# Rationally designed DNA-based scaffolds and switching probes for protein sensing

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## 1. ABSTRACT

The detection of a protein analyte and use of this type of information for disease diagnosis and physiological monitoring requires methods with high sensitivity and specificity that have to be also easy to use, rapid and, ideally, single-step. In the last ten years, a number of DNA-based sensing methods and sensors has been developed in order to achieve quantitative readout of protein biomarkers. Structure-switching DNA biosensors have played an important role in this effort, as detection specificity is greatly enhanced when protein detection is combined to a conformational change of the signaling probe at the nanoscale. As an alternative, DNA-based sensing platforms using DNA as a scaffold molecule for the attachment, with high accuracy and precision, of a wide range of non-nucleic acid molecules also represent a straightforward opportunity. Scaffold DNA biosensors have several advantages and potential, especially because different recognition elements (e.g. peptides, proteins, small molecules, antibodies, etc.) can be conjugated to the DNA scaffold in order to specifically interact with the target protein with high affinity and specificity. Our aim here is to provide an overview of the best examples of structure switching-based and scaffold DNA sensors, as well to introduce the reader to the rational design of innovative sensing mechanisms and strategies based on programmable functional DNA systems for protein detection.

## 2. INTRODUCTION

Detection of protein biomarkers is extremely important for the early diagnostics, treatment, and management of many diseases, as well as in fundamental research and other biomedical applications. Sensitive and accurate detection of proteins in biological fluids, as well the study of their interactions and their post-translational modifications, is a challenge for research and diagnostic purposes. The high cost and the complex operating protocols of protein analysis based on the use of laboratory-scale instruments are not able to meet the needs for frequent screening and/or continuous monitoring of patients in primary care, as well in remote health monitoring systems.

Starting from the 60's, specific detection of proteins has been achieved mainly through the use of a plethora of immunoassays (immunosorbent colorimetric, Immunoelectrophoresis, Western blot, etc.) capable of detecting and quantifying protein targets in biological fluids. To date, the enzyme-linked immunosorbent assay (ELISA)[1] and Western blot represent the most frequently used techniques. In the last twenty years, apart from these methods, a number of biosensing platforms using appropriate recognition elements (DNA, peptide, synthetic receptors, imprinted polymers, etc.) have been reported for the detection of protein in solution.[2–4] In particular, many efforts have been directed toward the development of point-of-care (POC) devices for sensitive and specific protein detection, similar to the glucometer in terms of ease-of-operation, response time, operating and equipment costs.

Among them, DNA-based biosensors appear particularly relevant because they harness the design ability of DNA-based systems to easily translate a specific binding event into a measurable signal. Watson-Crick-Franklin base pairing is indeed highly predictable and programmable, and DNA nanotechnologies can be engineered into molecular transduction systems and thus used in conjunction with a variety of materials interfaces.[5]

In the following Chapter, two main approaches developed in the context of protein-responsive nucleic acid-based biosensors are discussed. One is the design of structure switching DNA aptamers through which protein binding activity can be probed, processed, and converted into a measurable output. The second one is based on the use of DNA-based scaffold systems where DNA does not work as recognition element of the sensing platform, but it is merely employed as a structural unit over which responsive elements are rationally organized. Specifically, by using DNA scaffolds presenting different small and large recognition elements (peptide, proteins and antibody) accessible to protein binding, we highlight how it is possible to generate innovative biosensing platforms where a DNA scaffold can also be directly involved in the transduction of the binding activity into a measurable output. More

specifically, we first introduce the general principles and rules underlying the design of the target-induced structure-switching mechanism (Section 3 and 4). Then, we highlight some of the main examples of optical and electrochemical DNA-based biosensors that are based on structure-switching probes (Section 5). Finally, we introduce the concept of DNA scaffold (Section 6) showing some applications where simple single and double stranded DNA units can be harnessed as signalling scaffolds that generate a signal output only when in the presence of the specific target protein (Section 7).

## 3. THE MANY ADVANTAGES OF STRUCTURE-SWITCHING BIOSENSORS

Due to the extraordinary versatility, affinity and specificity of biomolecular recognition, biosensors[6] have seen significant research advances and commercial exploitation over the last years.[7–11] Besides the amazing recognition properties of biomolecules, however, a significant limitation in the development of biosensing technologies has been represented by the capacity of generating a measurable output directly associated to the binding of the target molecule.[12] For instance, immunosensors and many other enzyme- or nucleic acid-based biosensors simply rely on bioreceptors that can specifically interact with their targets without emitting electrons or photons upon target binding. To generate a signal in response to target binding most biosensors, as well many bio-analytical platforms, require subsequent washings/incubations steps, reagent-intensive procedures and/or the combination with a secondary recognition element labeled with a tag in order to generate a measurable output. On the contrary, "label-free" biosensors represent an alternative because they are capable of generating an analytical signal in response to the change of an intrinsic property of the receptor upon target recognition, such as subtle changes in molecular mass or charge. To monitor the small changes induced by the unlabeled components, these sensing strategies rely on the use of very sensitive and sophisticated equipment. Among label-free sensing techniques, most famous are the surface Plasmon resonance (SPR),[13] field effect transistors [14], quartz microbalances and microcantilevers[15]. However, they still suffer from a common limitation that is non-specific absorption of contaminants, especially when the sensing platforms are challenged in complex biological matrices.

To overcome this limitation, over millions of years of evolution Nature has optimized a specific class of biomolecules (i.e. switches) operating through the binding-induced structure switching mechanism that sense chemical/biological inputs and transduce the binding event into a specific biological function.[16] Structure switching generally provides superior specificity for the target analyte because the recognition event is mediated by the formation of many weak, non-covalent bonds (e.g. hydrogen bonding, hydrophobic effects, etc.) and thus is less sensitive to the presence of other interfering species in the solution. The peculiar behavior of biomolecular switches is often associated to conformational or oligomerization changes of the recognition elements, resulting in specific cascade functions, such as enzyme activation/inhibition, gene expression, or opening an ion channel.[17]

Inspired by this naturally occurring mechanism, significant efforts have been vested to take advantage of it for artificial biotechnologies. In particular, since structure switching can be easily

combined to the generation of a measurable signal output, biomolecular switches are perfect candidates for biosensing applications. The structural reconfiguration can be either combined to a relative change in FRET efficiency between a fluorophore and quencher pair,[18] or to the variation of electron transfer efficiency onto electrode surface, as well the activation of a catalytic activity.[19] As a consequence of structure switching mechanism, these biosensors are generally easy-to-use, reagentless and single step thus appearing particularly promising for the real time monitoring of binding events even in complex environments. Among them, engineered protein switches have been widely designed for synthetic biology and sensing applications, and they appear transformative for future point-of-need tests.[20, 21] On this respect, comprehensive reviews of protein switches can better describe their advantages and disadvantages for bioanalytical applications.[20]

Design and synthesis of oligonucleotides is still simpler and more affordable than those of protein. As a matter of fact, engineering protein-based recognition elements becomes a remarkably more challenging endeavor than engineering nucleic acid-based recognition elements. In this line, thanks to polymerase chain reaction technology (PCR) it is also possible to easily and quickly amplify the starting material with very little reagent requirements. Not to forget is the superior chemical robustness over time in a wider range of conditions that make oligonucleotide advantages way higher than compared to those of proteins. In addition, DNA is a low cost and stable biomolecule easy to synthetize using solid phase synthesis in an automated way.[22, 23] Altogether these features make nucleic acids more accessible and affordable to research labs spread all over the world. Therefore, the literature has experimented a large increase of examples of structure-switching DNA biosensors. Our focus here is on structure switching DNA biosensors for protein detection. More specifically, we introduce in this Section the basic concept underlying the design of structure-switching DNA receptors and then highlight some applications of structure-switching DNA recognition elements for either point of care applications[24] and real time continuous monitoring[25] of proteins.

## 4. ENGINEERING STRUCTURE SWITCHING DNA RECEPTORS

All the remarkable advantages that structure switching DNA-based recognition elements offer come at the cost of limited number of DNA sequences that undergo a conformational change upon target binding. Thus, structure switching has to be engineered into those responsive DNA elements that do not change their conformation in response to the binding of the target. Here we highlight some general rules to help the reader better understand how to design structure switching DNA receptors.

The physics of structure-switching recognition elements is well described by the population-shift model which provides a route to tune the useful dynamic range of such recognition elements more-or-less at will.[26] According to the population shift model, a DNA-based recognition element can populate two conformational states, i.e. a non-binding and a binding-competent state. The low-energy non-binding conformation of the DNA recognition element is a more stable conformation and it is always in equilibrium with a less stable, but binding-competent conformation. Target binding stabilizes the latter state thus shifting the equilibrium toward the bound state.[26, 27] Experimental validation of the free energy associated to the two equilibrium states is crucial to guide the engineering process, and experimental information can be easily obtained from urea[28] or temperature[29] denaturation curves. The equilibrium between the non-binding and binding-competent states is regulated by switching equilibrium constant ($K_s$, Figure 4.1) that can be properly tuned by simply working on the secondary structure of the DNA-based recognition element. As an example, a DNA-based recognition element with a $K_s$ lower than 0.1 describes a conformational equilibrium where 90% of the recognition element populates the non-binding competent state. This means that structure switching is generally associated to low signal background and large signal change upon target binding. However, stabilization of the non-binding conformation rises an energetic penalty that target binding must overcome in order to induce the switch. Hence, the concentration of target necessary to trigger the switch strongly depends on $K_s$ values, with lower $K_s$ associated to higher observed binding affinity. [26] Thus, it is very important to not over stabilize the non-binding conformation of the recognition element beyond values of $K_D$ required for the specific sensing application. Apparent binding affinities and switching constants can be related through the mathematical expression[26] displayed in Figure 4.1a. Generally, a good compromise between optimal signal-to-noise and good binding affinity is associated to $K_s$ values comprised between 0.1 to 1, with the latter that yields maximum signal gain of 50% (half population in the binding-competent state in absence of target) while decreasing the observer affinity of the recognition element ($K_d$) just 2-fold.[27]

