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Design of Specific Nucleic Acid-Based Biosensors for Protein Binding Activity

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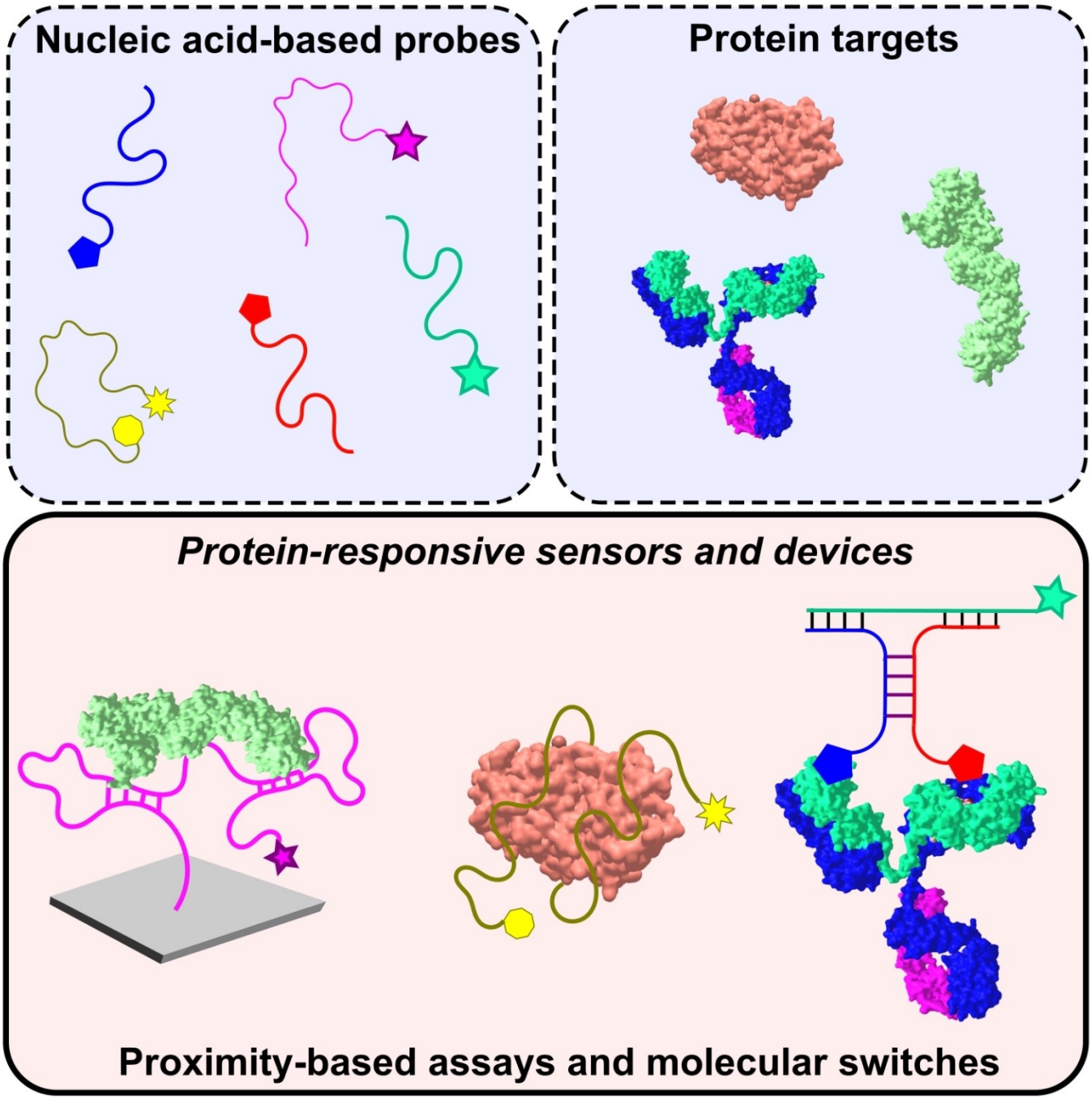

Nucleic acid-based biosensors for the detection of specific proteins combine the typical programmability of synthetic DNA systems with artificially controlled DNA-protein communication. The high-affinity interaction between a target protein and a specific ligand, such as an aptamer sequence, or a double stranded DNA domain, or a small peptide, is paired with a nature-mimicking molecular mechanism allowing for probing, processing, and translating protein binding activity into a measurable signal. In this Review, two main strategies developed in the context of protein-responsive nucleic acid-based biosensors are discussed. One is the design of proximity-based assays harnessing the spatial colocalization of functional probes within the volume of a multivalent protein. The other is the engineering of dynamic DNA structures that undergo a controlled conformational or structural change upon protein binding. Examples of applications from optical and electrochemical detection of antibodies in biofluids to fluorescence imaging of transcription factors in living cells are reported, and suggestions along with possible future directions in the field are discussed.

1. Introduction

Nucleic acid-based biosensors harness the programmability of DNA-based systems to translate a specific binding event into a measurable signal. Watson-Crick-Franklin base pairing is predictable and highly controllable, and DNA nanotechnologies can be engineered into molecular transduction systems and used in conjunction with a variety of materials interfaces.[1–3] When the molecule to detect is a DNA or an RNA sequence, e.g., a short non-coding RNA, or a circulating tumor DNA, a tailor nucleic acid biosensor can be developed through studying, controlling, and manipulating the thermodynamics and kinetics specific hybridization-based reactions, which are then coupled with a signal-generating process.[4–6] The design of nucleic acid-based biosensors that can recognize and measure a specific target protein requires instead the engineering of alternative binding-induced mechanisms through more creative approaches.[7–9] Inspired by nature, a smart strategy is to create artificial DNA-protein communication and then engineer mechanisms and interfaces through which protein binding activity can be probed, processed, and converted into a measurable signal (Scheme 1).

DNA-protein communication is central in nature. It underlies information processing and emergence of function in living systems. The whole genetic machinery, for instance, leverages reversible interactions between proteins, DNA and RNA, including transcription factors that bind to specific double stranded DNA motifs and regulate gene transcription.[10] Many other bioprocesses, from posttranscriptional gene regulation to cell replication, to mechanisms of adaptation and resistance, all rely on diverse interactions between specific nucleic acid sequences and proteins.[11,12] Artificial DNA-protein communication consists in programming and controlling the interaction between a target protein and a functional nucleic acid probe, and it can follow different routes. Direct protein-DNA communication can be pursued with DNA-binding proteins. As these proteins naturally recognize a specific double stranded DNA domain known as consensus sequence, they can be exposed to a synthetic copy of their cognate consensus sequence incorporated in a rationally designed DNA-based structure.[13,14] Heyduk and coworkers pioneered this concept and developed fragmented molecular beacons responsive to DNA-binding proteins.[15] Alternatively, high-affinity DNA aptamers are synthetic protein ligands that can be obtained through SELEX procedures and used for establishing a direct connection between a nucleic acid structure and a selected protein.[16,17] Conjugation of a small molecule or a peptide ligand to a DNA or RNA strand is another established strategy for creating artificial protein-nucleic acid communication.[18,19] An example is the chemical transducers developed by Margulies and coworkers: DNA structures modified with both an aptamer and small molecule ligands that enabled synthetic protein-protein communication.[20] Next, a strategy is needed through which one of the above specific binding events can be processed and translated into a measurable analytical output proportional to the concentration of the target protein. One possibility is to harness effective molarity to develop proximity-based assays. A multivalent protein can be used as a molecular platform to colocalize a set of protein-interacting nucleic acid probes in a confined volume.[21] The resulting increase in their local concentration triggers hybridization processes, otherwise negligible, designed to yield a measurable signal.[22] Alternatively, when multivalency is not available, a possible strategy is to design dynamic nucleic acid structures that undergo a programmable conformational or structural change upon their binding to a specific protein.[23] A protein-interacting element, such as a specific dsDNA domain or a DNA aptamer, is incorporated into a nucleic acid structure in equilibrium between different conformations, and the interaction with the target protein triggers a binding-induced conformational or folding change leading to the generation of an optical or electrochemical signal.[24] Here, we highlight and discuss these strategies that, in our opinion, have proven the most useful in

