

Engineering DNA quadruplexes in DNA nanostructures for biosensor construction

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Received: 16 July 2021 / Revised: 28 August 2021 / Accepted: 4 September 2021

ABSTRACT

DNA quadruplexes are nucleic acid conformations comprised of four strands. They are prevalent in human genomes and increasing efforts are being directed toward their engineering. Taking advantage of the programmability of Watson–Crick base-pairing and conjugation methodology of DNA with other molecules, DNA nanostructures of increasing complexity and diversified geometries have been artificially constructed since 1980s. In this review, we investigate the interweaving of natural DNA quadruplexes and artificial DNA nanostructures in the development of the ever-prosperous field of biosensing, highlighting their specific roles in the construction of biosensor, including recognition probe, signal probe, signal amplifier and support platform. Their implementation in various sensing scenes was surveyed. And finally, general conclusion and future perspective are discussed for further developments.

KEYWORDS

G-quadruplex, i-motif, DNA nanotechnology, biosensor

1 Introduction

The DNA secondary structure initially proposed by J. Watson and F. Crick was the right-handed B-form double-helix which complies with adenine (A)–thymine (T) and guanine (G)–cytosine (C) base pairing [1]. Later on, however, a variety of nucleic acid conformations have been revealed disobey the Watson–Crick canon, including G-quadruplexes and intercalated motif (i-motif) structures (Fig. 1) [2, 3]. They are four-stranded structures formed respectively by G-rich and C-rich sequences. DNA sequences with the potential to fold into G-quadruplex and i-motif structures are prevalent along the human genome. For instance, Waller et al. identified 5,125 sequences bearing four C-tracts of 5 cytosines from the genome, 637 of which are located in gene promoter regions [4]. Similarly, more than 700,000 distinct G-quadruplex structures were detected in the human genome by high-throughput sequencing, with a notable amount of which enriched within gene promoters and at loci often amplified in cancers [5]. Substantial evidence now exists to support that the two quadruplex structures play critical roles in gene expression regulation, telomere maintenance, and DNA repair [6, 7].

While Nature give birth to several different nucleic acid secondary structures, even more impressive DNA superstructures of higher structural complexity have been artificially designed and fabricated. This originated from the alternative view of considering DNA as building blocks for the construction of nanoscale architectures, rather than merely the genetic material of living organisms, which has been widely accepted in the last decades

since the pioneer work of Seeman on artificial nucleic acid lattices [8]. The big family of artificial DNA nanostructures now comprises many members, including a cube [9], a tetrahedron [10], polyhedral [11], large DNA “origami” folds [12] or assemblies [13, 14], and spherical nucleic acids (SNA) [15, 16]. The DNA cube, tetrahedron and polyhedra are cage-like structures containing double-helical edges and hollow faces. They are constructed from minimal numbers of DNA strands of designed sequence complementarity. In DNA origami, a long single-stranded DNA (ssDNA) is folded by hundreds of short ssDNAs along prescribed paths to form predesigned structures. And typical SNAs are core-shell structures, with a dense layer of nucleic acids covalently attached to spherical nanoparticle cores.

From the perspective of the DNA nanotechnology community, DNA quadruplex structures are no longer gene fragments executing biological functions, but rather reconfigurable structural units for the construction of functional nanostructures. Benefiting from excellent properties such as inherent biocompatibility, high enzymatic resistance, high mechanical rigidity, and near-atomic spatial organization capacity, DNA nanostructures provide a promising toolkit for engineering natural DNA quadruplex nucleic acids modules. This review revolves around the cooperation and interplay of the two groups of nucleic acid assemblies, i.e., naturally occurring DNA quadruplexes and artificial DNA nanostructures, in recent development of DNA-based biosensors, with an emphasis on examining their functionalities in every design. Firstly, biosensors can be readily constructed with DNA quadruplex acting as recognition probe and static DNA nanostructure as a platform or carrier. Secondly,