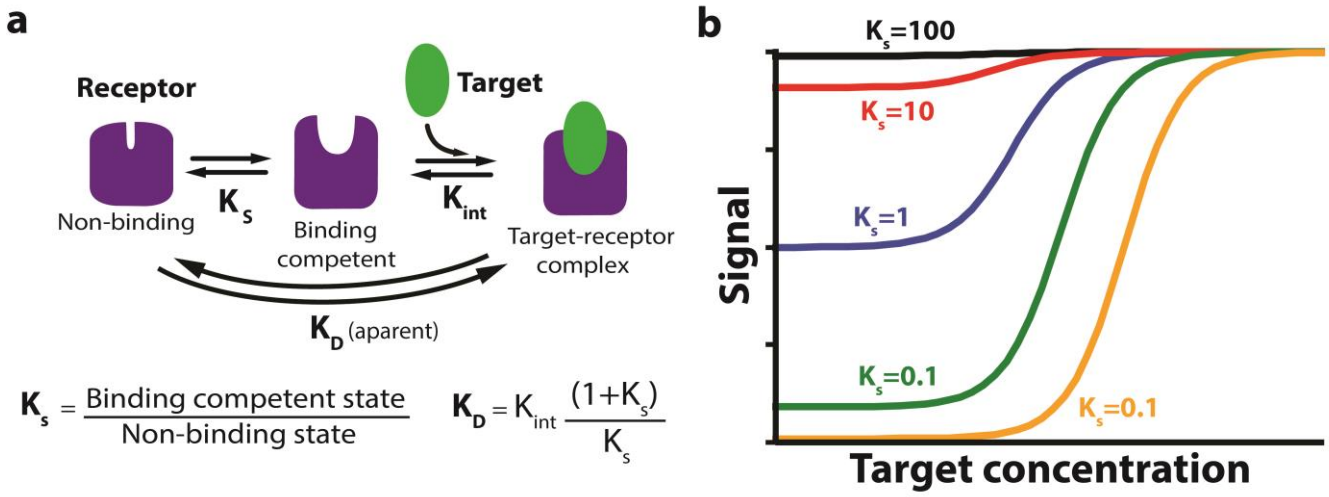


**Figure 4.1**: The population shift model describes biomolecular receptors operating through structure-switching mechanism. (**a**) Top, the working principle associated to the population-shift mechanism. The switching constant ($K_s$) determines the interconversion between the non-binding and the binding competent states of the recognition element. The target binding stabilizes the binding competent state. Bottom, the combination of $K_s$ and intrinsic constant ($K_{int}$) determines the final observed dissociation constant ($K_D$) and binding affinity of the recognition element for the target. (**b**) Recognition elements with high $K_s$ has a majority population of receptors in the binding competent state, which is translated in improved binding affinity (lower $K_D$) but also lower signal change upon target binding (black and red lines). Enhanced signal changes come at the cost of losing recognition element's affinity ($K_D$). As general rule, $K_s$ value comprised between 0.1 and 1 offer a good compromise in terms of binding affinity and signal gain.

Following these basic rules it is possible to design a conformational change mechanism in a DNA-based recognition element that naturally did not operate through structure switching, and also to tune the binding properties and/or signal transduction.[30] In the following subsections we highlight some of the most relevant strategies for the rational design of structure switching mechanisms for sensing applications.

### 4.1 – Formation of duplex motifs as regulators of structure switching.

This category describes the simplest and most straightforward strategy to incorporate a binding-induced structure switching mechanism into a DNA-based recognition element. The simple hybridization between a complementary strand (CS) and a DNA-based recognition element generates a duplex structure (i.e. non-binding state) that does not allow the binding of the target molecule. The target has

indeed to overcome a free-energy penalty to bind to the recognition element, and it will not be able to until reaching certain target concentrations. Using DNA aptamers as recognition element, this format is known with the name of “duplexed aptamers”.[31] This generic mechanism can be applied in biosensors with different architectures. For example, the CS can compete with the target for the free DNA recognition element labelled with a reporter molecule. In this approach the CS binds the recognition element that now populates a non-emitting conformation. When the target is present in a concentration sufficient to displace the CS, structure switching is associated to a significant signal change (Figure 4.2a and b). In an exhaustive and thorough study using optical characterizations, Lackey and co-workers[32] developed a methodology to study the kinetics associated to recognition events using duplexed aptamer sensors, and demonstrated the switching mechanism behind the L-tyrosinamide binding aptamer. Specifically, they found that the switching mechanism is consistent with a $S_N1$-like mechanism, in which the complementary strand of the aptamer must dissociate first before aptamer binding to its target. This mechanism, however, is not universal since other aptamers might follow other mechanisms such as induced-fit, in which the target molecule directly interacts with the duplexed recognition elements and facilitates the displacing of the complementary strand from the aptamer.[33] Another sensing architecture employs labelled CS bound to the DNA recognition element (low background signal) that when displaced by the formation of target-receptor complex generate a response. Dillen A. and co-workers[34] applied this strategy in an assay for thrombin detection and studied in detail the thermodynamics associated.

Tuning properly either CS concentration and relative binding affinity between CS and DNA recognition element is fundamental to deploy a working sensor. Essentially, the free energy of CS interaction with the DNA probe determines the $K_s$ value associated to the equilibrium between the non-binding and binding state. Therefore, it is important to properly select the length of the complementary motif, i.e. its binding affinity, and also CS concentration to achieve optimal signal gain upon target binding and adequate binding affinity. Besides, since the CS is an independent element added, it can be adapted without affecting intrinsic binding properties and selectivity of the recognition element, as opposed to other mechanisms explained later in the chapter. However, such assay formats are generally limited to end point measurements and are not easily transferrable to continuous monitoring platforms nor reusable biosensors.

The addition of a short self-complementary motif in the primary sequence of the selected DNA recognition element represents an alternative to the previously described approaches. The introduction of non-native Watson-Crick interactions generates a *distorted* non-binding conformation in the

recognition element. The self-complementary portion is usually contiguous to the binding motif of the recognition element and produces a stem-loop structure, where the loop is composed by only a few nucleotide long linker (e.g. poly thymine loop, polyethylene glycol). Even in this case, the presence of the target shifts the equilibrium toward the binding competent structure opening the duplex forming region, and therefore generating a signal output. [35, 36] This strategy has been successfully applied in the thrombin aptamer[37] and later adapted for electrochemical readout.[38]

**4.2 - Splitting DNA-based recognition elements into 2 or more independent fragments.**

Here the DNA recognition element is split into independent sequences not physically linked to each other. The structural change induced by the target binding guides the re-association of the two different subunits in one complex[39]. Not purely a structure switching, since the change in conformation is substituted for the assembly of the subunits, here the main requirement is the formation of secondary structures of the two subunits that still maintain the capacity to interact with the target molecule (Figure 4.2c and d). Even in this case, a remarkable advantage of this approach is associated to the generation of large signal gain upon target binding due to the relative high change in the distance between dissociated subunits (high relative distance) that comes into close proximity in the presence of the target being spatially confined at the sub-nanoscale. In comparison, typical unimolecular structure switching bioreceptors can only achieve small structure changes – and so limited signal change - that are basically comprised to the maximum distances within the recognition element itself. In principle, this approach could be applied to any aptamer as a generalizable mechanism of signal transduction[40], since it is just requires the correct splitting of the aptamer in different isolated subunits. Reality proves to be more complex and proper assessment of the splitting point in the aptamer is required. A general weakness of the strategy based on split aptamers is the partial re-assemble of the aptamer's subunits often occurring also in the absence of target. This phenomenon generally results in an increase of the background signal. Furthermore, the approach appears difficult to be generalized because splitting the aptamer can also significantly affect intrinsic binding properties and specificity toward the target. As a consequence, the repertoire of split aptamers available still remains limited[39]. Another main challenge can be the absence of structural information about the mechanism of target recognition. Development of new methodologies to study the kinetics and thermodynamics, such as the one reported by Morris and co-workers[41] will likely help to generalize this mechanism to more DNA-based recognition elements.

### 4.3 - Direct selection of structure switching DNA recognition elements.

During the selection process of DNA aptamers for non-nucleic acid target it is possible to directly select for structure-switching aptamers without the need of further engineering. Initial efforts in this area took advantage of immobilized DNA sequences to a solid surface using libraries containing a complementary docking motif (Figure 4.2e). Upon binding with the target, DNA sequences selected and further enriched are those detached from the anchoring sequence. That means that selected aptamers must have undergone a structural change upon target binding inducing strand displacement from the anchoring strand. Different groups have reported successful assays to specifically select small molecules[42] and proteins such as thrombin[43], proving the capacity to successfully select aptamers with structure-switching properties. However, a key limitation of this approach is represented by the fact that the equilibrium state for binding of the sequences to the beads is being constantly re-established with each wash, and thus some non-functional sequences are recovered during the target elution step.[44] Alternative structure-switching SELEX method using a homogenous isolation step can be also developed in order to distinguish between active and inactive library members, without the need to immobilize target or capture strand.[42]

### 4.4 - Modification of the DNA sequence, altering the binding pocket.

Small changes in the sequence of the recognition element can significantly affect the stability of the two states involved in the structure-switching mechanism. Such changes, either mutations, deletions (truncations) or additions of bases in the recognition element need to be done in a rational way, to guarantee the binding properties of the aptamer, or to avoid destabilizing the binding conformation (decreasing the $K_s$) so much that the apparent binding affinity of the recognition element would fall out of the detection range of interest for that target (Figure 4.2f). This approach has been extensively applied to aptamers for detection of small molecules[45–47], and for detection of proteins, for instance neutrophil gelatinase associated lipocalin (NGAL).[48] However, the small changes introduced with respect to the original binding recognition element, require the knowledge of extensive structural information to guide the process in a rational way. Due to the difficulties to obtain complete structural information for most of the aptamers, guidance in the process to select proper single point mutations is severely limited. Quick progress in the development of better algorithms and advances in bioinformatics

tools, as well the use of artificial intelligence will provide improved design of structure switching candidates. To date, still empiric try-and-error approaches followed by experimental analytical characterization represent the standard way to identify optimal aptamers. In some cases, spectroscopic techniques have been used to guide the process of inserting modifications into the recognition element,[47] facilitating a more rational process.[49]

