of a DNA aptamer-hemin complex, Chem. Biol. 5 (1998).

[69] I. Willner, B. Shlyahovsky, M. Zayats, B. Willner, DNAzymes for sensing, nanobiotechnology and logic gate applications, Chem. Soc. Rev. 37 (2008) 1153. doi:10.1039/b718428j.

[70] Y. Xiao, V. Pavlov, T. Niazov, A. Dishon, M. Kotler, I. Willner, Catalytic beacons for the detection of DNA and telomerase activity, J. Am. Chem. Soc. 126 (2004). doi:10.1021/ja031875r.

[71] Q. Guo, Y. Bao, X. Yang, K. Wang, Q. Wang, Y. Tan, Amplified electrochemical DNA sensor using peroxidase-like DNAzyme, Talanta. 83 (2010). doi:10.1016/j.talanta.2010.09.054.

[72] S. Deng, L. Cheng, J. Lei, Y. Cheng, Y. Huang, H. Ju, Label-free electrochemiluminescent detection of DNA by hybridization with a molecular beacon to form hemin/G-quadruplex architecture for signal inhibition, Nanoscale. 5 (2013). doi:10.1039/c2nr33471b.

[73] J. Kosman, Y.-T. Wu, A. Gluszynska, B. Juskowiak, N-Methyl-4-hydrazino-7-nitrobenzofurazan: A fluorogenic substrate for peroxidase-like DNAzyme, and its potential application, Anal. Bioanal. Chem. 406 (2014). doi:10.1007/s00216-014-8119-7.

[74] E. Golub, R. Freeman, A. Niazov, I. Willner, Hemin/G-quadruplexes as DNAzymes for the

fluorescent detection of DNA, aptamer-thrombin complexes, and probing the activity of glucose oxidase, Analyst. 136 (2011). doi:10.1039/c1an15596b.

[75] D.Y. Wang, B.H.Y. Lai, A.R. Feldman, D. Sen, A general approach for the use of oligonucleotide effectors to regulate the catalysis of RNA-cleaving ribozymes and DNAzymes., Nucleic Acids Res. 30 (2002) 1735–1742. doi:10.1093/nar/30.8.1735.

[76] D.Y. Wang, B.H.Y. Lai, D. Sen, A General Strategy for Effector-mediated Control of RNA-cleaving Ribozymes and DNA Enzymes, J. Mol. Biol. 318 (2002) 33–43. doi:10.1016/S0022-2836(02)00046-3.

[77] E. Mokany, S.M. Bone, P.E. Young, T.B. Doan, A. V. Todd, MNAzymes, a versatile new class of nucleic acid enzymes that can function as biosensors and molecular switches, J. Am. Chem. Soc. 132 (2010) 1051–1059. doi:10.1021/ja9076777.

[78] P. Zhang, Z. He, C. Wang, J. Chen, J. Zhao, X. Zhu, C.Z. Li, Q. Min, J.J. Zhu, In situ amplification of intracellular microrna with mnazyme nanodevices for multiplexed imaging, logic operation, and controlled drug release, ACS Nano. 9 (2015) 789–798. doi:10.1021/nn506309d.

[79] G. Adornetto, A. Porchetta, G. Palleschi, K.W. Plaxco, F. Ricci, A general approach to the design of allosteric, transcription factor-regulated DNAzymes, Chem. Sci. 6 (2015). doi:10.1039/c5sc00228a.

[80] J. Liu, Z. Cao, Y. Lu, Functional nucleic acid sensors, Chem. Rev. 109 (2009). doi:10.1021/cr030183i.

[81] M. Famulok, J.S. Hartig, G. Mayer, Functional aptamers and aptazymes in biotechnology, diagnostics, and therapy, Chem. Rev. 107 (2007) 3715–3743. doi:10.1021/cr0306743.

[82] M. Levy, A.D. Ellington, ATP-dependent allosteric DNA enzymes, Chem. Biol. 9 (2002) 417–426. doi:10.1016/S1074-5521(02)00123-0.

[83] C. Teller, S. Shimron, I. Willner, Aptamer-DNAzyme hairpins for amplified biosensing,

Anal. Chem. 81 (2009). doi:10.1021/ac901773b.