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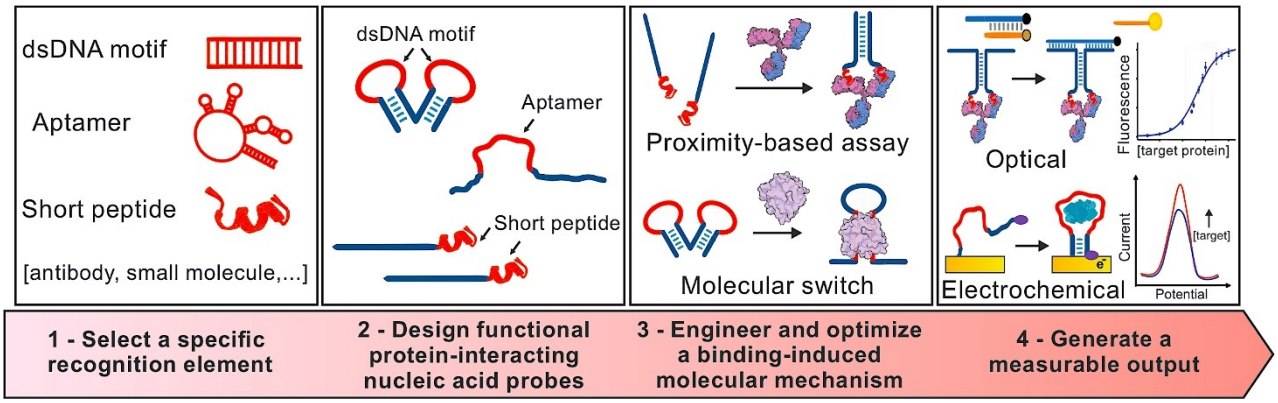

Scheme 1. A schematic roadmap for the design of specific nucleic acid-based biosensors for probing, processing and measuring protein binding activity.

the design of nucleic acid-based biosensors enabling the detection of proteins based on their binding activity, and report examples of works that demonstrate their potential in different fields, from quantification of protein biomarkers in bodily fluids to imaging of functional proteins directly in living cells.

2. Proximity-based assays using functional nucleic acid probes

Nucleic acid probes conjugated with specific affinity ligands can interact with multiple binding sites on a same target protein. This results in a drastic increase in their local concentration, an effect known as effective molarity, which can promote otherwise-unfavored hybridization processes. Direct or indirect quantification of the resulting proximity-driven output, such as a DNA strand displacement reaction or the self-assembly of a DNA structure, provides a measure of the binding activity of the target protein, and therefore of its concentration.

Simone Fortunati received his PhD in Chemical Sciences in 2020 and is currently a Research Assistant at the University of Parma under a "PON Ricerca e Innovazione" program of the Italian Ministry of University. His research work is focused on the development of electrochemical immunosensors and genosensors based on innovative bioreceptors for applications in Point-of-Care Testing and clinical diagnostics.

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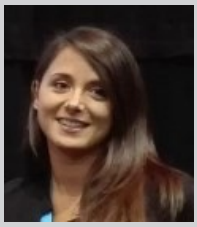
Lorena Baranda received her PhD early this year from the University of Rome Tor Vergata in the research group of Prof. Francesco Ricci. Her PhD project, in the frame of the Marie-Curie ITN project DNA Robotics, focused on achieving protein-templated reactions using DNA-based scaffolds. The last stage of her PhD research was funded by L'Oréal Italia-UNESCO "For Women In Science". Currently she is a post-doctoral researcher in the group of Prof. Andreas Walther at the University of Mainz in Germany, working on transmembrane signaling strategies using DNA tools.



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Alessandro Bertucci is an Assistant Professor at the Department of Chemistry, Life Sciences and Environmental Sustainability at the University of Parma. After earning his PhD in Chemistry in 2015, he worked as an Endeavour Research Fellow in Australia and then as a Marie Skłodowska-Curie Research Fellow in the USA and Italy. His research interests lie in DNA nanotechnology, biomolecular engineering and materials chemistry, aiming to develop hybrid systems and biomaterials with programmable behavior for applications in precision diagnostics and nanomedicine.

2.1. Detection, quantification and imaging of multivalent proteins

Proximity-based assays have enabled both fluorescence-based biosensors for protein detection, for instance leveraging binding-induced DNA annealing assays with a FRET readout,[25] and different electrochemical platforms supporting protein quantification in biofluids.[26–29] An important milestone has been the development of complex systems that leverage rationally designed binding-induced DNA assemblies, such as three-way DNA junctions or even more sophisticated DNA circuits.[30] Le and coworkers developed a binding-induced catalytic DNA circuit that differed from classical toehold-mediated DNA strand displacement reactions in that the release of a first output DNA strand was governed by a protein binding event. This DNA output was then used for promoting a second strand displacement reaction, serving as a reporter system (Figure 1a).[31] Tang et al. reported a protein-responsive catalytic hairpin assembly (CHA) system for the detection of thrombin and of cancer protein biomarkers. They demonstrated that two specific aptamer-tagged nucleic acid probes can self-assemble and form a new nucleic acid strand when they bind to the same target protein. This is used as a triggering input for a CHA process, which eventually produces a fluorescence output through a toehold-mediated DNA strand displacement reaction (Figure 1b).[32] An attractive feature of these assays is their high sensitivity, which can be greatly enhanced through amplification of the DNA output formed upon protein-templated assembly or ligation.[33] Fredriksson et al. demonstrated the potential of this approach in a seminal paper reporting a so-called "proximity ligation" method. They showed that a proximity-based mechanism in tandem with an enzymatic ligation could be used to form a DNA strand that is amplified via polymerase chain reaction (PCR).[34,35] Proximity ligation has since then become a widely used tool for protein analysis, proving useful in the development of many different diagnostic approaches. A recent work by Liu et al. reported, for instance, a highly specific and ultrasensitive method for the detection of serum COVID-19-associated antigens based on an aptamer-assisted proximity ligation assay (Apt-PLA) (Figure 1c).[36] With this method, it was possible to detect the SARS-CoV-2 nucleocapsid protein in 100% human serum with an LOD of 30.9 pg mL^{-1}. Strategies harnessing aptamer-based protein recognition followed by proximity-induced DNA assembly have also enabled *in vitro* detection and imaging of cell surface receptors.[37,38] With this approach, for instance, it has been