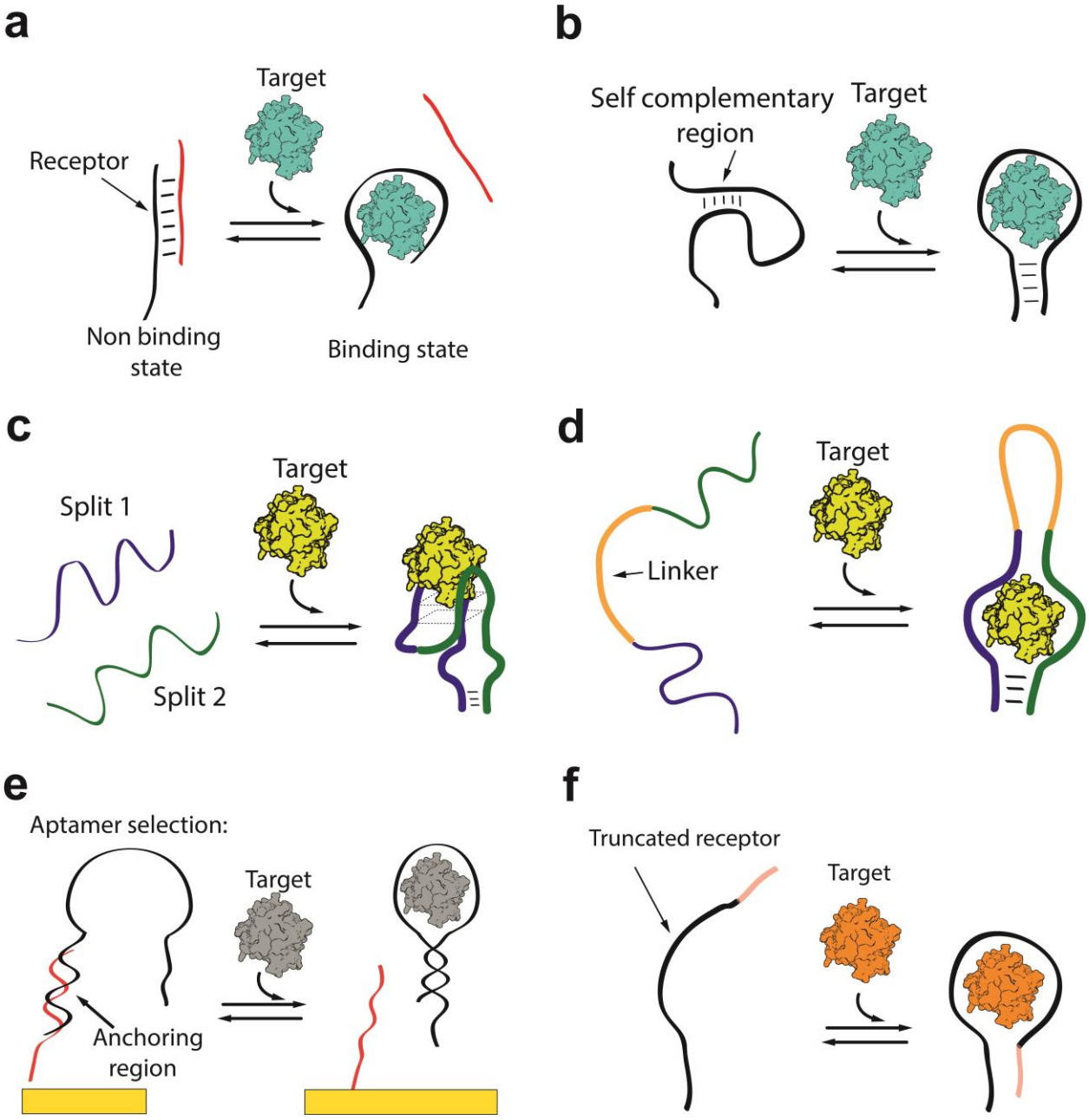


**Figure 4.2.** Different strategies to generate stricture switching aptamers. **(a)** Simplest strategy consists in the addition of a region complementary to the binding site in the recognition element. The target competes for the binding site of the

recognition element and disrupts the duplex in order to form the bound complex. **(b)** By introducing self-complementary motifs in the same DNA receptors it is possible to generate a non-binding state. [36] **(c)** Splitting of recognition element into two different subunits that rearrange and properly fold only in presence of the target. **(d)** In some cases, it can be advantageous to integrate the different split subunits in a single molecule by connecting them through a linker region. **(e)** Specific selection strategies in aptamer selection make possible to directly select structure switching aptamers that intrinsically operate through conformation change mechanism without further modifications. **(f)** More refined approaches to induce conformational change in a recognition element, such as an aptamer, can take advantage of truncations/single point modification of the probe sequence.

## 5. PROTEIN DETECTION USING STRUCTURE-SWITCHING DNA RECEPTORS

Target proteins can be detected by making use of rationally designed DNA-based recognition elements (i.e. aptamers) properly engineered to be responsive to their presence. To reach this goal, a simple strategy is based on the design of structure-switching probes that undergo a conformational change upon binding to the specific protein, thus producing a measurable signal output.

Aptamers are short DNA or RNA single strands (~ 12-80 nucleotides long) that fold into defined secondary and tertiary structures and can selectively bind to molecular targets, as small molecules, carbohydrates, proteins and live cells. In truth, aptamers are selected from libraries,[50] typical SELEX library starts with $10^{15}$ random sequences, of single stranded oligos. Through sequential steps of binding, eluting and amplification, DNA sequences capable of binding to the target of interest with high specificity and affinity are enriched within the library. After 5-15 selection cycles and counter selection steps against potential interferants, the enriched pool is sequenced, and aptamer candidates are identified from the sequencing results using bioinformatics analysis. The selected sequences are chemically synthesized and characterized by their binding ability and specificity to a target. In the last 30 years, more than 30 variants of their process selection (i.e. Systematic Evolution of Ligands by Exponential Enrichment (SELEX)), have been developed[51] and now it is possible to select aptamers that show specificity and affinity comparable to those of antibodies towards their targets. Aptamers, however, are smaller, low cost bioreceptor that are easier to produce and more straightforward to modify than antibodies.

As a result, many progresses have been done by the research community and pharmaceutical companies in developing aptamer-based technologies with applications ranging from diagnostics to therapeutics[52]. Recently, the first aptamer drug was approved by US Food and Drug Administration (pegaptanib sodium) and many others have showed promise in preclinical research and clinical trials. Among the myriad of aptamer-based biosensor technologies reported in scientific literature, some of them recently have been translated into commercially available diagnostic kit for food safety and biomarker detection.[53] Nevertheless, aptamer-based biosensors often share the same limitation of antibody-based detection platform, that is the limited capacity of transducing target recognition into a measurable signal without further addition of external reagents. To extend to aptamers all the advantages of the structure-switching binding mechanism previously described, different molecular designs have been proposed. Since only few aptamers present structure-switching functionality

rationally introduced directly in the selection[54–58] many strategies focused on how to re-create structure-switching behaviors into aptamers that do not undergo a conformational change upon binding to the specific target.

In this section we only report examples of sensing systems based on structure-switching DNA probes that presents a sequence or a motif (i.e. a synthetic aptamer or a consensus sequence) that recognizes the target protein. Nucleic acid-based sensing systems using RNA or other artificial mimic, as well systems where the recognition event is mediated by non-nucleic acid-based elements (i.e. peptides, small molecules, antibodies, etc.) will be discussed in the Section 6 and 7. To help the reader, we have separated subsections for the detection of proteins and transcription factors, also providing a brief overview of DNA-nanoswitches for the activity-based monitoring of repair enzymes.

### 5.1 Structure-switching DNA-based aptamers for the optical detection of proteins

Pioneer in this field was Ellington's group that designed of a new class of recognition elements named aptamer beacons.[37] By appending two complementary DNA motifs at the extremities of thrombin-binding aptamer, Ellington and co-workers generated a structure-switching "beacon-like" probe. In the absence of thrombin, the aptamer adopts a stem-loop structure that can be easy visualized by labelling the two termini of the sequence with a fluorophore/quencher pair resulting in a quenched fluorescence emission. Thrombin binding to the G-quadruplex motif triggers the conformation change of the aptamer that ultimately leads to a fluorescence signal increase. This molecular design has been successively developed by many other researchers for bioanalytical and biomedical applications. [38, 59–64] Following this design principles, more recently, a structure-switching aptamer has been selected by Tan and colleagues where a tyrosine kinase-7 (PTK7)-binding aptamer is integrated with a single-stranded pH-responsive i-motif allowing for selective binding of PTK-7 recognition elements on the surface of target CCRF-CEM cells.[65] By making use of a i-motif domain, the switching equilibrium is regulated by the pH of cellular microenvironment. At slightly acidic pH (i.e. in tumor microenvironment), the i-motifs enables the binding between the aptamer and the cognate target; at physiological pH (healthy cells) instead, the i-motif structure is switched in a random coil conformation that prevents the recognition event. Also Liu's group has reported on the detection of membrane protein PTK7 on living cells, specifically by employing "beacon-like" aptamers as Surface-enhanced Raman scattering (SERS) probe.[66] In the presence of the target protein, the structure switching exposes a DNA portion that triggers an enzyme-mediated signal amplification reaction that generate Cy5-labeled residual DNA

strands, which can further hybridize with the capture DNA strands immobilized on gold nanoparticles assembled on functionalized silicon substrate. By integrating SERS, aptamer conformational change, and Exo III enzyme amplification, the sensitivity is superior to that of the commercial ELISA method by several orders of magnitude. However, a common limitation of SERS-based assays showing high sensitivity is that such platforms sometimes exhibit limited accuracy and reliability for quantitative analysis.

Another useful strategy for the design of structure-switching DNA probes was developed by Szostak's group for the detection of ATP[67] and small molecules,[68] and later adapted for protein detection. The strategy consists of splitting the aptamer into two or more portions that can associate only in the presence of the specific target. Liu et al.[69] proposed a specific design for the detection of thrombin based on split aptamer-programmed self-assembly of quantum dots (QDs). Specifically, two fragments of the split aptamer are conjugated to different QD populations. The reassembling of the aptamer into a complex through sandwich-like assay induced by target thrombin leads to self-assembly of fluorescent QDs with a significant fluorescence quenching and band-shift due to exciton energy transfer between QDs. Thanks to the high sensitivity typical of the exciton energy transfer-based fluorescent detection, a limit of detection (LOD) of 15 pM is achieved. Chen et al., instead deployed a signal-on electrochemical aptasensor where one split portion of the thrombin aptamer is attached to a gold electrode and the second split sequence is modified with the redox tag ferrocene.[70] In the presence of the target, the two fragments assemble thus producing an electrochemical signal change at the electrode interface. The biosensing platform show a detection limit of 0.2 nM, higher sensitivity and lower background current compared to previously reported assays. Of note, the approach is not easy to generalize since in many cases it is possible that, even in absence of the target, the aptamer's subunits partially re-assemble, thus increasing significantly the background signal in the measurement. To mitigate this risk, Li's group[60] introduced the concept of strand displacement reactions as a mean of switching, where a partially complementary strand blocks one aptamer subunit from re-assembling into the signaling conformation.

