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Review Article

DNA Nanobiosensors: An Outlook on Signal Readout Strategies

Arun Richard Chandrasekaran

Confer Health, Inc., Charlestown, MA, USA

Correspondence should be addressed to Arun Richard Chandrasekaran; arunrichard@nyu.edu

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A suite of functionalities and structural versatility makes DNA an apt material for biosensing applications. DNA-based biosensors are cost-effective and sensitive and have the potential to be used as point-of-care diagnostic tools. Along with robustness and biocompatibility, these sensors also provide multiple readout strategies. Depending on the functionality of DNA-based biosensors, a variety of output strategies have been reported: fluorescence- and FRET-based readout, nanoparticle-based colorimetry, spectroscopy-based techniques, electrochemical signaling, gel electrophoresis, and atomic force microscopy.

1. Introduction

Biosensing is an area of research that has garnered attention due to its importance in medical diagnostics, biomolecular analysis, and studies involving molecular pathways. A variety of nanomaterials such as quantum dots, gold and silver nanoparticles, metallic nanowires, and carbon nanotubes have been developed for sensing applications [1–3]. These materials provide unique optical, electronic, chemical, and mechanical properties and contribute to both robust sensing and convenient and sensitive readout strategies. DNA is one other material that has found applications in biosensing. Over the past three decades, DNA has become a versatile material for bottom-up nanofabrication of two- [4] and three-dimensional lattices [5], nanoscale objects [6], and complex wireframe structures [7] with applications in biology [8], medicine [9], and materials science [10]. The structural aspects of DNA nanostructures combined with their ability to respond to stimuli have led to the creation of cost-effective and sensitive biosensors that are functional under a wide range of biologically relevant temperatures and conditions [11–14]. The recognition event in such biosensing approaches can be translated by specific readout strategies which are briefly discussed in this article in the following categories: (i) fluorescence, (ii) FRET, (iii) nanoparticle-based color change, (iv) electrochemical signaling, (v) gel electrophoresis, (vi) atomic force microscopy (AFM), and (vii) surface-enhanced Raman spectroscopy (SERS).

2. DNA Nanostructures for Biosensing

The field of DNA nanotechnology has seen the development of dynamic DNA machines [15] and devices [16] that respond to chemical or environmental stimuli. This concept is extended to DNA-based biosensors that rely on specific recognition events between a substrate and the target analyte (e.g., nucleic acid and protein detection) or programmed conformational changes (e.g., pH sensing). DNA nanostructures have some advantages for being used in biosensing. The intermolecular recognition of rationally designed DNA sequences allows highly precise design and construction of DNA nanostructures. The nanoscale dimensions of these structures provide large surface-to-volume ratios, thus resulting in large signal changes on target binding. DNA sequences can now be synthesized in large quantities in a cost-effective manner and can be chemically modified to enhance their functionality.

A majority of nucleic acid-based biosensors involve hybridization of a DNA or RNA strand to its complement (Figure 1(a)), or a complementary region in a stem loop (Figure 1(b)). Such stem-loop configurations can also be used to monitor global environmental changes such as changes in temperature by using a fluorophore-quencher pair on the ends of the strands. Biosensors for other stimuli such as pH changes are based on structures that involve triplex [17] (Figure 1(c)), i-motif formation [18] (Figure 1(d)), or poly-dA helix formation (Figure 1(e)) [19], all of which are