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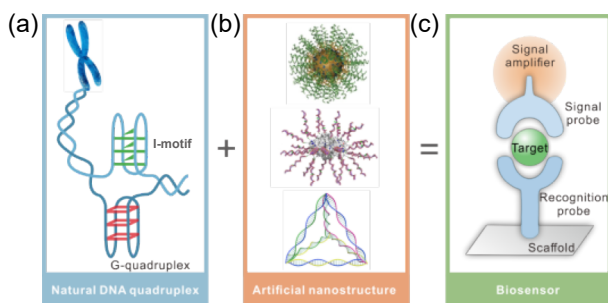


Figure 1 Biosensor construction using natural DNA quadruplexes and artificial DNA nanostructures. (a) DNA quadruplex conformations in human genomes. Base pairs in G-quadruplex and i-motif conformations were colored red and green, respectively. (b) Typical DNA nanostructures are shown here: spherical nucleic acids, and a tetrahedral DNA assembled from four strands. Reproduced with permission from Refs. [15, 16], © The American Chemical Society 2012 and 2020. (c) Schematic of a typical biosensor that consisted of four important components: a platform or scaffold, a recognition probe to capture the target, a signal probe to generate measurable signals, and a signal amplifier to improve detection limit of the signal. Both i-motif and G-quadruplex are able to act as recognition probe in a sensor, while G-quadruplex can function as signal probe when complexed with hemin. Further anchoring of multiple G-quadruplex-hemin complexes on a single DNA nanostructure forms a signal amplifier.

Table 1 DNA nanostructure-scaffolded DNA quadruplex sensors

Sensor components	DNA nanostructure	Functionality	Application	References
DNA nanostructure as carrier; DNA quadruplex as recognition probe	SNA	Dual-fluorophore-labeled monomolecular i-motif-forming strands delivered into cells	Intracellular pH sensor	[20]
		Monomolecular G-quadruplex delivered into cells and liberated from SNA by NIR irradiation	Intracellular K ⁺ sensor	[21]
		Thiazole orange molecules intercalated in folded i-motifs generated enhanced fluorescence	Intracellular pH sensor	[16]
	DNA tetrahedron	pH sensitivity of dimeric i-motif sensors was increased by preorganization of DNA tetrahedron	Intracellular pH sensor	[22]
	DNA triangular prism	Fluorescence change by conformational switching of monomolecular i-motif in acidic organelles	Intracellular pH sensor	[23]
DNA nanostructure as scaffold; DNA quadruplex-hemin complex as signal probe		Fluorescence enhancement in the coexistence of proton and ATP in lysosome	Subcellular imaging K ⁺ sensor	[24]
	DNA origami	Fluorescence change by K ⁺ -responsive structural change	sensor	[25]
	DNA tetrahedron	Regulation of electrochemical catalytic properties of heme-DNAzyme by DNA tetrahedron	Electrochemical sensor	[26]
	DNA nanotriangle	Formation of dimeric G-quadruplex incorporated in the edge of DNA tetrahedron depending on ATP	Electrochemical ATP sensor	[27]
	DNA rotaxane	Dimerization of DNA nanotriangle depending on K ⁺	Catalysis	[28]
Multiple DNA quadruplex-hemin complexes-loaded SNA as signal amplifier		Allosteric control of the formation of heme-DNAzyme by a DNA rotaxane nanostructure	Catalysis	[29]
		Signal amplification by heme-SNAzyme in chemiluminescence imaging of microRNA	Point-of-care diagnosis	[30]
	SNA	Signal amplification by heme-SNAzyme in electrochemiluminescence detection of microRNA	Early-stage diagnosis	[31]
		Target-induced construction of heme-SNAzyme in ultrasensitive chemiluminescence microRNA detection	Point-of-care diagnosis	[32]

cells [18, 19]. In the DNA nanotechnology field, they have often been utilized as functional elements owing to their peculiar conformations, properties and functionalities relative to canonical duplex. Firstly, they exhibit unique base pairs. DNA i-motifs adopt intercalated C – cytosinium (CH⁺) base pairs stemming from the protonation of N-3 on C, and G-quadruplexes are formed by alkali metal-induced stacking of G tetrads held by Hoogsteen base pairs. Secondly, they can exist in both intra- and inter-molecular conformations, which leads to three strategies to perform conformational switching upon proton or metal ions stimulation (Fig. 2): (a) Inducing a monomeric quadruplex to transition between intramolecular and random coil conformations. (b) Collision of two strands to form an intermolecular conformation, and their separation reforms random coils. (c) Introducing a partly complementary strand to destruct the folded intramolecular