Optical aptamer-based assays based on strand displacement reactions employ a binding aptamer modified with a fluorophore that is hybridized with an aptamer-complementary element (i.e. ACE) labelled with a quencher. In the absence of target, the two tags are in close proximity and the fluorescence signal is quenched. The binding event promotes the folding of the aptamer and the subsequent displacement of the quencher-tagged strand that ultimately results in a fluorescence signal increase. This "duplex-to-complex" strategy does not require any introduction of non-native interactions in the primary structure of the aptamer. Furthermore, the ACE can be designed of different length and

complementary to different aptamer's portions, thus allowing straightforward and programmable design features. These aptasensors are also commonly referred as *trans*-duplex aptamers,[31] to distinguish them from the *cis*-duplex aptamers where ACE and aptamer sequences are incorporated within the same nucleic acid sequence (e.g. unimolecular detection systems) and separated by a linker. The first example of this strategy was reported by Tan and co-workers that introduced a PEG linker to conjugate the binding aptamer with a short complementary DNA strand.[71] By doing so, they demonstrated human α-thrombin detection with faster kinetics and enhanced sensitivity in comparison to the previously reported assays. The *trans*- design, however, presents some advantages, such as easier probe production and simpler design.[31]

One of the limiting steps of the design of duplex aptamers is represented by the velocity of the signal transduction that are mostly governed by the hybridization kinetics of the ACE to the binding aptamer.[31] Recently, precise kinetic control has been reached by Zhang et al.[72] by combining protein-controlled DNA-nanoswitches with a strand displacement reaction to realize a kinetically controlled platform that transduces the presence of ligands (i.e. proteins, small molecules) into the release of a oligonucleotide. Here the aptamer sequence is designed to be inserted between a toehold and a displacement domain of an invading DNA strand (Figure 5.1 a). In the absence of the target, the toehold and displacement domains are separated by a long, unstructured aptamer and so the strand displacement reaction on the reporter system is very slow. Upon target binding and consequent aptamer folding, toehold and displacement domains come into closer proximity and so are able to trigger the strand-displacement reaction producing fluorescence signal increase. This strategy was successfully demonstrated for the detection of thrombin and PDGF achieving a LOD of 0.96 nM and 0.73 nM, respectively. Furthermore, by using this strategy, Zhang et al. reported on the capability to realize orthogonal transduction, logical operations, and DNA-based amplification reactions.

Recently, some *in vivo* biosensing platforms based on structure-switching aptamers have been also reported.[73–75] For example, photoacoustic imaging of thrombin in living mice has been reported by Zhang et al.[76] The authors showed that using thrombin-binding aptamer in combination with two DNA strands modified with a near-infrared fluorophore/quencher pair (IRDye 800CW/IRDye QC-1), an efficient contact quenching is obtained. With this simple strategy, the selective visualization and quantification of the target protein is reached with a dynamic range of 0–1000 nM and a limit of detection of 112 nM.

To this point, most of the assays illustrated are based on fluorimetric readout because of their higher sensitivity and facility of DNA labelling. Nevertheless, electrochemical readouts represent a more ideal

solution for POC assays, thanks to the high sensitivity, the rapid response time of analysis, the feasibility to miniaturise the platforms and to integrate them into low-cost, microfluidic devices. However, most electrochemical biosensors for protein detection do not operate in a sample-in-answer-out manner, especially when tested with unprocessed clinical samples but they generally require multiple washing steps and the addition of reagents to process the sample.

### 5.2 Structure-switching electrochemical Aptamer-based biosensors for protein detection

Pioneering works carried out by Plaxco's[38, 77, 78] using Electrochemical Aptamer-based biosensors (EAB sensors) have demonstrated how to overcome many of the issues mentioned above. EAB sensors have been reported as one of the most promising tools for both *in vitro* and *in vivo* monitoring of small molecules and proteins.[48, 79–86] This is principally due to the enhanced specificity associated to structure-switching mechanism of DNA aptamers and the ability of EAB sensors to perform well when placed in whole blood and in living body. EAB sensors are indeed rapid, reversible, reagentless, and can be easily adapted to new targets by simply changing the aptamer sequence. They are prepared in a very simple way using redox-reporter-and-thiol-modified DNA aptamers immobilized on a gold electrode.[87] In this way, as an example, it is possible to monitor the folding into a triple-stem conformation of the PDGF-responsive aptamer upon binding to PDGF (Figure 5.1 b).[88] As a result of the conformational change mechanism, the electrochemical tag comes closer to the electrode surface, generating an increase of the electrochemical signal due to improved electron transfer between the redox tag and the working electrode. The affinity and selectivity achieved by Plaxco's group using EAB sensors (i.e. 1 nM of PDGF directly in undiluted, unmodified blood serum) are generally high and often match to the window of target concentrations required to detect the protein analyte in the specific clinical sample. By adopting the same approach, the detection of thrombin has been also achieved, with a detection limit of 6.4 nM.[38] In this case, the binding event causes a conformational change that inhibits the electron transfer between the redox tag (i.e. methylene blue) and the electrode surface, generating a current decrease. Similarly, Liu and co-workers, showed the detection of interferon gamma in the presence of overabundant serum protein,[89] such as the detection of tumor necrosis factor-alpha (TNF-α) in blood samples (this last one by using an RNA-based aptamer).[89] The detection of epithelial tumor marker mucin 1 (MUC1) was reported by Ma et al. that demonstrated how the MUC1-responsive aptamer in the absence of the target, folds into a thermodynamically stable hairpin conformation, facilitating the direct electron transfer between the redox tag and the gold electrode, whereas in the presence of MUC1, the

aptamer no longer holds its hairpin conformation, moving the redox tag further away from the electrode surface.[89] In the same direction, Zhao et al. developed a folding-based EAB for detection of vascular endothelial growth factor (VEGF) in human whole blood.[89] Here, the cognate aptamer initially unfolded, upon the binding to VEGF, adopts a stem-loop structure that forces the redox label to be in the close proximity of the electrode. An outstanding detection limit of 5 pM was achieved in 50% of blood serum.

One of the main advantages of structure-switching EAB relies on their capacity of performing seconds-resolved, real-time measurements of molecules which makes the technology very attractive for clinical applications. In this respect, recently, Idili et al. demonstrated the single-step, rapid, reagentless, and quantitative measurement of the SARS-CoV-2 spike (S) protein in biological fluids (serum and artificial saliva).[90] Plaxco's group has also demonstrated the possibility to use this technology to achieve real-time monitoring of different small molecules and drugs in living rats.[46, 91, 92] Inspired by this approach, more recently, Parolo et al. achieved the first high-frequency, real-time detection with subminute time resolution of Neutrophil Gelatinase-Associated Lipocalin without any sample processing in human urine samples using urinary catheter.[25]

Although the unprecedented performance of EAB sensors *in vivo*, the platform still requires further optimization before it can see successful clinical application. Indeed, aptaswitches share some limitations that are commons to all the aptamer-based sensing technologies. In particular, the often-poor match between the affinity of binding aptamers for the specific target in the body where it is not possible to easily adjust the experimental conditions to tune the binding properties of the probe (pH, ionic strength, etc.). Additionally, sometimes the clinically relevant concentration of the analyte itself, as well the relatively shallow binding curves associated with "Langmuir-type" binding curves of single-site DNA recognition element, represent main limitations.[93] The last issue is particularly challenging for targets with a narrow clinical range. As an example, the windows of some target analytes (metabolites, drugs, etc.) *in vivo* is so narrow that the abilities of EAB biosensor technology do not still allow for their meaningful measurement. For example, to transition a noncooperative receptor from 10% occupied to 90% occupied requires an 81-fold change in target concentration. This reduces the ability of aptamer-based sensors to measure small differences in target concentration, limiting the precision of the assay and scope of such technologies in practical way. In the case of protein detection, an additional limitation is represented by the fact that EAB sensors require fast kinetics of target release to provide continuous, real-time *in vivo* monitoring but this is not possible because protein binding is generally associated to stronger interactions with the DNA probe.

Rationally-designed molecular approaches to overcoming these problems can be very tricky to optimize and difficult to predict, especially when the DNA aptamer is longer than 35-45 nt. The difficulties encountered to predict the aptamer folding upon binding to the target, as well the difficulty of understanding the dependence of conformational changes by the analyte also represent minor issues toward the development of general platform for POC application *in vivo*.

As already introduced, another general issue of the sensors based on structure-switching mechanism is represented by the limited sensitivity of affinity-based assays.[12, 94] Combining structure-switching to isothermal enzyme-free nucleic acid circuits may represent a valuable tool to overcome this limitation. The programmability of the molecular design, the easiness of the operations, the low cost associated to the amplification process make them particularly appealing for the integration in POC devices at the price of additional reagents and longer time of analysis. One of the most explored enzyme-free isothermal amplification process is represents by catalytic hairpin assembly (CHA), introduced by Yin et al. and engineered for multiple analytical applications by Ellington and co-workers.[95–97] Because CHA allows the direct detection of single-stranded oligonucleotides, the introduction of a transduction module able to convert protein binding into single-strand DNA catalyst of CHA is required. A general strategy has been reported by Tang's group to detect thrombin and cancer protein biomarkers.[98] The strategy combines the advantages of the structure switching aptamers with those of the proximity-based assays.[99, 100] Specifically, two nucleic acid probes were tagged with specific aptamers so that the target-induce co-localization of responsive aptamers induce the self-assembling and activates a CHA process which in turn generates a fluorescence output. A detection limit of 100 pM of thrombin was reached in 10-fold diluted human serum samples. This strategy is universal, highly versatile and robust, but the number of detectable target is quite limited by considering the fact that two binding sites, or at least a homodimer with two separate epitopes, is required. An attempt to detect proteins showing only one epitope has been reported by Li's group by introducing the use of DNA-templated photo-crosslinking in order to induce a proximity-dependent photo-crosslinking of a DNA strand to the target protein.[101] To increase sensitivity, also nanostructured materials deposited at the electrode interface (i.e. AuPNs, AgNPs, PtNPs, silica nanoparticles (Si NPs), QDs, magnetic nanoparticles (MNPs), carbon nanotubes (CNTs), graphene (GNs)) can be used.[89] For a more exhaustive description of the effects, advantages and disadvantages associated to the use of nanomaterials in combination with DNA biosensors, the reader can refer to specific article and reviews on this topic.[102–104]

### 5.3. DNA-based switches for the detection of transcription factors

Transcription factors (TFs) represent a particular class of proteins involved in the transcription of genes. The binding between the TFs and DNA is highly sequence-specific and play a key rule on cells differentiation and growth, such as on the interpretation of the genome information. By working as regulators and selector genes, their mutations and dysregulations are involved in many pathological processes.[105]

The rational design of DNA-nanoswitches for the detection of transcription factors (TFs) has been introduced by Vallée-Bélisle et al.[106] that in a pivotal work presented binding-activated fluorescent DNA probes, named transcription factor beacons, able to signal the presence of three transcription factors (TBP, Myc-Max, and NF-κB) directly in crude nuclear extracts. The transcription factor beacons contain a consensus, double-stranded DNA binding sequence able to recognize with extreme specificity the TFs target. These probes are designed in a way that they can populate two different conformations in equilibrium with each other: a stem-loop structure containing double-stranded consensus sequence for the specific binding of the protein, and a second structure where the consensus sequence is sequestered in a stem-loop "non-binding" state. By properly introducing a fluorophore/quencher pair, it is possible to monitor the binding of TFs as a means of fluorescence increase (Figure 5.1 c). The same strategy has been later adapted to an electrochemical format by simply attaching on a gold electrode the TF beacons labelled with a redox tag (i.e. methylene blue). The probe reconfiguration upon TF binding push the electrochemical tag away from the electrode surface generating a change of current.[107] By using a microfluidic devices, this technology make possible to quantify the proteins showing a detection limit of 4 nM for TATA binding protein in nuclear cell extracts. This feature together with the easiness of detection, attracted many other researchers that extended on this concept.[108] As an example, Bertucci et al. have successfully adapted the *in vitro* biosensors to intracellular imaging of nuclear factor kB (NF-kB) using living cancer cells and FRET-based readout.[109, 110] By using lipofectamine-based vectors, the nanodevice was successfully delivered to cultured PC3 cells. The transportation *via* endosomal escape and the successive diffusion into cytoplasm and nucleus was confirmed through stochastic optical reconstruction microscopy (STORM). The specificity of imaging was also confirmed by transferring the cells with siRNA to knockdown the expression of NF-kB. Recently Bertucci et al. also demonstrated the application of TF beacons as molecular translator for the actuation of strand displacement reactions by engineering a toehold-binding domain in the switch that can trigger the strand displacement reaction only upon switch reconfiguration induced by TFs.[111] Beside the

obvious biosensing applications, this approach pave the way to rationally control biomolecular communication pathways between nucleic acid-based systems and functional proteins. This approach can be of value for a variety of applications, ranging from synthetic biology to artificial transcription-transduction pathways and re-creation of cell-like behaviors in biomimetic systems.