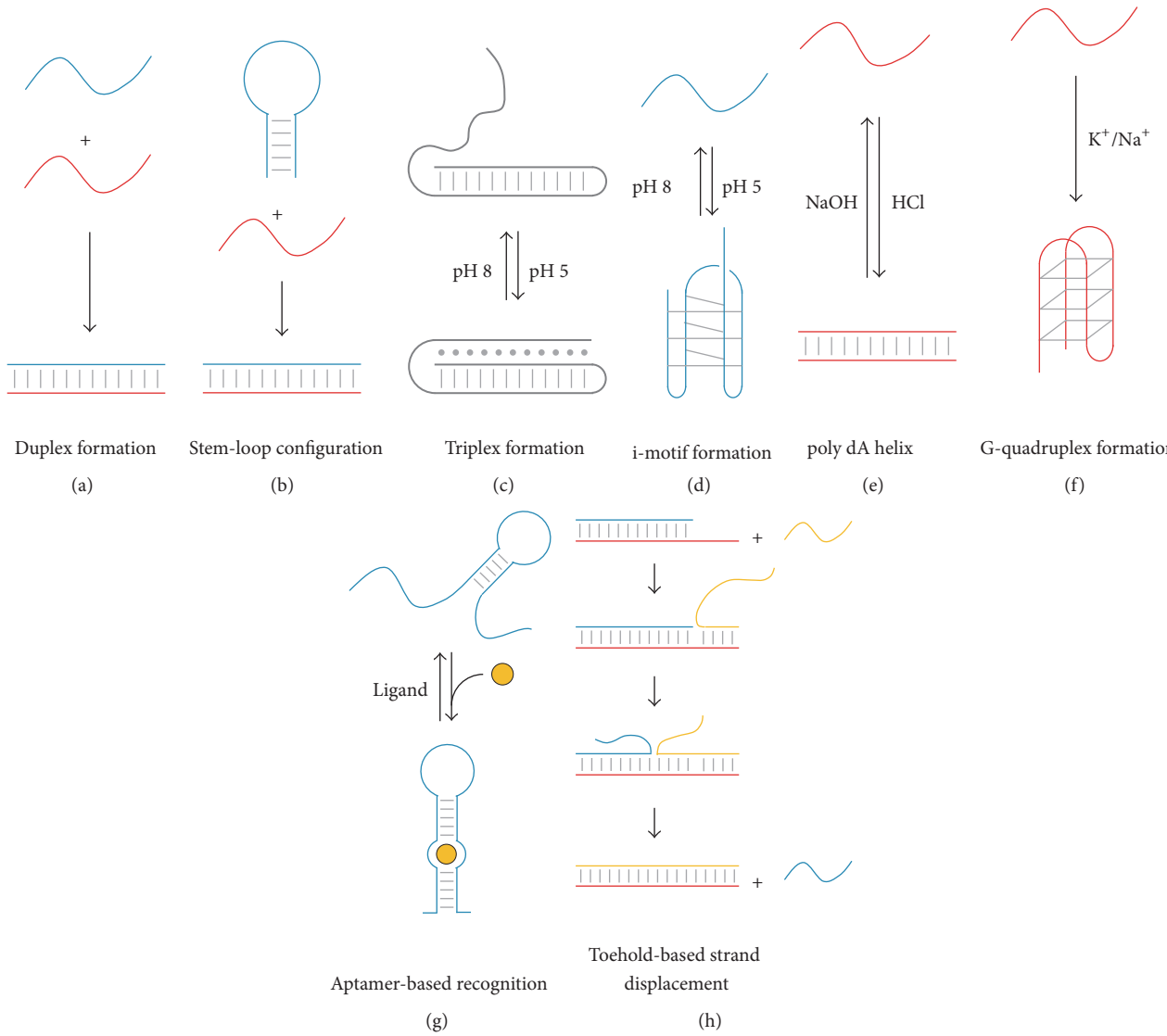

Figure 1: Concepts used in biosensing platforms. (a, b) Sequence-specific DNA hybridization in a duplex and stem-loop configuration, (c) triplex formation under acidic conditions, (d) i-motif formation under acidic conditions by C-rich DNA strands, (e) poly-dA helix formation under acidic conditions by poly-A strands, (f) G-quadruplex formation in the presence of K^+ or Na^+, (g) aptamer reconfiguration in the presence of specific ligands, and (h) toehold-based strand displacement.

sequence-specific and occur under acidic conditions. G-quadruplex formation is another sequence-specific conformational change that forms under specific ionic conditions such as the presence of K^+ or Na^+ [20] (Figure 1(f)). In addition, aptamer-based recognition has also been widely used in biosensors (Figure 1(g)). These are single-stranded oligonucleotides that can bind with high affinity to ions (e.g., K^+, Hg^{2+}, and Pb^{2+}), small organic molecules (e.g., ATP, amino acids, and vitamins), peptides, proteins (e.g., thrombin and growth factors), and even whole cells or microorganisms (e.g., bacteria) [21, 22], resulting in a secondary structure formation. Toehold-based strand displacement [23] (Figure 1(h)) has also been used for target readouts and signal amplification in many DNA-based biosensing platforms.