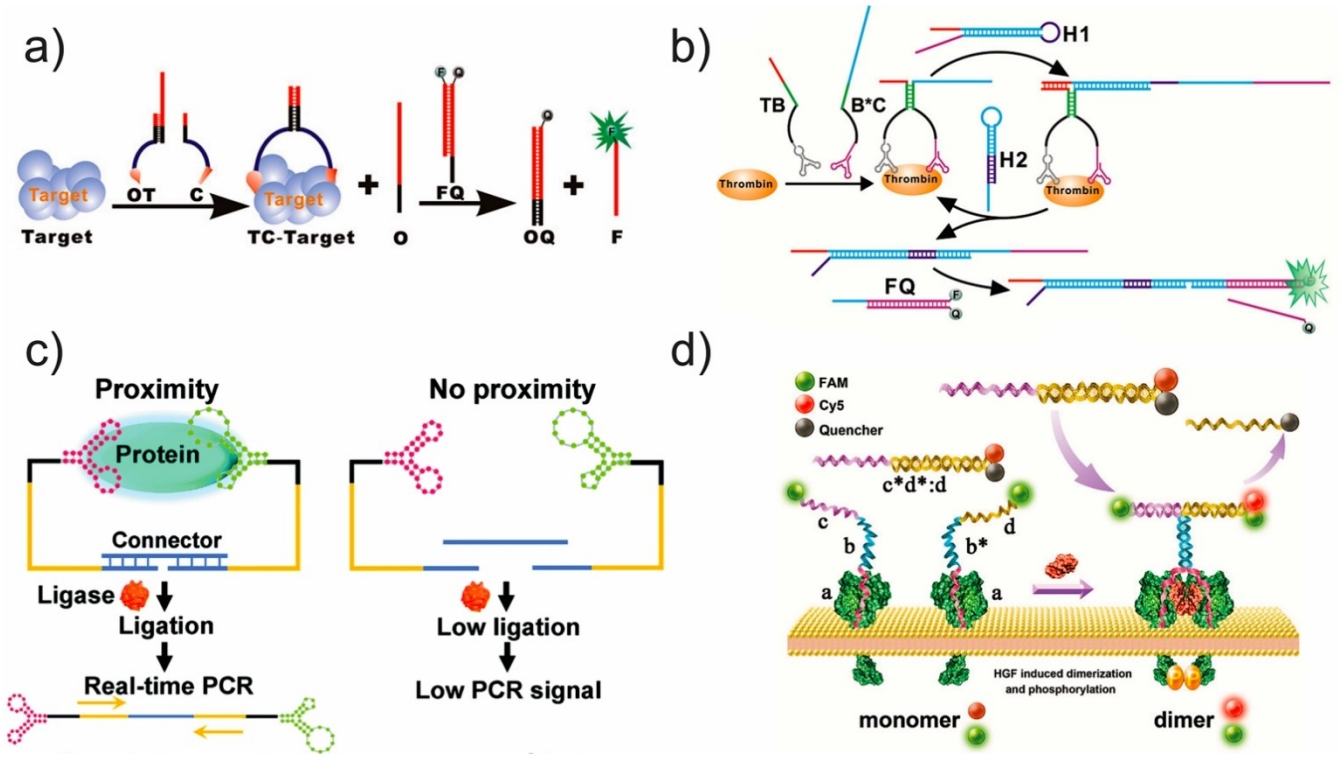

Figure 1. (a) A proximity-based DNA strand displacement reaction. The interaction between two affinity ligands and the target molecule brings the DNA probes into proximity. This results in the release of a DNA strand that can trigger a DNA-based reporter system. Reprinted from [31] Copyright 2013, American Chemical Society; (b) A protein-responsive catalytic hairpin assembly (CHA) triggered by a three-way junction input. The binding of two aptamers to the same thrombin molecule supports the formation of a protein-DNA complex that can catalyze a subsequent CHA reaction between two DNA hairpins. Reprinted with permission from [32]. Copyright 2015, American Chemical Society; (c) A protein-induced DNA proximity ligation assay with PCR amplification. Upon binding to the target protein, the free ends of the DNA aptamers are brought close enough to hybridize to a connector oligonucleotide. The enzymatic ligation of the two proximity probes generates a new DNA sequence, amplifiable via PCR. Reprinted from [36] under Creative Commons license; (d) A proximity-induced DNA assembly strategy for the detection of protein dimerization on the cell membrane. When the two receptors dimerize, two aptamer-tagged probes can assemble into a new DNA sequence, creating an input for a DNA strand displacement reaction as a fluorescent reporter system. Reprinted with permission from [39]. Copyright 2018, American Chemical Society

possible to the study in real-time the dimerization of mesenchymal epithelial transition receptors, as reported by Liang et al. When the receptors dimerize on the cell surface, two aptamer-tagged probes can assemble into a new DNA duplex, triggering a DNA strand displacement reaction for the generation of an optical signal (Figure 1d).[39] Despite the great potential of binding-induced DNA assembly in the design of programmable systems and protein-responsive sensors, a limitation remains that is the relatively small number of DNA aptamers and peptide ligands available to support such strategies. We note here that conjugating specific antibodies to functional DNA probes is a strategy that can contribute to a wider application of proximity-based assays to protein detection, and many examples of works based on this approach can be found in the literature.[28,29,40–42]

2.2. Antibody-controlled molecular operations

The bivalent nature and the typical Y-shaped structure of IgG antibodies make them ideal targets for DNA-based sensors harnessing proximity effects. Antibodies can be interfaced with DNA-based systems by programming and controlling their interactions with nucleic acid strands conjugated with specific recognition elements, i.e., antigen molecules (Scheme 1).[43–45] Two antigen-modified strands binding to the two paratopes on the same antibody undergo a significant increase in their local concentration, allowing for antibody-controlled DNA-based reactions. Tian and Heyduk pioneered this mechanism and demonstrated antibody-induced colocalization of a pair of FRET-labelled DNA oligos conjugated with antigen molecules.[46] However, despite its great potential in biosensor design, this approach suffered from a low signal-to-noise ratio and a significantly high background. Drawing on this principle, the Ricci group developed several different formats of dynamic DNA nanodevices for antibody detection. These are mostly based on the antibody-controlled assembly of functional DNA or RNA structures that can provide a concentration-dependent fluorescence signal.[47–50] Antibody-based colocalization of functional DNA probes can also be used for controlling more complex DNA-based circuits. The Merkx group developed DNA translator modules for translating antibody binding activity into a new, functional DNA strand, enabling the design of molecular logic circuits for complex signal processing (Figure 2a).[44] The new functional DNA strand can engage in a further downstream strand displacement reaction, generating a signal output. In addition, the same approach allowed the integration of multiple antibody inputs in molecular logic gates and to control the activity of enzymes using antibodies as regulatory inputs.

Later, Ranallo et al. developed an alternative design for antibody-controlled strand displacement reactions. Based on

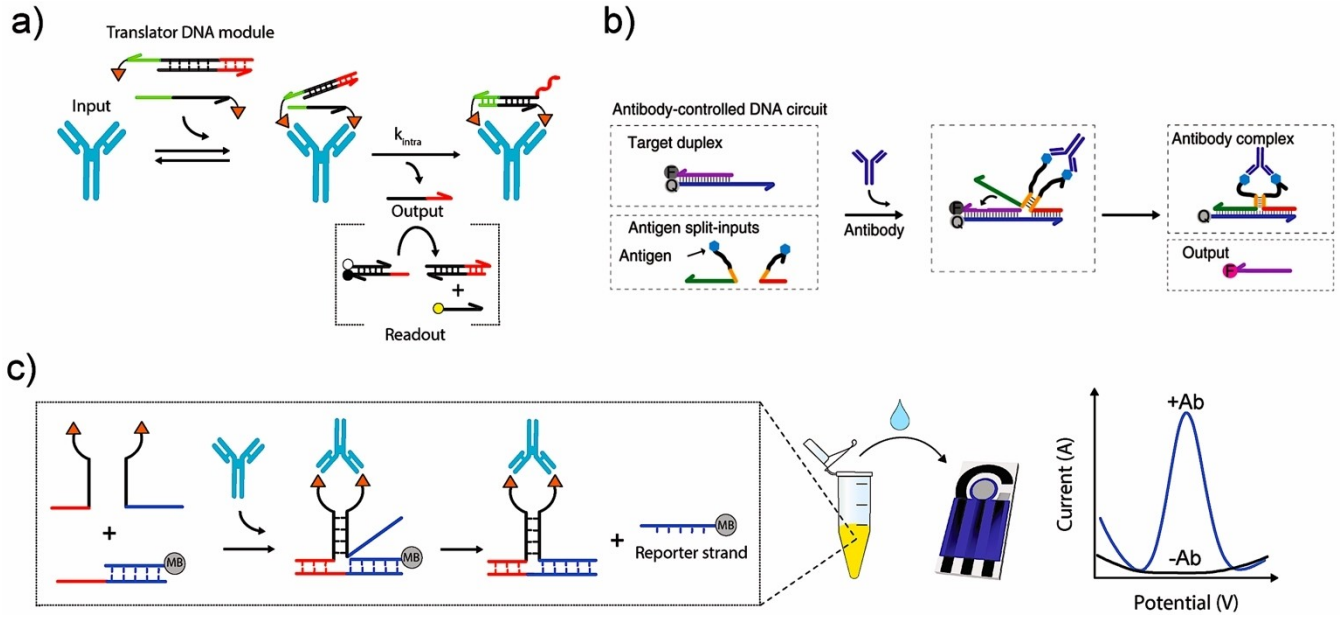

Figure 2. (a) Principle of antibody-templated strand exchange (ATSE). In the presence of a specific antibody (input), the translator DNA module is subjected to a strand exchange reaction mediated by the antibody-based mechanism, which releases a ssDNA (output). This DNA output in turn triggers another strand displacement reaction in which a fluorescence-tagged strand is released, generating an optical signal. Adapted with permission from Ref. [44] under Creative Commons license (b) An antibody-controlled DNA circuit supported by a proximity-based mechanism. The input strand responsible for a toehold-mediated displacement reaction is split into two portions, and these are each conjugated with a molecule (antigen) responsible for antibody recognition. The binding of the antibody to the two antigen-conjugated split portions leads to the reconstitution of the functional input strand through a colocalization effect, which can initiate the strand displacement reaction. Adapted with permission from Ref. [51] under Creative Commons license. (c) An antibody-mediated strand displacement reaction coupled to an electrochemical measurement. Binding of the target antibody to the two antigen-conjugated strands induces the formation of a functional complex able to activate a strand displacement reaction. This latter releases a redox-labelled reporter strand from a pre-hybridized duplex, which hybridizes with a DNA capture probe immobilized on the surface of a screen-printed electrode. An electrochemical Square Wave Voltammetry (SWV) signal is then measured. Adapted from Ref. [55] under Creative Commons license, American Chemical Society.