[84] J. Nie, D.-W. Zhang, C. Tie, Y.-L. Zhou, X.-X. Zhang, A label-free DNA hairpin biosensor for colorimetric detection of target with suitable functional DNA partners, Biosens. Bioelectron. 49 (2013). doi:10.1016/j.bios.2013.05.020.

[85] W. Zhou, X. Gong, Y. Xiang, R. Yuan, Y. Chai, Target-triggered quadratic amplification for label-free and sensitive visual detection of cytokines based on hairpin aptamer dnazyme probes, Anal. Chem. 86 (2014). doi:10.1021/ac403682c.

[86] G. Pelossof, R. Tel-Vered, J. Elbaz, I. Willner, Amplified biosensing using the horseradish peroxidase-mimicking DNAzyme as an electrocatalyst, Anal. Chem. 82 (2010) 4396–4402. doi:10.1021/ac100095u.

[87] D.-W. Zhang, J. Nie, F.-T. Zhang, L. Xu, Y.-L. Zhou, X.-X. Zhang, Novel homogeneous label-free electrochemical aptasensor based on functional DNA hairpin for target detection, Anal. Chem. 85 (2013). doi:10.1021/ac402295y.

[88] H. Zhang, B. Jiang, Y. Xiang, Y. Chai, R. Yuan, Label-free and amplified electrochemical detection of cytokine based on hairpin aptamer and catalytic DNAzyme, Analyst. 137 (2012). doi:10.1039/c2an15962g.

[89] Z. Zhou, L. Xiao, Y. Xiang, J. Zhou, A. Tong, A general approach for rational design of fluorescent DNA aptazyme sensors based on target-induced unfolding of DNA hairpins, Anal. Chim. Acta. 889 (2015) 179–186. doi:10.1016/j.aca.2015.06.036.

[90] R. Nutiu, Y. Li, Structure-switching signaling aptamers., J. Am. Chem. Soc. 125 (2003) 4771. doi:10.1021/ja028962o.

[91] J.C. Achenbach, R. Nutiu, Y. Li, Structure-switching allosteric deoxyribozymes, Anal. Chim. Acta. 534 (2005) 41–51. doi:10.1016/j.aca.2004.03.080.

[92] N. Lu, C. Shao, Z. Deng, Rational design of an optical adenosine sensor by conjugating a DNA aptamer with split DNAzyme halves, Chem. Commun. (2008). doi:10.1039/b810812a.

[93] X.B. Zhang, Z. Wang, H. Xing, Y. Xiang, Y. Lu, Catalytic and molecular beacons for amplified detection of metal ions and organic molecules with high sensitivity, Anal. Chem. 82 (2010) 5005–5011. doi:10.1021/ac1009047.

[94] P. Song, Y. Xiang, H. Xing, Z. Zhou, A. Tong, Y. Lu, Label-free catalytic and molecular beacon containing an abasic site for sensitive fluorescent detection of small inorganic and organic molecules, Anal. Chem. 84 (2012). doi:10.1021/ac203488p.

[95] Z. Xu, K. Morita, Y. Sato, Q. Dai, S. Nishizawa, N. Teramae, Label-free aptamer-based sensor using abasic site-containing DNA and a nucleobase-specific fluorescent ligand, Chem. Commun. (2009). doi:10.1039/b908345f.

[96] Y. Xiang, A. Tong, Y. Lu, Abasic site-containing DNAzyme and aptamer for label-free fluorescent detection of Pb2+ and adenosine with high sensitivity, selectivity, and tunable dynamic range, J. Am. Chem. Soc. 131 (2009). doi:10.1021/ja905854a.

[97] L. Gong, Z. Zhao, Y.-F. Lv, S.-Y. Huan, T. Fu, X.-B. Zhang, G.-L. Shen, R.-Q. Yu, DNAzyme-based biosensors and nanodevices, Chem. Commun. 51 (2015) 979–995. doi:10.1039/C4CC06855F.

[98] J.L. Vinkenborg, N. Karnowski, M. Famulok, Aptamers for allosteric regulation., Nat. Chem. Biol. 7 (2011) 519–527. doi:10.1038/nchembio.609.

[99] J. Kosman, B. Juskowiak, Peroxidase-mimicking DNAzymes for biosensing applications: A review, Anal. Chim. Acta. 707 (2011). doi:10.1016/j.aca.2011.08.050.