2.1. Fluorescence-Based. Conformational changes in a DNA nanostructure can be observed by tagging the component DNA strands with a fluorophore-quencher pair. For example, if a fluorophore and quencher are attached to two ends of a single strand forming a stem loop, the fluorescence is quenched in the stem-loop configuration due to the close proximity (<10–100 Å) of the fluorophore to the quencher (Figure 2(a), top). Change in the stem-loop configuration moves the fluorophore away from the quencher, thus increasing the fluorescence. This strategy has been used in a DNA-based beacon for the detection of antibodies and proteins [24]. This molecular switch was composed of a stem-loop system comprising a long strand that contained the loop and two short complementary strands with single-stranded tails. The ends of these tails were modified to contain an

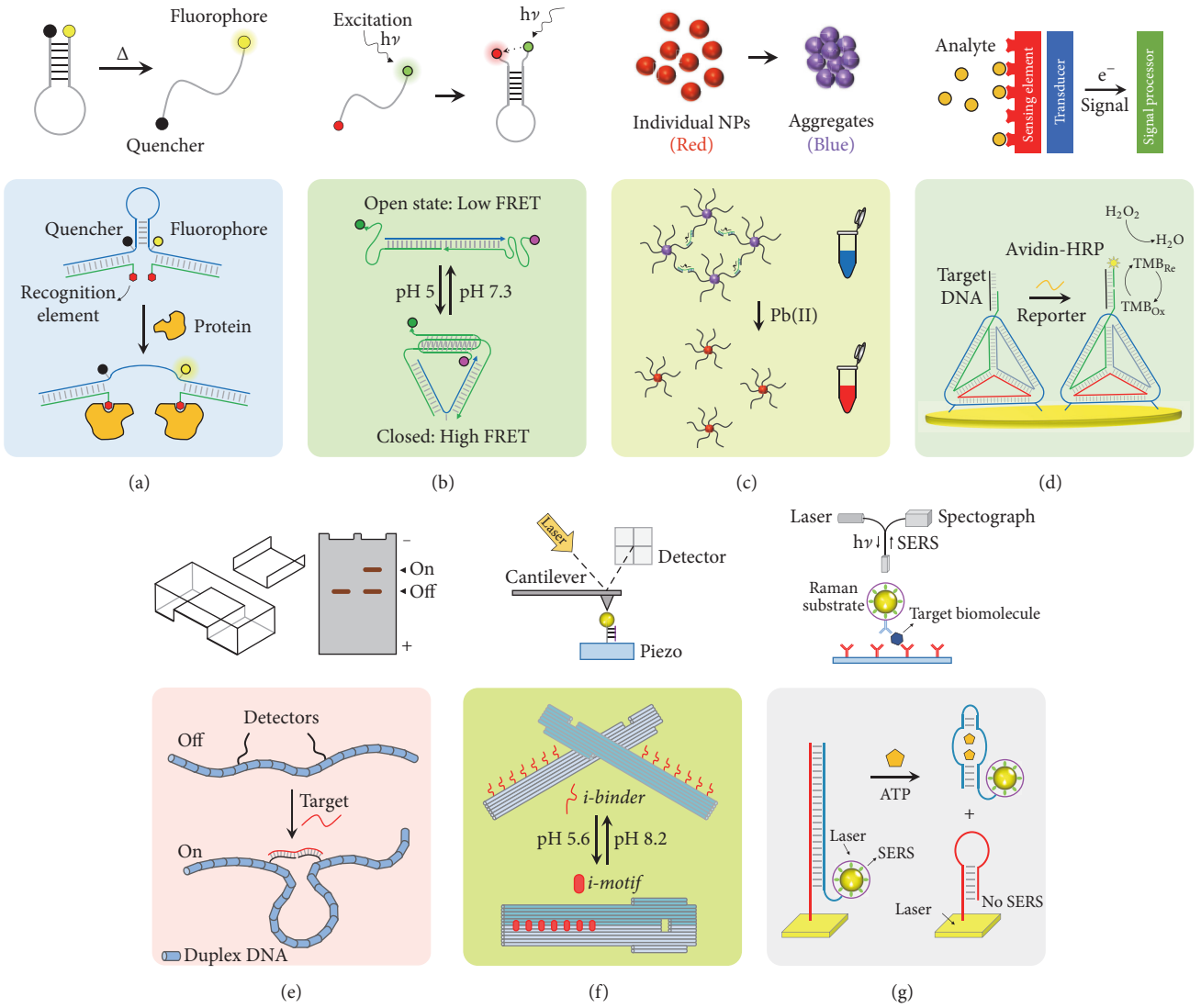

Figure 2: Readout strategies for DNA nanostructure-based biosensors. (a) Fluorescence-based readout: the example shown demonstrates the detection of proteins resulting in stem-loop reconfiguration leading to a fluorescent signal [24]. (b) FRET-based readout: The example shows a DNA nanodevice containing a FRET pair on opposite ends of a nicked duplex. The C-rich single-stranded extensions on either end of the duplex can form an i-motif at low pH resulting in a FRET signal [18]. (c) NP-based color change: nanoparticles aggregated via DNA strands and a DNAzyme are blue; presence or addition of Pb^{2+} ions causes cleavage of the DNAzyme resulting in nanoparticle disaggregation and a change in color to red [25]. (d) Electrochemical readout: DNA tetrahedra with single-stranded pendants can bind partially to target DNA. The remainder of the target DNA strand can bind a reporter strand that produces a HRP-based electrochemical readout [26]. (e) Gel electrophoresis: a DNA nanoswitch containing two single-stranded overhangs that are partially complementary to target DNA. Binding of target DNA to the two detectors causes the linear "off" state to change into a looped "on" state. The two states of the nanoswitch migrate differently on a gel, thus providing a digital on-off signal [27]. (f) AFM-based readout: DNA origami levers that contain C-rich single-stranded extensions can act as pH sensors. In acidic pH, the single-stranded extensions on each half of the lever can form an intermolecular i-motif causing a conformational change that can be visualized on an AFM [28]. (g) SERS-based readout: an ATP-binding aptamer is bound to a single-stranded probe on a gold surface. The presence of ATP triggers conformational change of the aptamer causing it to dissociate from the probe, resulting in a loss of the SERS signal [29].