the formation of a DNA invader strand templated by a specfic antibody, this approach was successfully used for controlling the dynamic assembly and disassembly of DNA nanostructures such as DNA nanotubes (Figure 2b).[51] All these strategies show a low background and a high versatility, yet the magnitude of the output signal is generally limited by stoichiometric antibody-controlled reactions. To enhance sensitivity, a signal amplification process can be placed downstream of one of the above mechanisms. An antibody-controlled DNA circuit can be coupled with the activation of an enzymatic reaction whose product provides a measurable output.[44] Jiang et al. harnessed antibody-controlled formation of a new DNA strand to trigger a hybridization chain reaction between dye-labelled DNA hairpins, demonstrating single-step, fluorescence-based detection of specific antibodies in the picomolar range.[52]

Recenlty, a highly specific and sensitive method for antibody detection was developed based on the antibody-controlled, RNA polymerase-based transcription of a fluorescent RNA aptamer using commercially available cell-free transcription kits.[53]

Proximity-based assays can also be adapted for electrochemical antibody detection by using redox-tagged nucleic acid probes. These electrochemical sensors combine the typical advantages of electrochemical assays, such as being reagent-free, rapid and single-step, with the high programmability of antibody-responsive DNA circuits. Possible designs include proximity-based DNA hybridization processes conducted directly on the surface of gold electrodes, or an off-line procedure in which a DNA strand resulting from an antibody-controlled DNA strand displacement reaction is subsequently detected by means of an electrochemical platform (Figure 2c).[54,55] The released redox-modified strand in this later strategy is transferred onto the surface of a DNA-modified gold electrode, and the resulting hybridization with the surface probes leads to an electrochemical signal. This platform exhibited a detection limit in the nanomolar range, and a high specificity towards the antibody of interest. These electrochemical assays use portable, compact devices and can efficiently operate in blood serum samples, which makes them particularly appealing for further optimization into point-of-care technologies for serological testing.

3. Protein-responsive DNA nanoswitches

Protein binding activity can be probed and quantified by using dynamic nucleic acid-based structures that provide a measurable signal when they undergo a binding- induced conformational or structural change.

Referred to as structure-switching probes or DNA nanoswitches, these dynamic structures are thermodynamically and kinetically controlled so that their intrinsic conformational equilibrium has a direct effect on their affinity towards a target protein, measured as their experimental dissociation constant.[23,56]

3.1. Optical molecular sensors for protein sensing and imaging

In a seminal work, Vallée-Bélisle et al. demonstrated that rationally desigend DNA nanoswitches could be used for the quantitative detection of different DNA-binding proteins in crude cellular extracts. These DNA nanoswitches are structures that incorporate a specific double-strand DNA domain recognized by a target transcription factor and are in equilibrium between a fluorescent and a non-fluorescent conformation.[57] Bertucci et al. then demonstrated for the first time the possibility to use an analogous DNA nanoswitch to probe the DNA-binding activity of a specific transcription factor, nuclear factor κB (NF-κB), directly in living cancer cells (Figure 3a).[58] This DNA nanoswitch, which includes the specific consensus sequence of NF-kB, can interconvert between a non-binding conformation (off-state) and a binding conformation (on-state). The non-binding conformation is a double-hairpin structure in which the two portions constituting the NF-kB consensus sequence are separated. Conversely, the binding conformation is a single-hairpin structure in which the NF-kB consensus sequence is fully formed and is recognized by the protein. The equilibrium between these two conformations is shifted toward the fluorescent on-state by the presence of NF-kB. Cellular delivery of these DNA nanoswitches to cultured PC3 cells by using lipofectamine-based vectors allowed for fluorescence imaging and relative quantification of overexpressed NF-kB in the cytosol (Figure 3b). Recently, a a similar DNA nanoswitch enabled the visualization of the intracellular dynamics of NF-kB in single cellular compartments by means of super resolution microscopy techniques.[59] Bertucci et al. then demonstrated DNA strand displacement reactions enabled by engineered DNA nanoswitches that could report on the binding activity of DNA-binding proteins, including the TATA binding protein (TBP) or the Myc-Max complex.[60] In the non-binding conformation of these nanoswitches, the toehold-binding portion of an invader strand is blocked in a duplex structure, unable to activate a strand displacement reaction (Figure 3c). Conversely, in the single-hairpin structure of the binding conformation, the same toehold portion is free to initiate a downstream strand displacement process, which can be monitored by using DNA strands labelled with a fluorophore and a quencher and can be finely modulated by varying the protein concentration from 100 to 300 nM. Looking ahead, more sensitive, quantitative detection of DNA-binding proteins could be achieved by integrating the above nanoswitches with molecular amplification systems such as those leveraging CHA-based reactions or more recent CRISPR-Cas-based platforms.[61] Zhang et al. showed that a similar strategy could be adapted for the detection of proteins that, like thrombin, recognize and bind to a specific aptamer ligand (Figure 3d).[62] In their design, the thrombin aptamer sequence and a short double stranded stem structure were placed between the toehold-binding and the invading domain of a DNA strand. In the absence of thrombin, the toehold and the invading domain remained separated, and no strand displacement reaction was observed. By increasing the concentration of thrombin from 0 to 500 nM, a fluorescence