[100] D. Wu, T. Gao, L. Lei, D. Yang, X. Mao, G. Li, Colorimetric detection of proteins based on target-induced activation of aptazyme, Anal. Chim. Acta. 942 (2016) 68–73. doi:10.1016/j.aca.2016.09.010.

[101] J. Zhu, T. Li, J. Hu, E. Wang, A novel dot-blot DNAzyme-linked aptamer assay for protein detection, Anal. Bioanal. Chem. 397 (2010). doi:10.1007/s00216-010-3802-9.

[102] K. Tram, J. Xia, R. Gysbers, Y. Li, An efficient catalytic DNA that cleaves L-RNA, PLoS

One. 10 (2015) 1–14. doi:10.1371/journal.pone.0126402.

[103] Y. Yang, J. Huang, X. Yang, K. Quan, H. Wang, L. Ying, N. Xie, M. Ou, K. Wang, Aptazyme-Gold Nanoparticle Sensor for Amplified Molecular Probing in Living Cells, Anal. Chem. 88 (2016) 5981–5987. doi:10.1021/acs.analchem.6b00999.

[104] Z. Zhang, D. Balogh, F. Wang, I. Willner, Smart mesoporous SiO2 nanoparticles for the DNAzyme-induced multiplexed release of substrates, J. Am. Chem. Soc. 135 (2013) 1934–1940. doi:10.1021/ja311385y.

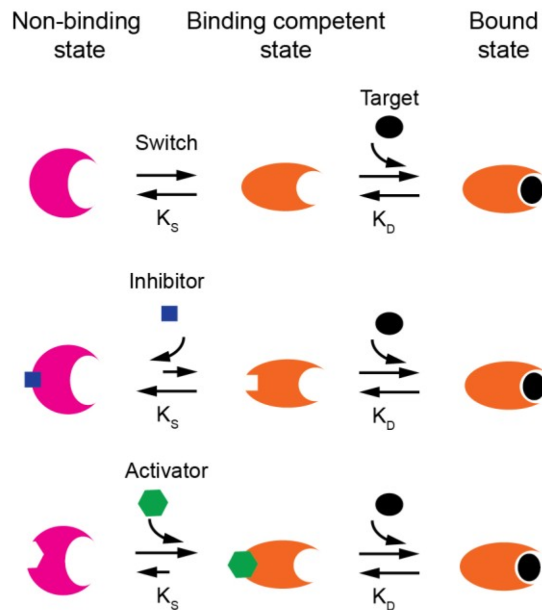


**Figure 1.** Many naturally occurring chemo-receptors work via a population-shift mechanism in which target binding pushes a pre-existing equilibrium between the *binding-competent* state and the *non-binding* state to the *bound* state (Top). In allosterically regulated DNA switches, the binding of 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.

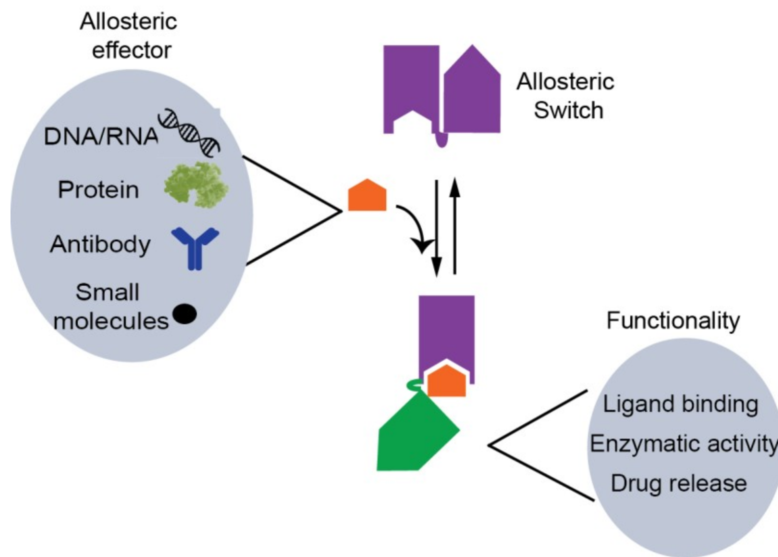


**Figure 2.** Allosterically regulated DNA-based switches. Different biological and chemical ligands (DNA, RNA, proteins, antibodies, small molecules) can act as allosteric effectors on DNA-switches. Their binding to the DNA-based receptor generates a conformational change that alters the DNA-switch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