appropriate target-specific recognition element (e.g., digoxigenin). The stem region contained a fluorophore/quencher pair and the fluorescent signal is quenched as long as the stem remains closed (Figure 2(a), bottom). Binding of a dig-specific antibody to both recognition elements (bivalent binding) pushes them away, thus opening the stem region and in turn causing enhanced fluorescence. This sensor was used to detect a variety of antibodies and protein targets including the HIV biomarker anti-p17 antibody.

Fluorescence-based biosensors have also been used for pH detection. One such example involves C-rich DNA strands containing a fluorophore on one end attached to a gold surface [30]. At acidic pH, the strands form an intramolecular i-motif, bringing the fluorophore closer to the

gold surface and essentially quenching it. At basic pH, the single strands can bind to a complementary strand pushing the fluorophore away from the gold surface, thereby enhancing the fluorescence. A similar example used a graphene surface instead of gold and worked on the basis of pH-dependent triplex formation [31]. In addition, solution-based triplex-forming nanoswitches have also been developed for pH detection [17]. This switch was designed so that the fluorophore-quencher pair remains closer when the switch forms a triplex, acting as an indicator of the pH range. Another example of fluorescence-based readout was DNA-tweezer nanostructures that were designed to contain restriction sites specific to endonucleases [32]. The presence of these endonucleases causes cleavage of component strands resulting in an increased fluorescent signal.

2.2. FRET-Based. Structural transitions in DNA nanostructures have been analyzed using Fluorescence Resonance Energy Transfer (FRET), in which fluorescence signals are generated for molecular association and separation in the 1–10 nm range (Figure 2(b), top) [33]. One such example is a DNA nanomachine based on an intramolecular i-motif that has been used as a pH sensor inside living cells [18]. The basis of this machine was a conformational change from an open linear structure under physiological conditions (pH 7.3, low FRET) to a closed triangular structure under acidic conditions (pH 5.0, high FRET) (Figure 2(b), bottom). This switch was effective in pH ranges 5.5 to 6.8 and was used to map spatial and temporal pH changes associated with endosome maturation in *Drosophila* hemocytes [18] as well as inside a multicellular organism *(Caenorhabditis elegans)* [34]. A similar strategy was also used to simultaneously map the pH gradients along two different but intersecting endocytic pathways inside the same cell [35]. In another example, the edges of a DNA tetrahedron were designed to contain dynamic sequences that are specific to adenosine triphosphate (ATP) [36]. These regions undergo a conformational change in the presence of ATP and were used to detect intracellular ATP via a FRET signal.