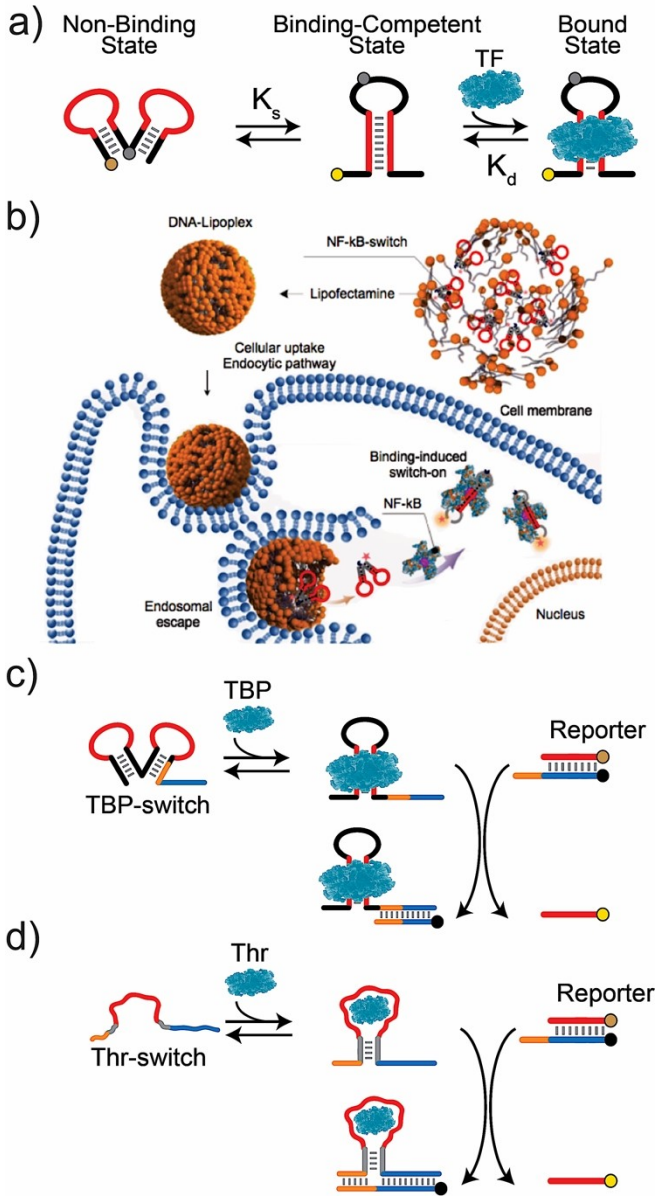

Figure 3. (a) A DNA-based nanoswitch for the quantitative detection of the transcription factor (TF) NF-κB. When the protein recognizes its binding domain embedded in the nanoswitch, a conformational change occurs that results in a fluorescence signal that can be used for intracellular imaging. The red portion in the nanoswitch is the specific double stranded DNA domain recognized by NF-κB. Reproduced from [58] with permission from the Royal Society of Chemistry. (b) DNA nanoswitches are packed into lipofectamine nanoparticles and delivered to the cytosol of living cells to perform intracellular protein imaging. Reproduced from [58] with permission from the Royal Society of Chemistry. (c) A DNA-based nanoswitch controlled by TATA-binding protein (TBP), which serves as a molecular translator for the actuation of strand displacement reactions. The red portion in the nanoswitch is the specific double stranded DNA domain recognized by TATA-binding protein. Adapted with permission from [60]. Copyright 2020, Wiley-VCH GmbH. (d) A DNA-based platform enabling the detection of aptamer-binding proteins like thrombin (Thr), through the activation of binding-induced strand displacement reactions. The red portion in the nanoswitch is the specific aptamer sequence recognized by thrombin. Adapted from [62] under Creative Commons license.

signal was instead progressively generated from a strand displacement reaction initiated by a fully formed, thrombin-templated invader strand. This method was reported to have a limit of detection (LOD) of 0.96 nM and to enable detection of thrombin in a complex matrix such as 1% serum.

In principle, this strategy could be extended to many other proteins of interest by simply changing the specific aptamer sequence in the final design. DNA nanoswitches have also supported antibody detection. Ranallo et al. engineered modular, antigen-modified, structure-switching DNA beacons that can undergo a structural change upon simultaneous binding to the two paratopes of a target antibody. To improve sensitivity, Pfeiffer et al. incorporated a similar antibody-responsive DNA modular structure into the plasmonic hotspot of a DNA origami nanoantenna, demonstrating single-molecule antibody detection.[63]

3.2. Electrochemical sensors based on DNA nanoswitches

DNA nanoswitches have also been applied to the development of electrochemical biosensors, harnessing a binding-induced conformational or structural change that generates an electrochemical signal.[64–66] This approach affords sensors that are reagentless, rapid, cheap and capable of working in complex matrices such as biological fluids. In a seminal work, Barton and coworkers studied the response of a DNA-based sensor designed to probe the binding activity of *HhaI* methylase and TBP. They monitored how the binding of these base-flipping proteins to a double stranded DNA (dsDNA) caused a disruption of the nucleobase stacking and reduced the electrochemical signal of a conjugated daunomycin tag by hampering charge transfer (CT) effect through the DNA double helix (Figure 4a).[67] Based on this working principle, it was possible to detect the presence of both proteins within tens of seconds at low micromolar concentration. Gorodetsky et al. reported a biosensing platform for the detection of TBP based on a dsDNA immobilized on gold microelectrodes and modified with the electrochemical tag nile blue.[68] Also in this case, the working principle leveraged a reduction in CT through the double helix caused by the interaction between TBP and the DNA probes. This work highlighted the advantages in terms of sensing performance achieved using a microelectrode, in comparison with an analogous system built on macroelectrodes. Excision of an electrochemically tagged dsDNA was also employed in a following work by Muren and Barton, in which the methyltransferase activity of both bacterial (*SssI*) and human (*Dnmt*1) methylase was detected by protecting a dsDNA from cleavage from a restriction enzyme through site-specific methylation.[69] This assay was developed on a multiplexed chip and showed a similar LOD for *SssI* (2 nM) and *DnmtI* (5 nM), with a dynamic range of 5–50 nM for *DnmtI* and of 2–8 nM for *SssI*.

A direct interaction between a protein and a dsDNA-based system is not, however, the only modality supporting the development of an electrochemical biosensor.[70] There are many examples of biosensing platforms that yield an electrochemical output by leveraging a protein-responsive conformational change in an engineered single stranded DNA structure.[71]

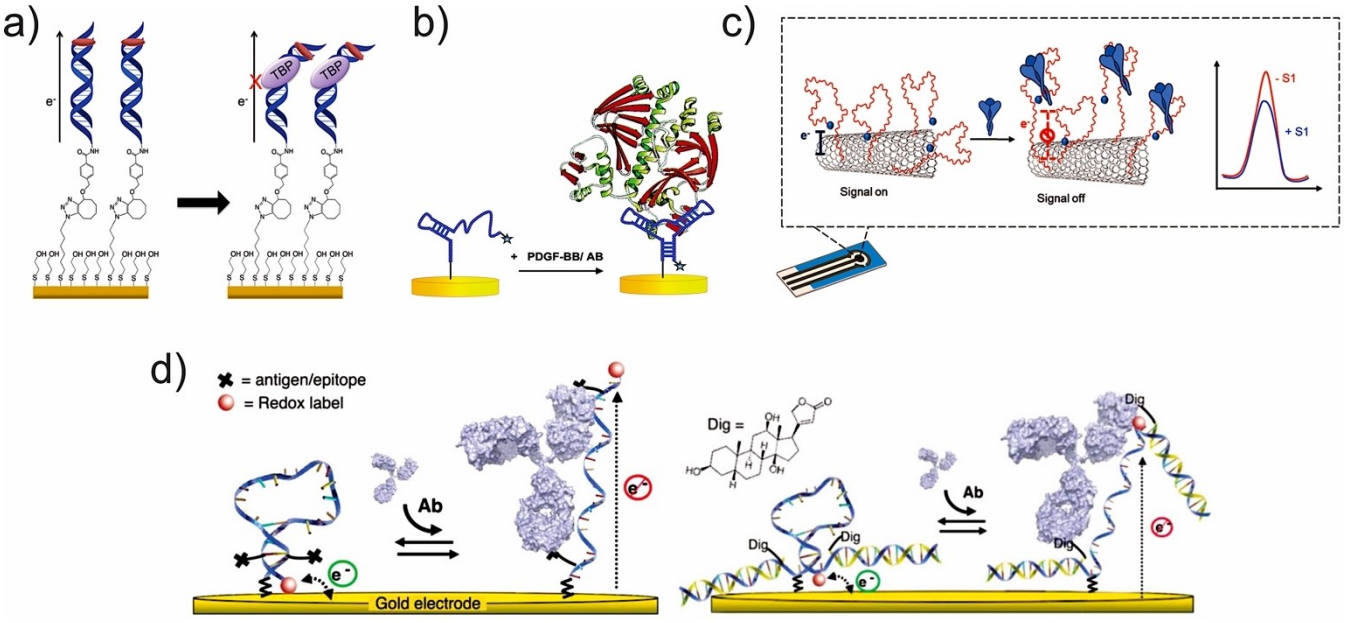

Figure 4. (a) Detection of TATA-binding protein (TBP) through disruption of a charge transfer process. Binding of TBP to a dsDNA probe causes a change in the nucleobase stacking and hampers the charge transfer process responsible of the electrochemical reduction of a daunomycin tag. Reprinted with permission from [65]. Copyright 2013, American Chemical Society; (b) Aptamer-based detection of Platelet-Derived Growth Factor (PDGF). Upon binding to PDGF, a methylene blue-modified aptamer structurally rearranges and folds into a triple-stem conformation that brings the electrochemical tag in proximity to the electrode surface, thereby allowing for efficient electron transfer. Reprinted with permission from [76]. Copyright 2007, American Chemical Society; (c) Signal-off detection of the S1 protein of SARS-CoV-2 using a redox-tagged aptamer immobilized on SWCNT-SPEs. When the redox-tagged aptamer specifically binds to the target S1 protein, the resulting conformational change moves the electrochemical tag away from the surface of the SWCNTs, leading to a decrease in the measured current. Adapted from [78] under Creative Commons license; (d) Detection of anti-DNP antibodies using an electrochemical DNA switch modified with a redox tag and two DNP haptens (left). Binding of the target antibody results in the opening of the initial DNA structure and formation of a single-stranded DNA that moves the electrochemical tag away from the gold electrode surface, enabling signal-off-based antibody detection. A modular version of the same assay is where the haptens are included in an initial DNA structure through hybridization with two complementary DNA probes (right). Reprinted with permission from [79]. Copyright 2012, American Chemical Society.