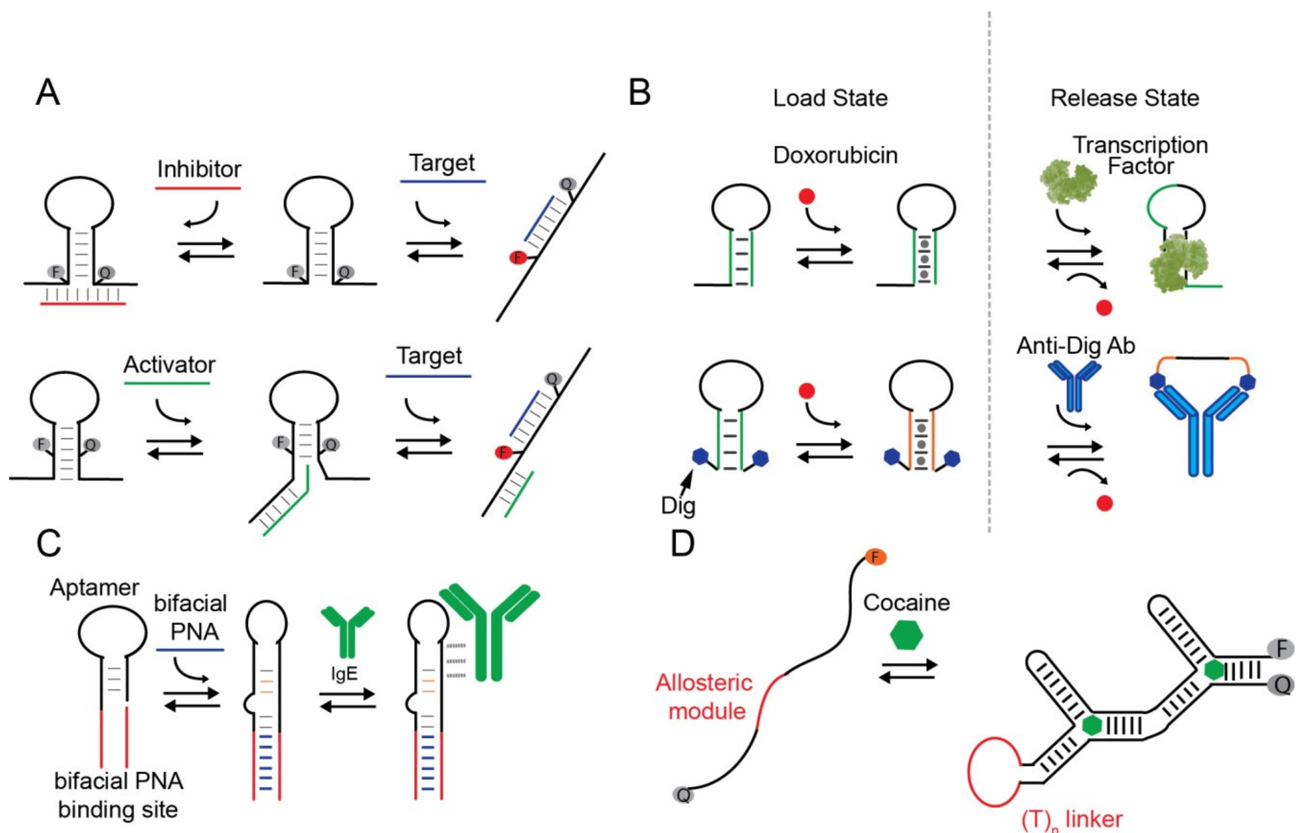


**Figure 3.** Examples of allosterically regulated DNA-based molecular beacons and DNA-aptamers. A) Molecular beacons are allosterically regulated via the introduction of single-stranded tails on each of the beacon's two termini. Two single-stranded "tails" added to a molecular beacon serve as allosteric binding site. The binding of the inhibitor (red) to the two tails bridge 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 sequence binding to this tail 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). doi: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 the allosteric effector

concentration. Adapted from Ref. 52 with permission from the Royal Society of Chemistry. C) Aptamers are allosterically regulated by bifacial PNA (bPNA). The binding of bPNA switches-on the aptamer binding affinity for the protein. The stem structure of a protein-binding DNA aptamer that binds immunoglobulin E (IgE) is ablated by replacement with two unstructured oligo-T strands, which are bPNA binding sites. The addition of bPNA serves as a folding "splint" to structure 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). doi: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, which, in contrast occurs at a preformed site. Adapted with permission from A.J. Simon, A. Vallée-Bélisle, F. Ricci, K.W. Plaxco, Proc. Natl. Acad. Sci. 111 (2014) 15048–15053. doi:10.1073/pnas.1410796111.