We have also designed TF beacons with enzyme-like activity (DNAzymes) by introducing allosteric control over the switching mechanism.[112] The TF-controlled nanozyme shows peroxidase-like catalysis and this provides a means to quantify enzyme activity through simple colorimetric readout by adding TMB. The bioassay is cheaper than many other assays based on the use of expensive reagents or labelled molecules. However, the catalytic efficiency of this class of DNAzymes is low and the reaction times limit its applications. Following the TF-induced switching mechanism, recently Zhou's group developed an allosteric DNA-Silver nanocluster (DNA-AgNCs) switch.[113] The fluorescence of DNA-AgNCs is regulated by a guanine-rich enhancer sequence (GRS) that enhances its fluorescence 500-fold when it is close to the DNA-AgNCs. With this strategy, the label-free quantification of NF-κB p50 has been reported showing a LOD of 2 nM.

### 5.4. Activity-based sensors: DNA-based switches for the monitoring of repair enzymes

Cells are continuously exposed to endogenous and exogenous mutagens which induce chemical alterations of the DNA structure (i.e. “DNA damage”) impacting human health.[114] In response to this, Nature has elaborated a DNA repair machinery reversing the mutagens-induced DNA damage[115] such as base excision repair (BER),[116] mismatch repair,[117] nucleotide excision repair,[118] and direct damage reversal[119] that collectively represent protective processes blocking entry of cells into carcinogenesis. An increased risk to develop cancer is generally associated with low DNA repair capacity,[120–122] and interindividual variations in DNA repair capacity are thought to determine different susceptibilities to cancer. To date, DNA repair capacity can be assessed indirectly at the level of transcription - for selected genes involved in the different repair pathways - translations (proteomics), and also using single-nucleotide polymorphism (SNPs) screening.[122] However, enzyme activity often does not correlate with the rate of transcription/translation, and not even with the amount of protein present, whereas SNPs in repair genes are not always informative when the gene is not expressed.[123] As an alternative, a wide variety of cell-based assays have been reported but they generally requires labor-intensive analysis that makes difficult their implementation in a clinical setting. As an example, in vitro Comet assays (also in a high-throughput format) have been proposed to indirectly assess rate of

repair in cells and tissues,[123] but requires careful experimental design to specifically target a repair activity in the presence of other cellular processes affecting the DNA damage levels. The host cell reactivation (HCR) assay requires the transfection of a DNA plasmid into a target cell population in order to monitor successful repair activity as a means of reporter gene expression. HCR is also cumbersome, indirect and limited by the need for separate experiments to measure different repair activities.[124] Cell lysate-based measurements of repair capacity in vitro represent a valid alternative but they are not prone to multiplexing, requiring for separate assays for measurements of more than one repair pathway. Although an incredible number of studies comparing DNA repair capacities have been used to assess the response to chemotherapy or radiation, laboratory-friendly toolkit for real-time and direct monitoring of a specific enzyme repair activity have lagged behind. In this regard, chemically-modified nucleic acid probes appear promising as they offer many advantages over the traditional methods mentioned above, such as, possibility of imaging DNA repair directly in cells and tissues,[125] and adaptability for high-throughput assays in biological media. In this respect we address the reader to the review by Kool's group to learn more about the topic.[125]

DNA nanoswitches can also be used to directly monitor the activity of enzymes involved in DNA repair. Most of these assays employ structure-switching DNA probes that populate a different secondary structure as result of enzymatic activity, generally a G-quadruplex motif, that is further used to generate a measurable colorimetric, chemiluminescent or fluorescent output. Pivotal in the field of DNA switch sensors for the detection of the DNA repair enzymatic activity was the work led by Ren and co-workers, that designed a label-free quadruplex-based functional molecular beacon for the real-time monitoring of UDG activity.[126] The molecular design here makes use of a blocking strand that prevents the proper folding into a G-quadruplex conformation. Specifically, an oligonucleotide presenting several uracil residues binds to a molecular beacon, precluding its folding in a G-quadruplex structure. Upon the removal of uracil residues by UDG, the G-rich portion can fold in a G-quadruplex motif that is finally bound by the N-methyl mesoporphyrin IX (NMM) dye, generating a ~14-fold enhancement in fluorescence, given the strong interaction between NMM and the folded G-quadruplex. Following this work, several subsequent UDG probes[127, 128] and a T4 PNK probe[129] based on the use of blocking strand have been developed. Ma and co-workers reported the first luminescent DNA-switch based assay for the detection of nicking endonuclease activity.[129] They synthetized different luminescent Ir(III) complexes presenting a weak affinity toward a DNA duplex and high affinity toward G-quadruplex, that in turns means low and high luminescence signals, respectively. In their assay, the cleavage induced by Nb BbvCI enzyme causes the release of a guanine-rich sequence that folds into a G-quadruplex,

enhancing the luminescence of the Ir(III) complexes. The assay was proved in cell extracts showing its potential as user-friendly and cost-effective switch-on platform. Successively, other complexes were synthesized for the detection of protein tyrosine kinase-7 (PTK7) in aqueous solution.[129] Here the specific binding between the PTK7 and the sgc8 aptamer triggers the release of the G-quadruplex-forming sequence.

A new class of DNA-nanoswitches for monitoring the activity of methyltransferase activity has been recently reported by our group.[130] We rationally designed folding-upon-repair DNA nanoswitches that can switch from a duplex conformation (low FRET efficiency) into an FRET-active triplex DNA structure upon human methyltransferase (hMGMT) activity. hMGMT converts O6-methyl-guanine (O6-MeG) nucleobases in the DNA duplex into repaired guanine nucleobases restoring triplex DNA (Figure 5.1 d). Molecular Dynamics simulations confirm that a methyl group at the guanine O6 position in the duplex portion of the nanoswitch hampers the triplex formation, so that the folding into triplex structure is strictly associated to the repair activity. The activity of different methyltransferase enzymes has been evaluated also in the presence of several enzyme inhibitors and the assay has been further adapted for drug screening. Another new strategy based on the use of DNAzymes for the monitoring of demethylase activity have been just reported by Jiang's group. They designed fluorogenic DNAzymes, named RADzymes (i.e. repaired and activated DNAzyme), containing single methyl lesion in specific positions that inhibit the catalytic activity of the engineered DNAzymes.[131] The reparation of MGMT-mediated O6-MeG lesion leads to the restoration of the catalytic activity and produces a fluorescence output. The repair activity has been assessed in different cells and under drugs treatment, giving promising results. In the same work, the monitoring of ALKBH2 activity was also achieved by engineering a fluorogenic RADzyme containing 3MeC lesions. Successively, epigenetically modified DNAzymes (EMOzymes), were engineered and combined with a CRISPR/Cas12a amplification for the quantification of active GMT,[132] showing LOD of 0.054 nM.

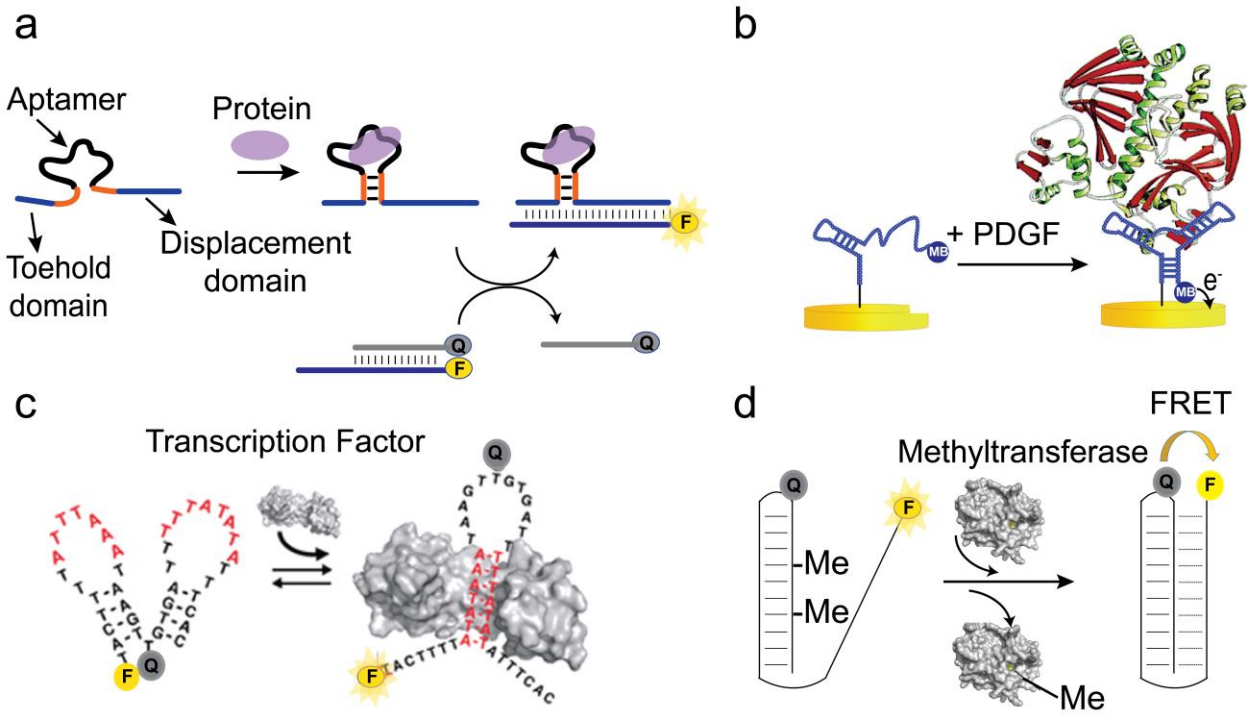


**Figure 5.1. (a)** Protein- induced strand displacement reaction. The displacement is triggered by the conformational change induced by the presence of protein. Adapted with permission from ref. [72] (Copyright © 2021, Springer Nature). **(b)** Electrochemical aptamer-based (E-AB) sensor for the detection of platelet-derived growth factor (PDGF). The PDGF-binding aptamer is attached onto the gold surface and labeled with a redox tag (i.e. methylene blue, MB). Upon target binding, the aptamer folds into a stable three-way junction leading the MB label close to the electrode surface, allowing a more efficient electron transfer. Adapted with permission from ref. [88]. (Copyright © 2007, American Chemical Society). **(c)** Transcription factor beacon signaling the presence of specific DNA-binding proteins. The binding of the TF induces a conformational change that can be detected by properly modifying the beacon with a fluorophore/quencher pair at the extremities of one of the two stems associated to the nonbinding state. Adapted with permission from ref. [106] (Copyright © 2011, American Chemical Society). **(d)** Transcription factor-responsive DNAzyme. The presence of TF induces a conformational change whose result is the activation of the catalytic activity of the DNAzyme. Adapted with permission from ref. [112] (Copyright © 2015, Royal Society of Chemistry).