This working principle has been extensively used in aptamer-based biosensors. An electrochemical signal is generated when a binding-induced change in the structural folding of a redox-tagged aptamer determines a change in the electron transfer process between the redox tag and the electrode.[72,73] Xiao et al. developed an electrochemical platform for the detection of thrombin by using a methylene blue-modified DNA aptamer immobilized on a gold electrode surface.

This aptamer undergoes a binding-induced structural change when in the presence of thrombin, causing a decrease in the registered current signal.[74] Remarkably, this sensor was capable of detecting thrombin in the nanomolar range, spanning more than two orders of magnitude within minutes. However, this system is based on a signal-off detection mechanism, which may result in limitations in terms of signal gain (i.e., 100% signal suppression) and false positives caused by degradation of the aptamer structure. Thrombin detection via an aptamer-based platform was also proposed by O'Sullivan and coworkers. The thrombin aptamer was shortened to a length of 15 bases, and its binding-induced folding was used to bring a ferrocene electrochemical tag closer to the electrode surface, thus enhancing the current signal.[75] Following this approach, it was possible to obtain an LOD of 0.5 nM. Lai et al. developed a signal-on electrochemical aptasensor for the detection of Platelet-Derived Growth Factor (PDGF). They used a methylene blue-modified aptamer that, upon binding to PDGF, conformationally changed into a new structure composed of three double stranded stems, which could bring the electrochemical tag near to the electrode surface (Figure 4b).[76] A comparison with analogous optical aptasensors demonstrated that this electrochemical method could achieve an enhanced sensitivity and a far better selectivity. Recently, electrochemical aptasensors have been developed in response to emerging needs, such as rapidly detecting the SARS-CoV-2 virus.[77] Curti et al. reported a novel electrochemical platform in which a DNA aptamer targeting the SARS-CoV-2 S1 protein was immobilized on single-walled carbon nanotube screen-printed electrodes (Figure 4c).[78] This biosensor, which leveraged a folding-based signal-off mechanism, showed a LOD of 7 nM and could efficiently work in an artificial viral transport medium such as that used for the collection of nasopharyngeal swabs, showing no cross-reactivity when in the presence of other viral proteins. Another advantage offered by the use of oligonucleotides as synthetic receptors is the ease with which electrode surface functionalization can be achieved, as well as the variety of chemical modifications that can be inserted in a DNA sequence. This feature was exploited by Plaxco and coworkers to develop a bioelectrochemical switch based on a single stranded DNA labelled both with a redox tag and two haptens.[79] In the absence of the target protein, this single-strand DNA maintains a closed hairpin conformation that keeps the methylene blue tag in proximity to the electrode surface.

The addition of a specific antibody binding to the two haptens results in the disruption of the DNA probe conformation, which moves the redox tag away from the electrode surface (Figure 4d). This signal-off mechanism was applied to the determination of anti-2,4-dinitrophenol (DNP) antibodies using a DNA probe functionalized with DNP haptens. Tetra-modification of a single-strand DNA could be challenging, though. A modular version of the same DNA-based probe was proposed in which the two haptens were conjugated to two different oligo strands, which were then hybridized with a third methylene blue-modified DNA strand. This mechanism was successfully employed for the determination of model anti-dioxigenin (DIG) antibodies and of the anti-HIV antibody AF5 directly in whole blood in a clinically relevant working range (Figure 4d).

4. Summary and Outlook

The design of molecular transduction systems and interfaces that can process and report on protein binding activity can greatly benefit from the programmability and versatility of synthetic nucleic acids. By artificially recreating protein-DNA communication, sensing technologies can be engineered that enable both a qualitative and a quantitative analysis of target proteins. Many different target proteins can in principle be detected by simply changing a specific protein-interacting element integrated in the sensing system. Advances in DNA nanotechnology can further spur on this approach by enabling new ways of processing information through programmable DNA-based reactions.

Advanced forms of signal processing can be for instance obtained applying DNA-based logic operations, circuit computation, and thresholding mechanisms, which allow for a more complex and digital elaboration of the measured signal.[44,80–82] The integration with high-performing amplification methods, such as those based on CRISPR-Cas effectors or other isothermal enzyme-based techniques, can also provide new (bio)chemical tools for ultrasensitive protein detection.[83] In this Review, we have highlighted how nucleic acid biosensors can be rationally designed to probe protein binding activity, yielding an optical or an electrochemical output that is ultimately a function of the concentration of the target protein. We have discussed the two main strategies successfully applied to the development of molecular sensors and biosensing devices -that is, proximity-based assays harnessing the confinement of multiple functional probes within the volume of a target protein, and structure-switching DNA devices that undergo a binding-induced conformational change. We have showcased several works that demonstrate detection of specific proteins in model biological samples, in real complex matrices, and directly in living cells. Table 1 summarizes the characteristics and the analytical performances of some of the most representative works. We note, however, that some challenges remain. Incorporating a specific dsDNA or aptamer sequence into a functional nucleic acid probe requires a careful rational design of the different sequences composing the sensing/transduction system. This may prove especially challenging when using relatively long nucleic acid strands or sequences that fold into specific secondary or tertiary structures. Protein-DNA communication is also limited by the number of currently available aptamer and peptide ligands. This calls for progresses in the discovery of new affinity ligands, for example through SELEX (aptamers) and phage display (peptides).[84,85] The ability of a biosensor to work in complex matrices and in crude biological samples should also be assessed, parallelized by focused studies on specificity and possible false positives caused by degradation effects or interfering agents. Looking ahead, we believe that many exciting opportunities will create

Table 1. Detection strategy and analytical performance of sensing platforms based on proximity-based assays or structure-switching DNA devices.

Mechanism	Target species	Signal transduction	Limit of detection	Response time	Matrix	Reference
Proximity-based catalytic hairpin assembly	Thrombin	Optical	100 pM	150 min	10% serum	32
Proximity-based, ligation-dependent qPCR amplification	SARS-CoV-2 Nucleocapsid protein	Optical	37.5 pg/mL	120 min	Undiluted serum	36
Proximity-based, antibody-responsive strand exchange	Anti-HA, Anti-HIV-p17 antibodies	Optical	23 pM (Anti-HA)	60 min	NA	44
Proximity-based, antibody-responsive strand displacement	Anti-Dioxigenin, anti-HIV, anti-hemagglutinin antibodies, cetuximab	Electrochemical	9 nM (Anti-DIG)	30 min	90% serum	55
Conformational change in a DNA nanoswitch	NF-kB Transcription factor	Optical	NA	4 h	*In vitro* live cells	58
Aptamer-based nanoswitch	ATP, Thrombin	Optical	930 nM (ATP) 0.96 nM (Thrombin)	60 min	10% serum	62
Induced folding of an aptamer-based nanoswitch	SARS-CoV-2 S1 Spike protein	Electrochemical	7 nM	60 min	Viral Transfer Medium	78
Modular DNA-based nanoswitch	Anti-Dioxigenin, Anti-HIV AF5 antibodies	Electrochemical	1 nM (Anti-DIG) 10 nM (Anti-HIV)	$<$10 min	Undiluted whole blood	79

NA = Not Assessed.

from interfacing and integrating the design of protein-responsive nucleic acid biosensors with materials chemistry and device engineering.[86,87] The use of nanomaterials as delivery platforms will help translate new protein sensing technologies from *ex-vivo* to *in-vivo*, enabling the analysis of protein expression directly in living organisms. Incorporating nucleic acid-based biosensors into engineered material platforms will support the development of new generations of electrochemical devices and wearable technologies for the monitoring of specific proteins in real-time.