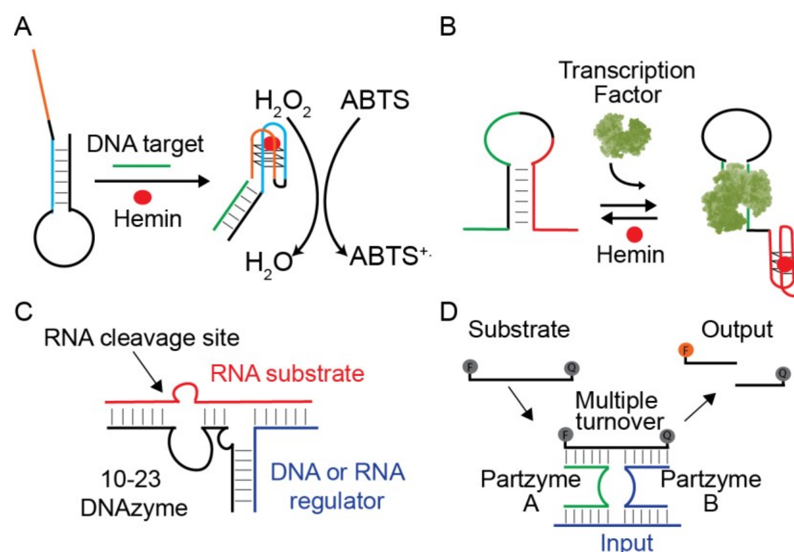


**Figure 4.** Examples of DNAzymes whose enzymatic activity is regulated through allostery. A) Peroxidase-mimicking enzymatic activity of DNAzyme allosterically controlled by combining tailored molecular beacons and hemin/G-quadruplex DNAzymes. When the light blue segment is hybridized in the hairpin structure, 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). doi: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. 79 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. At the three-way junction a bulge composed of two adenosine bases was introduced in the DNAzyme strand to enhance stability of the junction. The arrow 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) DNAzyme allosterically activate through the use of nucleic acid "assembly facilitator". The DNAzyme is splitted 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 at the presence of the nucleic acid assembly facilitator, such as a target nucleic acid molecule that act as an allosteric effector. The assembly facilitator provided the "input" that directed the assembly of partzymes into an MNAzyme with a functional catalytic core capable of cleaving multiple substrates into products thus providing an "output". By labelling the substrate on each side of the cleavage site with a fluorophore (F) and a matched quencher (Q), the cleavage can be monitored by an increase in fluorescence. 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. doi:10.1021/ja9076777. Copyright (2010) American Chemical Society.