2.3. Nanoparticle-Based. Metallic nanoparticles (NPs) have been shown to exhibit defined color changes between individual nanoparticles and aggregated clusters [37, 38]. This characteristic has been used in colorimetric assays based on DNA-functionalized gold nanoparticles (AuNPs) that provide an optical readout (visual color change) (Figure 2(c), top). One such example is the DNAzyme-mediated self-assembly of AuNPs that was used in the detection of Pb(II) ions [25]. The DNAzyme used in this case consists of an enzyme strand and a substrate strand. The substrate strand was extended on both ends with sequences complementary to the single strands on the AuNPs. Hybridization of these two DNA strands results in aggregation of the AuNPs (blue color). On addition of Pb(II), the enzyme strand catalyzes cleavage of the substrate strand resulting in disaggregation of the AuNPs, thereby resulting in a color change to red (Figure 2(c), bottom). This visual color change acts as an indicator of the presence or absence of Pb(II) ions. Another example that uses NP-based visual color change is a lateral flow nucleic acid biosensor for detecting nucleic acid sequences [39]. In this case, AuNPs were modified to contain biotin-tagged DNA strands that were complementary to a target DNA strand. Target-bound DNA-AuNP conjugates get accumulated on a streptavidin coated test line, thus resulting in a color change (red) of the test line.

2.4. Electrochemical Readout. DNA-based electrochemical sensors use nanoscale interactions between the target and a recognition layer and the signal is transduced (e.g., by enzyme activity) via a solid electrode surface (Figure 2(d), top). DNA tetrahedron-based biosensors combined with surface-based assays have been used for electrochemical detection of nucleic acids [26]. In this design, the bottom three vertices of the tetrahedral DNA probe were bound to a gold electrode surface via thiol modifications. The fourth vertex was designed to contain a DNA strand that is complementary to part of the target (Figure 2(d), bottom). When part of the target binds to the probe, a biotinylated reporter probe binds to the remaining part of the target. This hybridization event is then transduced into electrochemical signals through the specific binding of an avidin-HRP (horseradish peroxidase) conjugate to the biotin, leading to enzyme turnover-based signal transduction. A similar strategy, combined with multi-branched hybridization chain reaction (mHCR) for improved sensitivity, was used for cancer cell detection [40]. Moreover, by conjugating the DNA tetrahedral probe to an antibody (e.g., tumor necrosis factor alpha), the strategy has been redesigned for immunological sensing as well [41]. In another example, electrochemical sensing of HIV DNA has been done using long-range self-assembled DNA constructs [42]. In this case, the output signal was based on the accumulation of hexaammineruthenium(III) chloride (RuHex) on the negatively charged phosphate backbone of the DNA via electrostatic forces.

Electrochemical aptamer-based sensors have been developed for the detection of proteins, small molecules, and inorganic ions [43]. In this case, an aptamer probe containing an electrochemical redox reporter molecule is attached onto a gold electrode. Target binding induces a conformational change of the aptamer, thus altering the position of the reporter relative to the electrode, yielding a measurable current change. Such a strategy has been used to construct a real-time biosensor capable of continuously tracking doxorubicin (a chemotherapeutic) and kanamycin (an antibiotic) in live rats and in human whole blood [44, 45]. This strategy has also been used to detect specific proteins [46] and antibodies [47] directly in undiluted blood serum.

2.5. Gel Electrophoresis-Based. Gel electrophoresis is the most ubiquitous technique in a biology or biochemical laboratory (Figure 2(e), top). DNA nanoswitches have been designed for analysis of biomolecular interactions such as biotin-streptavidin, antibody-antigen, peptide ligation, and restriction enzyme cleavage [48]. These events result in a conformational change of the nanoswitch that can be analyzed through gel electrophoresis. This strategy was recently adapted for the detection of specific nucleic acid sequences [27]. The *off* state of the nanoswitch is a linear duplex formed

by a single-stranded M13 scaffold and a set of staple strands. Two of the staple strands were modified to contain single-stranded extensions (detectors), each of which binds to parts of the target. Hybridization of the target oligonucleotide to the detectors reconfigures the switch to form a loop, thus changing it to the *on* state (Figure 2(e), bottom). The "off" and "on" states of the DNA nanoswitches migrate differently on an agarose gel. Gel-shift assays are routinely used in laboratories, and this strategy provides a relatively easy and one-step method to detect target nucleic acids by the appearance of the "on" band.