## 6. PROGRAMMABLE DNA NANOSTRUCTURES AS SCAFFOLDS FOR BIOMOLECULE DETECTION

In this section we discuss the use of DNA as a structural material for building scaffolds supporting signaling transduction mechanisms. DNA provides an extraordinarily versatile material for self-assembly and DNA nanostructures have been demonstrated to be useful for biosensing applications. Since DNA thermodynamics is highly predictable, using sequences of limited length (less than 100 nucleotides), it is possible to build nanostructures with precision almost at the level of Amstrongs.[133–135] Another interesting feature of DNA is the good stability and resistance to degradation compared to other biomolecules such as enzymes or antibodies. Moreover, it is possible to control the formation and rearrangement of the DNA structure by tuning experimental conditions (temperature, ionic strength, pH, etc.). Generally, DNA nanostructures take advantage of the canonical Watson-Crick-Franklin base pairing but they can also make use of other types of base pairing, such as non-canonical DNA-RNA base pairing, or Hoogsteen interactions that control the formation of triple helixes.[136] In this context, the capability to build spatially controlled DNA structures at the nanoscale is discussed with regard to biosensing. In particular, in Section 7 we focus the discussion on simple, minimal DNA scaffolded systems[137] where the DNA is not used as the recognition element of the platform but merely as a dynamic scaffold.

### 6.1. Simple single and double stranded DNA scaffolds for sensing applications

Single or double stranded DNA sequences modified by non-nucleic acid recognition elements represent the simplest DNA scaffold structure that can be employed for biosensing. We highlight here single and double stranded DNA-based scaffolds used in three different classes of bioassays, specifically i) platforms based on steric hindrance effects, ii) platforms associated to scaffolds operating through structure switching mechanisms, and iii) those that take advantage of proximity-based effects to generate a signal output. In the first category, i.e. biosensors based in steric hindrance effects (Figure 6.1 a), signal generation is directly related to the limited hybridization of the DNA scaffold when it is bound to a large biomolecular target. Specifically, the presence of the target alters the intrinsic mobility and hybridization efficiency with a complementary nucleic acid probe, thus consequently inducing a change in the kinetics and consequently into a measurable output. The second group comprises structure-switching DNA scaffolds, where DNA scaffold plays the role of a dynamic reconfigurable unit populating

different conformations in the presence and in the absence of the target protein. The interaction of the recognition element covalently conjugated to the DNA scaffold with the target protein induces a structural reconfiguration of the DNA scaffold. In contrast to the sensors based in structure switching recognition elements, in this case, the target needs to induce a rearrangement into the DNA scaffold. Proximity-based DNA assays (Figure 6.1 b and c) represent the third group of biosensors using single or double stranded DNA's structures as target-responsive scaffold elements. In these assays, the DNA scaffolds are also coupled to a reporter system that generates an output signal only when the two DNA elements are co-localized onto the same target acting as a template molecule. Based on the principle of local concentration[138] , also referred as co-localization effect, the simultaneous interaction of the two DNA scaffolds with the same target bring them closer together, thus increasing extraordinarily the effective molar concentration. To be successful, the DNA scaffold strands must be designed in a way that their relative binding affinity is poor when they free in solution, whereas only upon target binding they can hybridize inducing scaffold interaction and signal generation. Interestingly, thanks to DNA's intrinsic properties, the co-localization based detection can be connected to a signal amplifying reaction, such as DNA amplification via enzyme-based amplification producing ultrasensitive platforms.

### 6.2 DNA as building block of complex nanostructures

Beyond the use of short duplex DNA structures, DNA can be used to build structures in the nano- and microscale when designed and arranged in the proper way. Pioneered by Nandrian C. "Ned" Seeman when introducing the nucleic acid junctions[139], structural DNA nanotechnology rapidly expanded the possibility to properly design complex DNA-based nanostructures[140] settling the basis for a variety of applications[133]. Most of the mechanisms used to create DNA scaffolds and nanostructures rely in the control and programmability introduced through the use of "sticky ends" to control tile-based self-assembly or the design of more complex origami DNA.

A sticky end is a short single-stranded overhang protruding from a double stranded DNA molecules. Examples of tile-based self-assembly are DNA based nanotubes,[141] where few strands of DNA assemble forming a small structure or "tile", representing the basic building unit of the nanostructure, with a 2 dimensional shape similar to a rectangle. Each tile presents short protruding oligos out of the four "corners" of the tile (i.e. sticky ends). The sequence of these sticky ends can be designed in such a way that it hybridizes with other tiles and assemble into bigger DNA based nanostructure. These initially 2 dimensional structures can curve and fold over themselves forming a cylinder/rod like structure open

at both ends. Thanks to DNA's programmability it is possible to build nanotubes with different decorations and homogeneous or heterogeneous composition of its building blocks[142] up to the size of micrometers, so they can be visualized under fluorescence microscope. By modulating the sticky ends' interaction, it has been also reported the possibility to induce assembling or disassembling of such structures. Ricci and his group have developed many examples where DNA nanotubes are used as supramolecular scaffolds for sensing purposes.[143]

W.K. Rothemund instead reported a simple method named DNA origami where a set of properly designed short DNA strands determine the correct folding of a long single strand DNA into desired 2D shapes[144]. Using DNA origami is possible to assemble DNA strands of diverse geometrical shapes. This work settled the basis for later studies where a number of complex and stimuli-responsive 2- and 3-D DNA-based nanostructures have been reported (Figure 6.1 d).[135, 145] These DNA-based nanostructures can incorporate a diversity of modifications and components to introduce a wide range of functionalities with applications well beyond biosensing. Therefore, DNA origami design and applications fall out of the scope of this chapter, for further reading of other applications few relevant references have been included: drug delivery applications[146], cargo and release,[147, 148] enzymatic activity control,[149] and even pore formation on lipidic membranes.[150, 151]

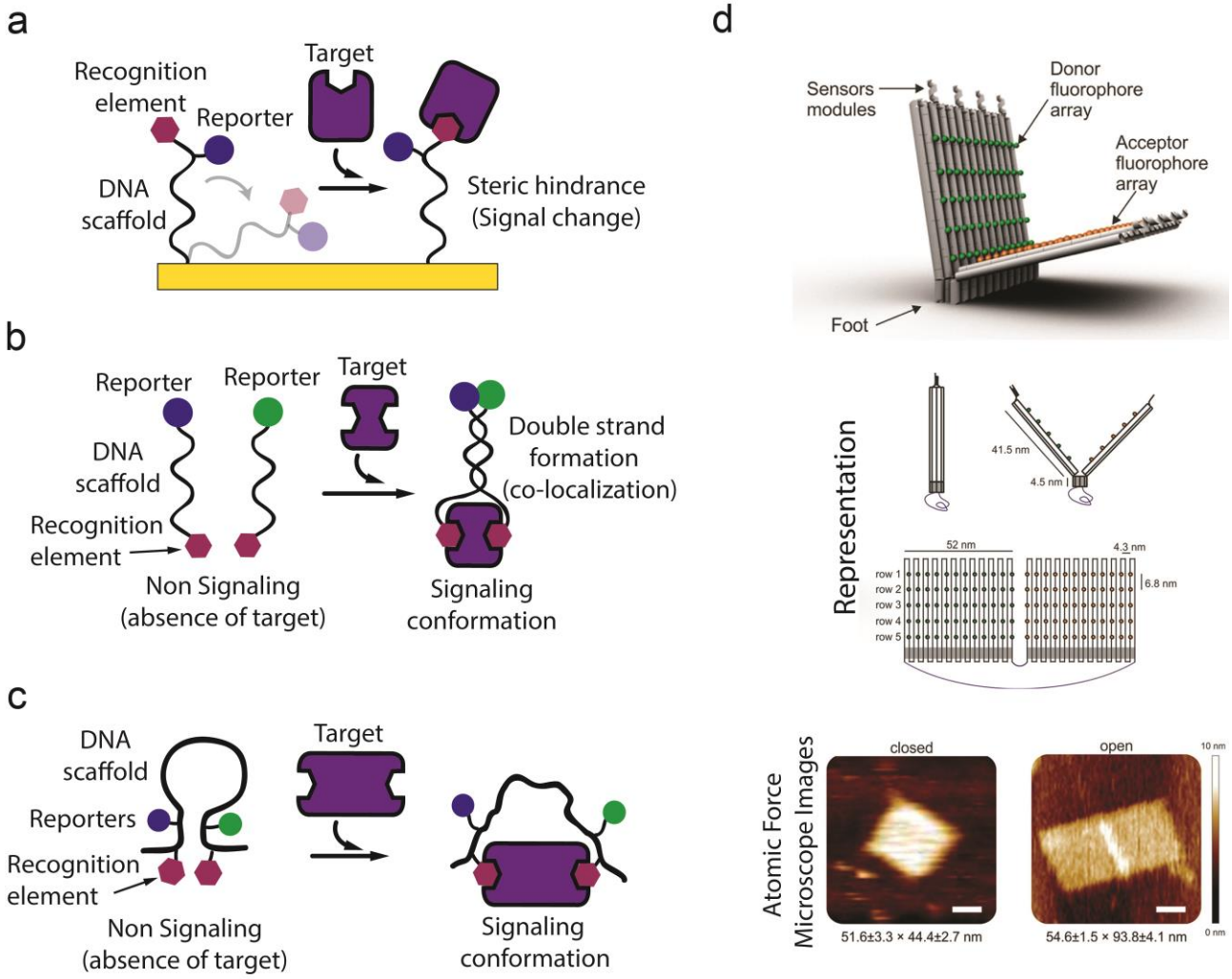


**Figure 6.1** Sensing approaches supported by DNA-based scaffolds. **(a)** DNA can be used as a scaffold molecule holding both recognition and reporter elements. When the target recognizes the non-nucleic acid recognition element, the efficiency of the scaffold to properly collide to the electrode interface significantly decreases producing current signal change. **(b)** DNA scaffold systems for sensing based on proximity effect. **(c)** The target binding can also induce the structural reconfiguration of a hairpin DNA scaffold resulting in a change of FRET signal. **(d)** Target-responsive folding of complex DNA nanostructures of hundreds of nm. In the middle, schematic view of the design of an origami "giant" beacon applied for biosensing applications. Microscopy images correspond to Atomic Force Microscope (AFM) imaging, scale bars correspond to 20 nm. Adapted with permission from ref. [152] (Copyright© 2018 American Chemical Society).