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Conflict of Interest

The authors declare no conflicts of interest

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[1] J. Chao, D. Zhu, Y. Zhang, L. Wang, C. Fan, *Biosens. Bioelectron.* **2016**, *76*, 68–79.
[2] D. Ye, X. Zuo, C. Fan, *Annu. Rev. Anal. Chem.* **2018**, *11*, 171–195.
[3] S. Ranallo, A. Porchetta, F. Ricci, *Anal. Chem.* **2019**, *91*, 44–59.
[4] R. Huang, N. He, Z. Li, *Biosens. Bioelectron.* **2018**, *109*, 27–34.
[5] A. R. Chandrasekaran, J. A. Punnoose, L. Zhou, P. Dey, B. K. Dey, K. Halvorsen, *Nucleic Acids Res.* **2019**, *47*, 10489–10505.
[6] D. Y. Zhang, G. Seelig, *Nat. Chem.* **2011**, *3*, 103–113.
[7] E. E. Watson, S. Angerani, P. M. Sabale, N. Winssinger, *J. Am. Chem. Soc.* **2021**, *143*, 4467–4482.
[8] D. K. Agrawal, R. Schulman, *Nucleic Acids Res.* **2020**, *48*, 6431–6444.
[9] W. Engelen, B. M. G. Janssen, M. Merkx, *Chem. Commun.* **2016**, *52*, 3598–3610.
[10] A. J. M. Walhout, *Genome Res.* **2006**, *16*, 1445–1454.
[11] N. Y. Tretyakova, A. Groehler, S. Ji, *Acc. Chem. Res.* **2015**, *48*, 1631–1644.
[12] Y. Zou, Y. Liu, X. Wu, S. M. Shell, *J. Cell. Physiol.* **2006**, *208*, 267–273.
[13] G. Badis, M. F. Berger, A. A. Philippakis, S. Talukder, A. R. Gehrke, S. A. Jaeger, E. T. Chan, G. Metzler, A. Vedenko, X. Chen, H. Kuznetsov, C.-F. Wang, D. Coburn, D. E. Newburger, Q. Morris, T. R. Hughes, M. L. Bulyk, *Science* **2009**, *324*, 1720–1723.
[14] F. Praetorius, H. Dietz, *Science.* **2017**, *355*.
[15] T. Heyduk, E. Heyduk, *Nat. Biotechnol.* **2002**, *20*, 171–176.
[16] Z. Huang, L. Qiu, T. Zhang, W. Tan, *Matter* **2021**, *4*, 461–489.
[17] M. Famulok, G. Mayer, *Chem. Biol.* **2014**, *21*, 1055–1058.
[18] N. Stephanopoulos, *Bioconjugate Chem.* **2019**, *30*, 1915–1922.
[19] W. Engelen, K. Zhu, N. Subedi, A. Idili, F. Ricci, J. Tel, M. Merkx, *ACS Cent. Sci.* **2020**, *6*, 22–31.
[20] R. Peri-Naor, T. Ilani, L. Motiei, D. Margulies, *J. Am. Chem. Soc.* **2015**, *137*, 9507–9510.
[21] M. Rossetti, A. Bertucci, T. Patiño, L. Baranda, A. Porchetta, *Chem. Eur. J.* **2020**, *26*, 9826–9834.
[22] H. Zhang, F. Li, B. Dever, C. Wang, X.-F. Li, X. C. Le, *Angew. Chem. Int. Ed.* **2013**, *52*, 10698–10705; *Angew. Chem.* **2013**, *125*, 10894–10902.
[23] A. Vallée-Bélisle, F. Ricci, K. W. Plaxco, *Proc. Nat. Acad. Sci.* **2009**, *106*, 13802–13807.
[24] S. Bissonnette, E. Del Grosso, A. J. Simon, K. W. Plaxco, F. Ricci, A. Vallée-Bélisle, *ACS Sens.* **2020**, *5*, 1937–1942.
[25] E. Heyduk, T. Heyduk, *Anal. Chem.* **2005**, *77*, 1147–1156.
[26] J. Hu, T. Wang, J. Kim, C. Shannon, C. J. Easley, *J. Am. Chem. Soc.* **2012**, *134*, 7066–7072.
[27] L. Zhang, K. Zhang, G. Liu, M. Liu, Y. Liu, J. Li, *Anal. Chem.* **2015**, *87*, 5677–5682.
[28] J. Hu, Y. Yu, J. C. Brooks, L. A. Godwin, S. Somasundaram, F. Torabinejad, J. Kim, C. Shannon, C. J. Easley, *J. Am. Chem. Soc.* **2014**, *136*, 8467–8474.
[29] S. M. Traynor, G. A. Wang, R. Pandey, F. Li, L. Soleymani, *Angew. Chem. Int. Ed.* **2020**, *59*, 22617–22622; *Angew. Chem.* **2020**, *132*, 22806–22811.
[30] F. Li, Y. Lin, X. C. Le, *Anal. Chem.* **2013**, *85*, 10835–10841.
[31] F. Li, H. Zhang, Z. Wang, X. Li, X.-F. Li, X. C. Le, *J. Am. Chem. Soc.* **2013**, *135*, 2443–2446.
[32] Y. Tang, Y. Lin, X. Yang, Z. Wang, X. C. Le, F. Li, *Anal. Chem.* **2015**, *87*, 8063–8066.
[33] D. A. Di Giusto, W. A. Wlassoff, J. J. Gooding, B. A. Messerle, G. C. King *Nucleic Acids Res.* **2005**, *33*, 1–7.
[34] S. Fredriksson, M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gústafsdóttir, A. Östman, U. Landegren, *Nat. Biotechnol.* **2002**, *20*, 473–477.
[35] M. Famulok, *Nat. Biotechnol.* **2002**, *20*, 448–449.
[36] R. Liu, L. He, Y. Hu, Z. Luo, J. Zhang, *Chem. Sci.* **2020**, *11*, 12157–12164.
[37] X. Chang, C. Zhang, C. Lv, Y. Sun, M. Zhang, Y. Zhao, L. Yang, D. Han, W. Tan, *J. Am. Chem. Soc.* **2019**, *141*, 12738–12743.
[38] Y. Li, X. Zhang, W. Pan, N. Li, B. Tang, *Anal. Chem.* **2020**, *92*, 11921–11926.
[39] H. Liang, S. Chen, P. Li, L. Wang, J. Li, J. Li, H.-H. Yang, W. Tan, *J. Am. Chem. Soc.* **2018**, *140*, 4186–4190.
[40] A. Klaesson, K. Grannas, T. Ebai, J. Heldin, B. Koos, M. Leino, D. Raykova, J. Oelrich, L. Arngården, O. Söderberg, U. Landegren, *Sci. Rep.* **2018**, *8*, 5400.
[41] R. Y. Nong, D. Wu, J. Yan, M. Hammond, G. J. Gu, M. Kamali-Moghaddam, U. Landegren, S. Darmanis, *Nat. Protoc.* **2013**, *8*, 1234–1248.
[42] Y. S. Ang, J. J. Li, P.-J. Chua, C.-T. Ng, B.-H. Bay, L.-Y. L. Yung, *Anal. Chem.* **2018**, *90*, 6193–6198.
[43] B. M. G. Janssen, M. van Rosmalen, L. van Beek, M. Merkx, *Angew. Chem.* **2015**, *127*, 2560–2563; *Angew. Chem. Int. Ed.* **2015**, *54*, 2530–2533.
[44] W. Engelen, L. H. H. Meijer, B. Somers, T. F. A. de Greef, M. Merkx, *Nat. Commun.* **2017**, *8*, 14473.
[45] W. Engelen, C. Sigl, K. Kadletz, E. M. Willner, H. Dietz, *J. Am. Chem. Soc.* **2021**, *143*, 21630–21636.
[46] L. Tian, T. Heyduk, *Anal. Chem.* **2009**, *81*, 5218–5225.
[47] A. Porchetta, R. Ippodrino, B. Marini, A. Caruso, F. Caccuri, F. Ricci, *J. Am. Chem. Soc.* **2018**, *140*, 947–953.
[48] S. Ranallo, M. Rossetti, K. W. Plaxco, A. Vallée-Bélisle, F. Ricci, *Angew. Chem. Int. Ed.* **2015**, *54*, 13214–13218; *Angew. Chem.* **2015**, *127*, 13412–13416.
[49] M. Mocenigo, A. Porchetta, M. Rossetti, E. Brass, L. Tonini, L. Puzzi, E. Tagliabue, T. Triulzi, B. Marini, F. Ricci, R. Ippodrino, *ACS Sens.* **2020**, *5*, 3109–3115.
[50] A. Bertucci, A. Porchetta, F. Ricci, *Anal. Chem.* **2018**, *90*, 1049–1053.
[51] S. Ranallo, D. Sorrentino, F. Ricci, *Nat. Commun.* **2019**, *10*, 5509.
[52] N. Li, L. Liu, M.-H. Xiang, J.-W. Liu, R.-Q. Yu, J.-H. Jiang, *Chem. Commun.* **2019**, *55*, 4387–4390.
[53] A. Patino Diaz, S. Bracaglia, S. Ranallo, T. Patino, A. Porchetta, F. Ricci, *J. Am. Chem. Soc.* **2022**, *144*, 5820–5826.
[54] M. Rossetti, S. Brannetti, M. Mocenigo, B. Marini, R. Ippodrino, A. Porchetta, *Angew. Chem. Int. Ed.* **2020**, *59*, 14973–14978; *Angew. Chem.* **2020**, *132*, 15083–15088.
[55] S. Bracaglia, S. Ranallo, K. W. Plaxco, F. Ricci, *ACS Sens.* **2021**, *6*, 2442–2448.
[56] K. Wang, Z. Tang, C. J. Yang, Y. Kim, X. Fang, W. Li, Y. Wu, C. D. Medley, Z. Cao, J. Li, P. Colon, H. Lin, W. Tan, *Angew. Chem. Int. Ed.* **2009**, *48*, 856–870; *Angew. Chem.* **2009**, *121*, 870–885.
[57] A. Vallée-Bélisle, A. J. Bonham, N. O. Reich, F. Ricci, K. W. Plaxco, *J. Am. Chem. Soc.* **2011**, *133*, 13836–13839.
[58] A. Bertucci, J. Guo, N. Oppmann, A. Glab, F. Ricci, F. Caruso, F. Cavalieri, *Nanoscale* **2018**, *10*, 2034–2044.