2.6. AFM-Based. Atomic force microscopy (AFM) is frequently used to analyze two-dimensional constructs made from DNA (Figure 2(f), top). The DNA origami technique [49] provides a convenient route to the assembly of such two-dimensional platforms that allow the arrangement of functional moieties. For example, DNA origami sheets containing single-stranded DNA probes complementary to a target sequence can act as molecular chips for detecting the presence of the target oligonucleotide [50]. Hybridization of the probe tiles to the target in solution was detected using AFM, based on the difference in elastic properties of single-stranded (probes without target) and double-stranded DNA (probes bound to target). AFM readout is more pronounced in structures that change conformation or lead to a visual marker on target interaction. In one such example, DNA origami was used to construct a "nanoplier" containing C-rich sequences (i-binders) on each lever of the plier [28]. Under acidic conditions, these sequences form an intermolecular i-motif, thereby bringing the two levers together (Figure 2(f), bottom). This structural transition can be visualized using AFM. In another example, DNA origami tiles with specific topological markers were used to detect single nucleotide polymorphisms (SNPs) producing a direct visual readout of the target nucleotide contained in the probe sequence [51]. The platform contained graphical representations of the four nucleotides A, T, G, and C, and the symbol containing the test nucleotide identity disappears in the presence of the target.

2.7. SERS-Based. Surface-enhanced Raman scattering (SERS), a variation of standard Raman spectroscopy, provides a significantly enhanced Raman signal through electromagnetic interaction between the analyte molecules and metal surface [52, 53]. In typical SERS assays, Raman reporters are attached to the surface of metallic nanoparticles (the SERS substrates) and covered by a protective shell (Figure 2(g), top) that prevents leaching out of the Raman reporters and improves water solubility and stability [54, 55]. In one such example, an aptamer-based biosensor was designed to detect ATP using SERS [29]. This study used malachite green isothiocyanate as the Raman reporter which was sandwiched between a gold nanostar core and a silica shell. The aptamer probe, specific to ATP, was immobilized on a gold surface by hybridization to complementary single-stranded DNA that is attached to the gold surface (Figure 2(g), bottom). Binding of ATP to the aptamer causes it to fold, thereby detaching from its complementary strand. This duplex dissociation causes a reduction in the SERS signal, thus acting as a detection mechanism for ATP molecules.

Spatial control of plasmonic nanoparticles using rigid DNA nanostructures allows the creation of distinct structure-dependent optical features [56]. One such example is the use of a DNA tetrahedron to control the positioning of AuNPs [57]. By using thiol-modified DNA strands that can self-assemble into a tetrahedron, the structure can be used to recruit 20 nm AuNPs on each of its four vertices. In addition, the DNA tetrahedron was designed to contain Cy3 molecule, a Raman active dye, on one of its edges. This structure was further coated with silver to form Ag-Au nanoshells. The system was used to detect single-stranded DNA that was complementary to the component strands of the tetrahedron. Target addition causes formation of duplexes by hybridization of the component strands with the target, resulting in disassembly of the NP cluster, therefore causing a different SERS signal for the duplexes. Another example is a DNA origami platform that was used to assemble 40 nm AuNP dimers with sub-5 nm gaps between them [58]. The origami platform provides a strong plasmonic coupling between the NPs and this system was used to attain SERS measurements of specific single-stranded DNA molecules. Depending on the sequences of the single-stranded DNA that is coated on the surface of the NP dimers, specific SERS spectral peaks are attained that can be used to detect or identify specific DNA sequences.

3. Discussion and Outlook

Multidimensional DNA nanostructures have been shown to be useful as frameworks for precisely programmed arrangement of functional molecules such as ligands, enzymes, and chemical groups [59, 60]. These strategies involve sequence-specific recognition of a DNA nanostructure [61] or covalent linkage of a functional moiety on the DNA strand [62]. Such site-specific positioning of biomolecules allows these structures to be used as biosensing platforms for a variety of target analytes. Moreover, the ability to design triggered responses to a variety of external chemical and biological stimuli makes DNA-based devices versatile for biosensing. Such stimuli responsive structures can be not only used as biosensors but also configured to react to specific biomarkers and release cargos from macromolecular containers [63]. For example, a recent enzyme-powered DNA-AuNP nanomachine was used to release payloads while also serving as a biosensor for nucleic acid detection [64]. Such nanostructures that can act according to stimuli can be used as "sense-and-treat" devices for theranostic applications [65]. Sensing capabilities can also be combined with computing platforms for this purpose [66].