## 7. DNA SCAFFOLD-BASED SENSORS FOR PROTEIN DETECTION

### 7.1. Electrochemical DNA scaffold sensors

To detect antibody and protein biomarkers, a number of publications reported on the use of redox-tagged DNA signaling scaffolds modified with a non-nucleic acid recognition element, and covalently attached to an interrogating electrode. Specifically, this sensing approach utilizes a rigid but dynamic double stranded DNA scaffold composed by one DNA strand terminally modified with a redox reporter that hybridize with a second complementary DNA covalently linked to a recognition element.[153–157] In many examples, such DNA architecture is anchored onto the surface of a working electrode via a flexible linker which allows the redox reporter to strike the electrode. The binding of a target protein to the recognition element significantly affects the collisional kinetics of redox-tagged DNA scaffold at the electrode interface (Figure 7.1a and b). Key parameters that need special attention here are the size of the recognition element and the target molecule, with particular importance of their relative ratio. In this respect, one of the main limitation is that high molecular weight recognition elements results in a high steric hindrance effecting the intrinsic dynamics of the DNA scaffold, even in the absence of the target molecule. The signal change is instead maximized using a small recognition element having relatively lower molecular weight in comparison to the that of the target protein. Specifically, Kang and co-workers[156] recently demonstrated that as the recognition element increases in size (more than 70 kDa), the steric bulk reduces the electron transfer also in the absence of the target molecule. By doing so, the current signal is already suppressed to a level that no further signal change upon target binding can be detected. However, this issue was elegantly solved by reducing the size of the recognition elements to the minimal unit interacting with the target,[156, 157] i.e. when detecting an antibody target using just its hapten as recognition elements. An alternative strategy was designed by Plaxco's group to achieve antibody detection in untreated whole blood.[158] Here a stem-loop DNA scaffold labelled at the end with a redox tag (i.e. methylene blue) and internally modified with two identical recognition elements (i.e. small haptens or polypetide epitopes) acts as signaling scaffold. Specifically, in the absence of the antibody, the redox tag is in close proximity to the electrode surface generating high current signal. When the target binds the two recognition elements, it forces the opening of the stem-loop structure leading to increased distance between the redox tag and the electrode surface, resulting in a lower current signal. Despite these platforms have been successfully deployed for the detection of

proteins and antibodies in untreated biological samples, the approach cannot be easily generalized to target proteins requiring the presence of high molecular weight biorecognition elements. The thermodynamic optimization of the stem-loop DNA scaffold is not trivial, especially considering the fact that electrode-bound DNA probes show significant differences with respect to the simulated free energy in solution collected using standard bioinformatics tools (i.e NUPACK, mFold, etc.)[159, 160].

Using the same concept of scaffolded DNA sensors, but in a complementary approach referred as "molecular pendulum", Kelley's group reported on reagentless DNA-based scaffold sensors addressing some of the above mentioned limitations.[161] The method relies on measuring the sensor's electric field-mediated transport using electron-transfer kinetics of a reporter molecule attached to DNA. By measuring changes in falling time of the negatively charged DNA-probe to the electrode surface upon target binding via chronoamperometry, the presence of a protein bound to the sensor complex can be tracked continuously in real time. This approach provides a generalizable means of monitoring of a range of physiologically relevant proteins directly in a variety of body fluids, as well as in living animals. Furthermore, this assay allows direct detection of whole SARS-CoV-2 virions and its associated spike protein in untreated saliva samples of COVID-19 affected patients within minutes.[162]

Mimicking the structure of natural Immunoglobulin G antibodies, Idili et al.[163] designed a Y-shaped DNA scaffold containing two redox reporters and two recognition elements at the ends of the DNA arms. The bivalent binding of the antibody target to Y-shaped DNA scaffold produces high steric hindrance effects and the use of two reporters a stronger electrochemical signal. Thanks to this rationally designed configuration, the authors detected IgG antibody and streptavidin, a multivalent protein, in serum and artificial saliva with detection limits in the low nanomolar range of concentration. Mahshid and co-workers[164] took advantage of steric hindrance effect for the simultaneous one-step detection of large macromolecules (e.g., proteins and antibodies) in whole blood. In this assay, hybridization kinetic of a redox-active signaling strand to a surface-bound capturing strand was inhibited due to the steric hindrance caused by binding of a large macromolecule to the recognition element on the signaling strand. As a result, electrochemical signal was suppressed in proportion to the size and concentration of the target macromolecule. In a follow-up work, a collaboration between Kelley and Vallée-Bélisle groups[165] reported the use of nanostructured microelectrode (NME) as the interface for the steric-hindrance based assay. High curvature structures of NMEs allows immobilization of a longer capturing probe at higher surface density. This improves the detection limit of the platform compared to the previous one (LOD of 10 pM vs LOD of 100 nM for streptavidin as model analyte). They also demonstrated improvements in response time and detection sensitivity as a result of the size-

dependent hybridization rates and morphology-induced blocking effects on the electrode surface. These platforms were also used to detect small molecules through a competitive antibody-based assay directly in complex matrices.[166, 167]

### 7.2. Proximity-based DNA scaffold sensors

To create a highly selective and more generalizable way to quantify proteins and antibodies, target-induced reconfiguration of signaling DNA scaffolds controlled by proximity effects can be harnessed in order to generate a measurable signal output. In this regard, the typical Y-shaped structure of bivalent IgG antibodies makes them a perfect candidate for the development of proximity-based DNA sensors, as demonstrated by our group.[168, 169] As a matter of fact, the hinge region that links the Fc and Fab portions of an antibody is a flexible tether that allows a quite independent movement of the two Fab arms, thus making the distance between the two binding sites (present at the end of the Fab arms) quite variable. Despite this, the common y-shaped structural view of an IgG or IgE antibody shows the two binding sites separated by approximately 10−12 nm. This distance can be taken as a reference for the rational design of DNA-based units that can interact only when they are co-localized on the same target antibody at the nanoscale volume.

In this regard, our group reported on the rational design of programmable structure-switching DNA-based scaffolds that combine the advantageous features of DNA-based nanoswitches with those of co-localization-based methods. Specifically, the co-localization on the same target antibody of the stem-loop scaffold and its complementary input DNA strand leads the two units into close proximity, forcing the opening of the stem-loop DNA scaffold with consequent increase of the FRET signal [168, 169]. This design allows one-step and orthogonal detection of clinically relevant IgG and IgE antibodies in human blood serum and plasma samples in a no-wash format within less than 10 minutes. The platform has been successfully applied to monitor the immune response of HIV-positive patients treated with a peptide-based (AT20 peptide) phase-I therapeutic vaccine, and also proved its capability to differentiate unvaccinated positives from vaccinated patients.[169] One of the major advantages of this design is the capability to recognize any bivalent molecular target for which a recognition element can be conjugated to a nucleic acid strand. This modular and versatile approach has been also used in a follow-up work to detect a monoclonal antibody (i.e. trastuzumab) only by changing the Ab-recognizing epitope.[168] Trastuzumab, a growth-inhibitory humanized monoclonal anti-HER2/neu, is currently used for passive immunotherapy in the treatment of breast cancer. The method exhibited a linear range of 10 to 75 nM

with a change in $K_{1/2}$ from 100 ± 10 to 30 ± 5 nM in the absence and presence of trastuzumab, respectively. Given the minimum trastuzumab concentration required for the therapy is 137 nM, we were able to monitor the immune response of breast cancer patients treated with trastuzumab-based therapy with comparable results with ELISA in terms of positive/negative discrimination. By adapting the strategy to a modular design, we also reported on the detection of small molecule (i.e. antigen) using the same assay through a competitive format.[170]

Taking advantage of the proximity effect, Ricci's group also used proteins and bivalent antibodies to control DNA-templated chemical reactions.[171, 172] In their first reported strategy, bivalent binding of a specific IgG antibody to a pair of antigen-conjugated DNA templating strands colocalizes reactants to trigger a chemical reaction.[171] The method was characterized by versatility and orthogonality, moreover by synthesizing the thrombin-binding aptamer, a molecule with potential therapeutic, the authors demonstrated potential utility of their strategy to control the synthesis of functional molecules with specific antibodies. In a follow up work, the authors also demonstrated the generalizability of their strategy by using protein to control a set of DNA-templated reactions.[172] Following the same concept, our group has recently designed an electrochemical device for the rapid, single-step and multiplex detection of clinically relevant antibodies in human serum sample.[173] As shown in Figure 7.1 c, the simultaneous recognition of bivalent antibody target by two antigen-conjugated DNA scaffolds provide a means to induce the proximity-dependent DNA hybridization at the electrode interface. By doing so, the redox-labeled DNA scaffold free in solution comes into close proximity to the electrode surface producing an enhanced current signal that is proportional to the antibody concentration. This molecular design clearly demonstrates the impact of the proximity effect on hybridization efficiency, as the binding curve between the two scaffold DNAs show a significant lower binding affinity (higher Kd) in the absence of the antibody, whereas improved relative binding affinity between the two strands can be detected in the presence of saturating concentration of target antibody.