[59] A. Glab, A. Bertucci, F. Martino, M. Wojnilowicz, A. Amodio, M. Venanzi, F. Ricci, G. Forte, F. Caruso, F. Cavalieri, *Nanoscale* **2020**, *12*, 15402–15413.
[60] A. Bertucci, A. Porchetta, E. Del Grosso, T. Patiño, A. Idili, F. Ricci, *Angew. Chem. Int. Ed.* **2020**, *59*, 20577–20581; *Angew. Chem.* **2020**, *132*, 20758–20762.
[61] R. Aman, A. Mahas, M. Mahfouz, *ACS Synth. Biol.* **2020**, *9*, 1226–1233.
[62] Q.-L. Zhang, L.-L. Wang, Y. Liu, J. Lin, L. Xu, *Nat. Commun.* **2021**, *12*, 4654.
[63] M. Pfeiffer, K. Trofymchuk, S. Ranallo, F. Ricci, F. Steiner, F. Cole, V. Glembockyte, P. Tinnefeld, *iScience* **2021**, *24*, 103072.
[64] A. J. Bonham, K. Hsieh, B. S. Ferguson, A. Vallée-Bélisle, F. Ricci, H. T. Soh, K. W. Plaxco, *J. Am. Chem. Soc.* **2012**, *134*, 3346–3348.
[65] A. L. Furst, M. G. Hill, J. K. Barton, *Langmuir* **2013**, *29*, 16141–16149.
[66] A. L. Furst, N. B. Muren, M. G. Hill, J. K. Barton, *Proc. Natl. Acad. Sci. USA* **2014**, *111*, 14985–14989.
[67] E. M. Boon, J. E. Salas, J. K. Barton, *Nat. Biotechnol.* **2002**, *20*, 282–286.
[68] A. A. Gorodetsky, A. Ebrahim, J. K. Barton, *J. Am. Chem. Soc.* **2008**, *130*, 2924–2925.
[69] N. B. Muren, J. K. Barton, *J. Am. Chem. Soc.* **2013**, *135*, 16632–16640.
[70] F. Ricci, G. Adornetto, D. Moscone, K. W. Plaxco, G. Palleschi, *Chem. Commun.* **2010**, *46*, 1742.
[71] F. Ricci, A. J. Bonham, A. C. Mason, N. O. Reich, K. W. Plaxco, *Anal. Chem.* **2009**, *81*, 1608–1614.
[72] Y. Xiao, B. D. Piorek, K. W. Plaxco, A. J. Heeger, *J. Am. Chem. Soc.* **2005**, *127*, 17990–17991.
[73] C. Parolo, A. Idili, G. Ortega, A. Csordas, A. Hsu, N. Arroyo-Currás, Q. Yang, B. S. Ferguson, J. Wang, K. W. Plaxco, *ACS Sens.* **2020**, *5*, 1877–1881.
[74] Y. Xiao, A. A. Lubin, A. J. Heeger, K. W. Plaxco, *Angew. Chem.* **2005**, *117*, 5592–5595; *Angew. Chem. Int. Ed.* **2005**, *44*, 5456–5459.
[75] A.-E. Radi, J. L. Acero Sánchez, E. Baldrich, C. K. O'Sullivan, *J. Am. Chem. Soc.* **2006**, *128*, 117–124.
[76] R. Y. Lai, K. W. Plaxco, A. J. Heeger, *Anal. Chem.* **2007**, *79*, 229–233.
[77] A. Idili, C. Parolo, R. Alvarez-Diduk, A. Merkoçi, *ACS Sens.* **2021**, *6*, 3093–3101.
[78] F. Curti, S. Fortunati, W. Knoll, M. Giannetto, R. Corradini, A. Bertucci, M. Careri, *ACS Appl. Mater. Interfaces* **2022**, *14*, 19204–19211.
[79] A. Vallée-Bélisle, F. Ricci, T. Uzawa, F. Xia, K. W. Plaxco, *J. Am. Chem. Soc.* **2012**, *134*, 15197–15200.
[80] W. E. Arter, Y. Yusim, Q. Peter, C. G. Taylor, D. Klenerman, U. F. Keyser, T. P. J. Knowles, *ACS Nano* **2020**, *14*, 5763–5771.
[81] L. Qian, E. Winfree, J. Bruck, *Nature* **2011**, *475*, 368–372.
[82] D. Han, Z. Zhu, C. Wu, L. Peng, L. Zhou, B. Gulbakan, G. Zhu, K. R. Williams, W. Tan, *J. Am. Chem. Soc.* **2012**, *134*, 20797–20804.
[83] Y. Tang, L. Gao, W. Feng, C. Guo, Q. Yang, F. Li, X. C. Le, *Chem. Soc. Rev.* **2021**, *50*, 11844–11869.
[84] M. Darmostuk, S. Rimpelova, H. Gbelcova, T. Ruml, *Biotechnol. Adv.* **2015**, *33*, 1141–1161.
[85] J. Pande, M. M. Szewczyk, A. K. Grover, *Biotechnol. Adv.* **2010**, *28*, 849–858.
[86] A. P. Liu, E. A. Appel, P. D. Ashby, B. M. Baker, E. Franco, L. Gu, K. Haynes, N. S. Joshi, A. M. Kloxin, P. H. J. Kouwer, J. Mittal, L. Morsut, V. Noireaux, S. Parekh, R. Schulman, S. K. Y. Tang, M. T. Valentine, S. L. Vega, W. Weber, N. Stephanopoulos, O. Chaudhuri, *Nat. Mater.* **2022**, *21*, 390–397.
[87] A. Clifford, J. Das, H. Yousefi, A. Mahmud, J. B. Chen, S. O. Kelley, *J. Am. Chem. Soc.* **2021**, *143*, 5281–5294.

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