The dynamic nature of such programmable DNA devices plays a major role in the development of robust and sensitive molecular sensing that is functional at the nanoscale while providing a convenient signal readout. The potential use of a specific biosensing strategy is reliant on factors such as assay/readout time, skill required to perform the assay, the amount of sample required and the dynamic range and sensitivity, and the cost of the method. Considering these factors, the use of AFM potentially limits the practicality

of such biosensors due to the equipment cost and the requirement of skilled personnel. Furthermore, AFM can only be used to read out surface-based assays or those involving 2D DNA nanostructures and requires visualizing multiple fields of the sample (usually deposited on a mica surface) to yield quantitative results. The gel-based readout such as the one using DNA nanoswitches provides a simple assay for research laboratories to identify the presence of a target nucleic acid without requiring multiple mixing steps or enzymatic amplification. This method is currently limited to laboratory usage but can be extended to point-of-care testing by using bufferless gel systems and portable electrophoresis units. Moreover, the cost of gel-based assays is much cheaper as it only requires already existing equipment in a lab. Nanoparticle-based assays, both optical and SERS, have been very successful in developing point-of-care diagnostics with a relatively easier assay and quicker readout. One notable platform is the lateral flow assay which has been frequently used in clinical diagnostics with a simple visual readout (a colored test line) compared to a standard (a control line). These tests do not require any equipment and provide the end user with a "yes or no" answer.

One limitation of current biosensing strategies is the use of multiple steps for signal generation and amplification which increases the time required for detection. In addition, DNA nanostructures used for sensing purposes have to withstand the different solution conditions while being intact during detection of biomarkers in vivo [67]. Specifically, these structures are prone to degradation by nucleases in biological systems. Recent chemical strategies that provide a solution to this problem include the use of a phosphorothioate backbone [68], locked nucleic acids (LNA) [69], L-DNA [70], $5'/3'$ modifications including hexaethylene glycol (HEG), hexane diol (C6) and $5'$-phosphate (P) [71], and other xenonucleic acids [72]. Previous research has shown DNA nanostructures to be stable in cell lysates [73] and the integrity of these structures in tissue culture environment has been analyzed [74]. It would be useful to analyze the stability of these structures in a variety of environments (e.g., different cell types, normal versus tumorous tissues) so that the biosensors can be tailored for optimal responses. Future work on DNA-based chemical and biological sensors will especially aid in the characterization and development of these structures for in situ sensing. With recent developments in DNA-PAINT (a variation of point accumulation for imaging in nanoscale topography) [75, 76], it is possible to create DNA nanostructures that can signal the presence of specific biomarkers in vivo. Other recent developments in this front include DNA nanothermometers based on DNA clamp architectures that are useful for temperatures in the range of 30°C to 85°C [77]. In addition, DNA origami structures have been combined with solid-state nanopores for detection of λ-DNA molecules [78], opening up a new route to single molecule detection of biomolecules.

Self-assembly techniques, especially DNA origami, have made the construction of nanoscale objects easier. In addition, the cost involved in the preparation of DNA nanostructures has reduced in recent times with synthetic oligonucleotides being able to be simply ordered from a company. Recent research has shown that the cost of synthetic DNA can be reduced further to as low as \$0.001 per base pair [79]. Moreover, custom-tailored DNA scaffolds now allow the construction of DNA origami structures of different sizes and are not limited to the frequently used M13 single strand [80]. With the aid of suitable purification methods [81–85], these nanostructures can be prepared in pure forms that provide enhanced sensitivity. DNA, being a biomolecule, also provides an advantage of being biocompatible [86] and can be useful for biosensing in combination with biomimetic approaches. Thus, designed DNA architectures provide a route to the creation of highly sensitive biosensors, with minimal cost and high assembly efficiency, with a selection of output strategies for varying purposes.

Conflicts of Interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

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