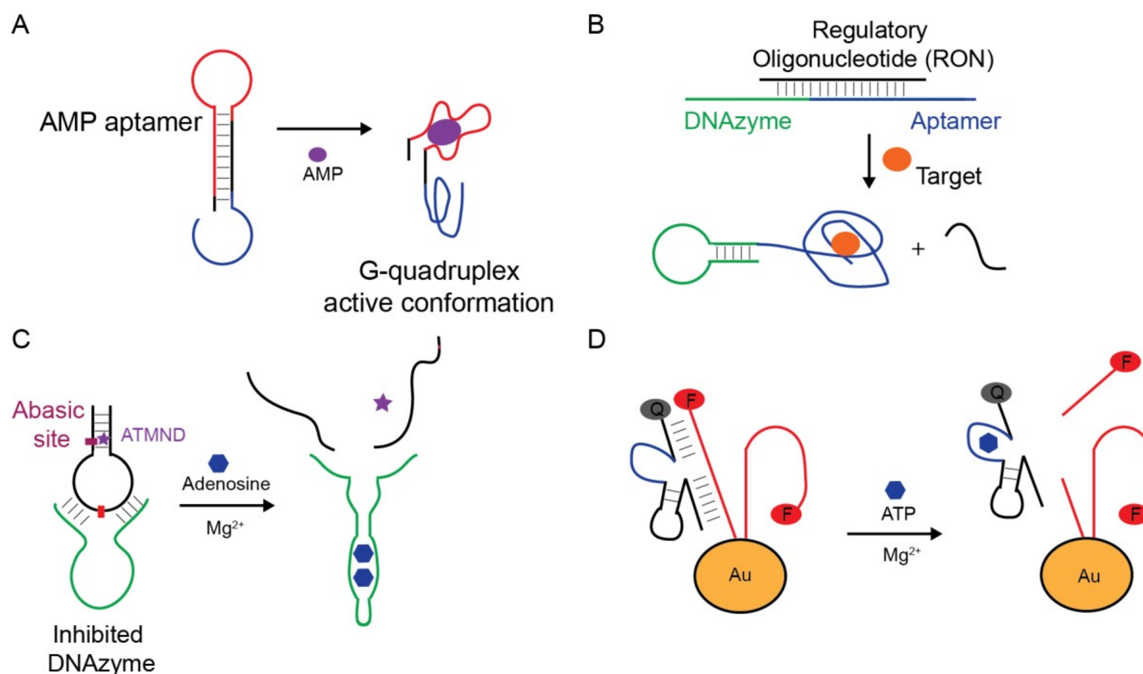


**Figure 5.** Examples of allosterically regulated DNA- aptazymes.

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 through the formation of the AMP-aptamer complex, resulting in the self-assembly of the active HRP-mimicking DNAzyme. Adapted with permission from C. Teller, S. Shimron, I. Willner, Anal. Chem. 81 (2009). doi:10.1021/ac901773b. Copyright (2009) American Chemical Society. B) Structure-switching aptazymes. A DNA aptamer (blue sequence) is linked to a DNAzyme (green sequence). In the absence of ligand, a complementary oligonucleotide sequence, called regulatory oligonucleotide (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 regulatory oligonucleotide. 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. doi:10.1016/j.aca.2004.03.080. C) General strategy of catalytic and molecular beacon. The DNAzyme substrate is incorporated in the loop of a molecular beacon having an abasic site in the hybridized stem region. To do that, the substrate of the DNAzyme is extended at both extremities to form the molecular beacon. 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 domain alone can not form a stable and active structure to catalyze the cleavage of the hairpin structured substrate, even in the presence of $Mg^{2+}$ ions. Upon addition of adenosine, instead, the aptamer region binds to adenosine and form a compact structure, which activates the DNAzyme by restoring the stem-loop structure of the DNAzyme and its function. The signal is given by the external fluorophore ATMND whose fluorescence is quenched when it binds to the abasic site in hybridized molecular beacon stem region of the substrate (inactive DNAzyme) and it is restored when the substrate is cleaved and ATMND is release in solution from the stem region. Adapted with permission from P. Song, Y. Xiang, H. Xing, Z. Zhou, A. Tong, Y. Lu, Anal. Chem. 84 (2012). doi:10.1021/ac203488p. Copyright (2012) American Chemical Society. D) Aptazyme−Gold Nanoparticle Sensor for Amplified Molecular Probing in Living Cells. The ATP-specific aptazyme strand, composed of $Mg^{2+}$ -dependent 10−23 DNAzyme and ATP aptamer, and the relative substrate are immobilized on gold nanoparticles (AuNP). The substrate strands labelled with a fluorophore hybridize the ATP-specific aptazyme strands functionalized with a quencher. 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 activate the aptazyme. The activated aptazyme cleaves the fluorophore-labeled substrate strand, releasing the shorter fluorophore-labeled DNA fragment. The

active aptazyme can then bind to another substrate strand on the AuNP, causing the cleaving of another substrate strand. Thus, these cleaved fluorophore-labeled DNA fragments are separated from both BHQ-2 and AuNP, resulting in fluorescence enhancement. During this cyclic process, a very small number of ATP molecules can initiate the cleavage of many fluorophore-labeled substrate strands from the AuNP surface, 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. doi:10.1021/acs.analchem.6b00999. Copyright (2016) American Chemical Society.