Similarly, electrochemical proximity assays relying on simultaneous binding of two antibody-modified nucleic acid probes to the same protein can be developed to bring a redox reported into close proximity to the electrode surface. Using this approach, thrombin and insulin were monitored down to the picomolar and femtomolar ranges of concentration, respectively.[174, 175] Furthermore, different proximity assays have been deployed into electro chemiluminescent,[176] photoelectrochemical[177] and electrochemical[178] assays to measure alpha-fetoprotein, insulin, prostate specific antigen proteins, respectively. Although proximity effect offers high selectivity and enhanced sensitivity and allows to monitor a broad range of proteins, the identification of two distinct binding sites on the same

target, the signal decline at high target-to-probe ratios and the challenging conjugation of certain ligands to the DNA scaffold still represent criticalities.[99]

### 7.3. Tetrahedral DNA nanostructure (TDN)- based sensors

As an example of relatively more complex DNA scaffolds to be used for biosensing, we briefly highlight here one of the most studied DNA nanostructures, i.e. Tetrahedral DNA nanostructure (TDN). While most aptamers and DNAs are inclined to degradation *in vivo* by nuclease, TDN is widely delivered into cells for diagnostic and therapeutic applications due to the its high cell permeability, enhanced nuclease resistance and intrinsic biocompatibility.[179, 180] By incorporating functional sequence into TDN edges, reconfigurable DNA tetrahedra have been developed to respond to various biomolecules both *in vivo* and *in vitro*. Moreover, TDN represents a well-defined scaffold for arraying multiple fluorophores at precise positions and distances in which provides a parallel and multiplexed sensing platform. Benefiting from this property Zhou et al. designed a TDN, containing three different fluorophore-quencher pairs and predesigned hairpin structures at edges.[181] By the appropriately engineering of hairpin units as recognition elements, different miRNAs (i.e., miRNA-21, miRNA-221, and miRNA-155) and endonucleases (i.e., EcoRI, Nt.BbvCI, and HindIII) were analyzed in parallel. The generality of the platform was further demonstrated by multiplex monitoring of proteins (i.e., ATP, VEGF, and thrombin). To achieve this, the edges of TDN were stretched by the respective aptamer strands. Protein-aptamer binding induces the reconfiguration of the edges into fluorophore-quenched state, leading to multiplexed detection of proteins. In addition, the authors demonstrated the applicability of their strategy to image MCF-7 and HepG2 cancer cells and their discrimination from normal MCF-10A breast cells. Using reconfigurable DNA tetrahedra nanostructures containing dynamic sequences, various logic gates have also been constructed to intracellular diagnostics of different targets including small molecules, metal ions, microRNAs and coronaviruses. [182–184]

Another strategy takes advantage of a TDN reconfiguration to detect repair enzyme activities as enzyme substrate.[185–187] With this concept, in one study, Tong et al. incorporated an rU base at one of the TDN-forming strands which was dual-labeled with a fluorophore and a quencher to generate a structure collapse-based “off-on” fluorescent probe, operating as substrate for real time intecellular imaging of RNAse A.[186] In the absence of RNAse A, the TDN presents a fluorophore-quencher pair in close proximity and so the nanostructured probe populates its “Off” state. When the rU base is specifically recognized and digested by RNAse A, TDN structure collapses sand fluorescence recovery is

observed. Using this approach, a detection limit of 0.09 pg $\mu L^{-1}$ was achieved for RNAse A which was improved to 0.005 pg $\mu L^{-1}$ in the presence of a natural compound as RNase A activity stimulator. Similar strategy was reported by Zhou et al. to monitor methyltransferase activity down to 0.045 $UmL^{-1}$ and screen its various inhibitors.[187]

Other applications of TDN-based approaches have been reported, in the last ten years, especially in the field of electrochemical DNA scaffold biosensors. As a matter of fact, classic DNA electrochemical biosensors suffer from nonspecific adsorption, disordered probe organization, and segregation of DNA probes on the electrode surface, that in turn prevents the formation of a homogeneous receptor interface. This heterogeneity might limit the capacity of immobilized DNA probes to properly recognize the target molecule, a fact that is a major obstacle in the development of ultrasensitive DNA electrochemical sensors. In this regard, interface engineering strategies including thiolate small molecules backfilling, polyadenine-based DNA assembly, and DNA assembly on inorganic nanoparticles have been employed to address these shortcomings.[188] Fan's group pioneered the use of TDNs to modulate the interface of electrochemical DNA sensors.[189] The high mechanical rigidity and structural stability of TDNs provide several advantages compared to single or double stranded DNA probes, including well-controlled density and orientation of capture probes, and minimization of nonspecific adsorption on the electrode surface. These features result in reduced background signal and increased target accessibility to the capture probe in the complex matrices. In addition, the hollow structure of the tetrahedra enables redox molecules to easily penetrate into the thick layer, supporting sensitive electrochemical detection. The ability to program the nanoscale lateral distance between capture probes, and thus the detection limit using TDNs of different sizes, is another important feature of the TDN-based biosensing interface. Lin and co-workers[190] demonstrated an inverse proportion between the sensitivity of the biosensor and TDN size, by showing improved detection limit from 10 pM to 1 fM by tuning the size of TDN. TDN-based electrochemical platforms have been tailored to detect multiple targets, including target proteins (i.e. antibodies). In one communication, Fan's group combined the advantages of the TDN-based platform to present highly sensitive and regenerative electrochemical immunosensing of a variety of protein markers (e.g., tumor necrosis factor-α (TNF-α), prostate-specific antigen, and α-fetoprotein).[191] Specifically, to monitor TNF-α, a well-known tumor biomarker, TDN conjugated with TNF-α antibody was robustly anchored to the gold electrode surface through the three thiol groups on its three vertices.[192] Using a sandwich-like strategy, TNF-α binding to the TDN-tethered antibody was detected using a biotinylated anti TNF-α antibody as reporter module and horseradish-peroxidase conjugated avidin (avidin-HRP), that generated an electrocatalytic signal that

allowed ultrasensitive protein detection (Figure 7.1d). Similarly, also aptamers have been anchored on TDN-decorated interface to specifically detect thrombin,[193] cancerous exosomes[194] and small molecules[195] with sensitivities three to four orders of magnitude higher compared to the single-stranded aptamer-based sensors. Given all these advantages, these highly sensitive platforms could also be adapted in POC diagnostic sensors as well as hold great promise for intracellular and in vivo biosensing owing to their efficient performance and stability.

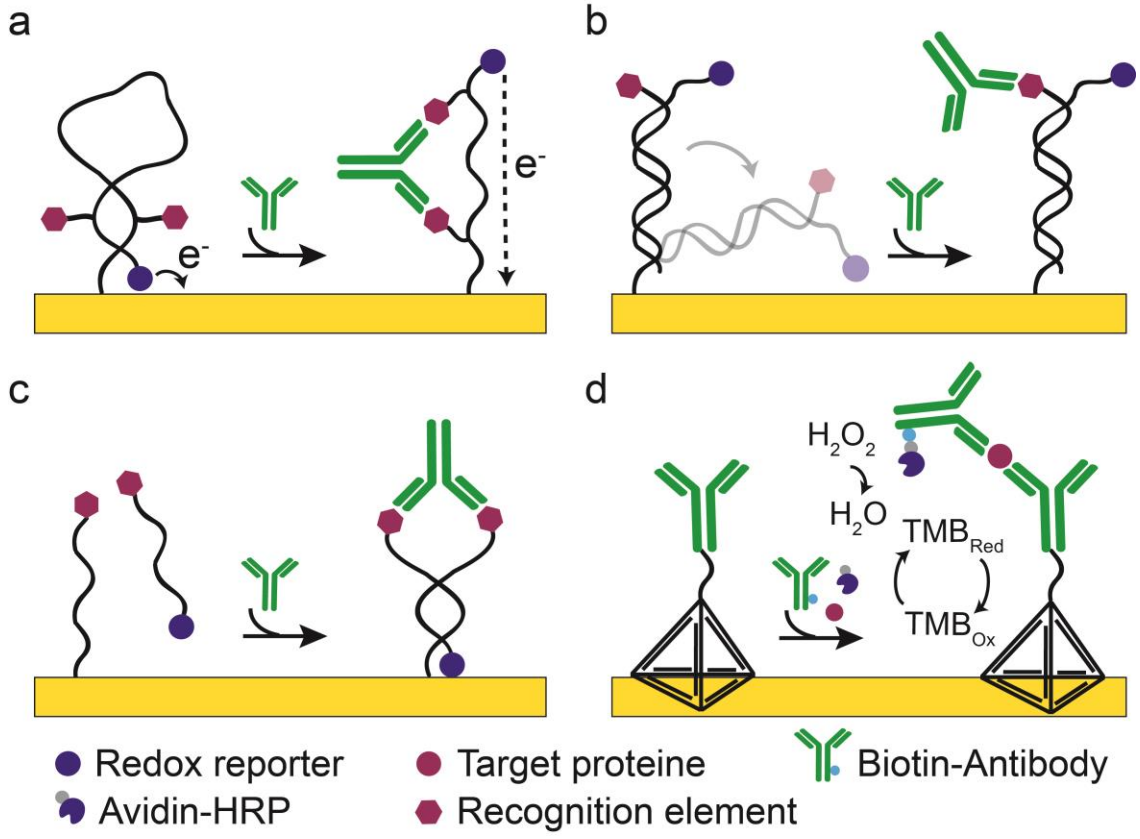


**Figure 7.1**: Example of DNA-based electrochemical sensors for protein detection. **(a)** Structure switching-based electrochemical assays. Conformational change associated to the structural reconfiguration of the DNA probe upon antibody binding brings the redox reporter away from the electrode, resulting in reduced electron transfer between the electrode and the redox reporter. Adapted with permission from Ref. [158] (Copyright © 2012, American Chemical Society). **(b)** Steric hindrance-based electrochemical assay. Steric bulk hindrance of the target antibody hinders collisional activity of the DNA probe and hampers electron-transfer. Adapted with permission from Ref. [153] (Copyright © 2009, American Chemical Society). **(c)** Proximity-based electrochemical assay. Co-localization of the two antigen labelled DNA strands on the same target antibody, brings the redox reporter closer to the electrode surface and enhances electron transfer. Adapted with permission from Ref. [173] (Copyright © 2020, Wiley). **(d)** Tetrahedral DNA nanostructure (TDN)-based electrochemical assay. Target protein binding to the TDN-tagged antibody leads to enzyme turnover-based signal transduction. Adapted with permission from Ref. [192]. (Copyright © 2011, Royal Society of Chemistry).

## 8. CONCLUSIONS

The rational design of functional nanostructures and structure-switching probes can be harnessed to develop biosensing platforms for protein detection. Taking advantage of the predictability of DNA interactions and of recent progress in the chemistry of nucleic acids, especially with respect to biomolecule and small molecule conjugation, we are now able to finely control the spatial organization of responsive molecular components into a DNA-based scaffold, as well to predict the dynamic behavior of switchable DNA-based elements upon target binding.

Here we showed a number of structure-switching DNA probes and programmable DNA scaffold systems with different levels of complexity (2D DNA nanostructures, DNA origami, tetrahedral DNA, beacons, etc.) employed to organize molecules (i.e., enzymes, antigens, and peptides) with nanometer precision for biosensing. We believe that in the near future it will be possible to translate such analytical assays into real and commercially available sensing platforms.

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