G-quadruplex can exist as signal probe when complexed with hemin. Thirdly, anchoring multiple hemin-complexed G-quadruplexes on a DNA nanostructure can give rise to the function of signal amplification. Besides, in addition to merely acting as carrier, DNA nanostructures are capable of modulating the catalytic/sensing property of hemin-complexed G-quadruplex via spatial arrangement of its position. An overview of biosensors fabricated by DNA quadruplexes and artificial DNA nanostructures in the past few years is summarized in Table 1. These biosensors were built toward targets ranging from ions, small molecules to bio-macromolecules working in specific scenes both *in vivo* and *in vitro*. In addition, these approaches of cooperation between DNA quadruplexes and artificial DNA nanostructures hold wider implications for constructing biosensors using materials beyond nucleic acids.

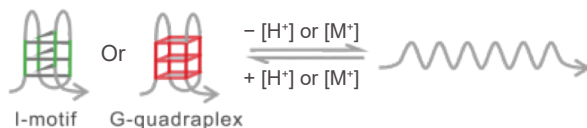
2 Characteristics of naturally occurring DNA quadruplexes and artificial DNA nanostructures

DNA quadruplex conformations have been historically first found by fiber diffraction, characterized *in vitro* via nuclear magnetic resonance (NMR) spectra and X-ray crystallography [17], and more recently visualized in the nuclei of living human

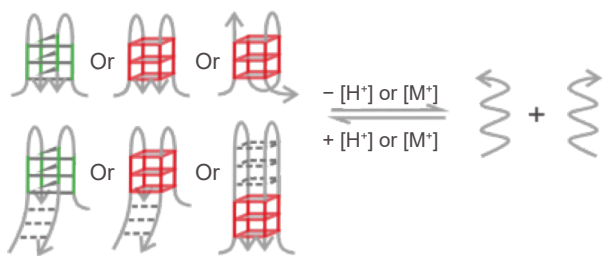
conformation, and its release reforms the intramolecular conformation. It's noteworthy that in strategy b, short duplex or hairpin need to be incorporated in the intermolecular quadruplexes, under some circumstances, to endow them with enhanced hybridization directionality and thermal stability, as well as to prevent the formation of undesired structures. These conformational switching strategies of DNA quadruplex provide appropriate structural changes in a biosensor for a specific output mechanism. Thirdly, multiple folding arrangements (relative orientations for adjacent strands) can be adopted by these multi-stranded nucleic acids. G-quadruplexes display at best the topological complexity: either two, three or all of the four strands can orient in the same direction, and the rest in the opposite. I-motifs, however, adopt a fixed antiparallel orientation to allow the intercalation of two parallel-stranded duplex components.

Detailed descriptions on topologies of i-motif and G-quadruplex have been summarized in some excellent literatures [33–35]. Lastly, their stabilities are dependent on their sequence compositions (e.g., stem's composition and length, loop's size, composition and connectivity, and possible additional interactions among bases and phosphodiester backbones) [36–39], and meanwhile influenced by a number of external factors, such as pH [4, 40], temperature [41], ionic strength [42, 43], noble metals [44, 45], and molecular crowding [46–48]. The high degree of DNA quadruplex conformational polymorphism and topological diversity, and their dependences on multifold factors greatly enriched the toolbox of the ever-developing DNA nanotechnology field.

(a) Intramolecular conformation-random coil switching:



(b) Intermolecular conformation-random coil switching:



(c) Intramolecular conformation-duplex switching:

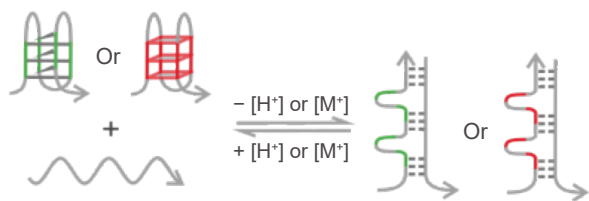


Figure 2 Three strategies of inverting DNA quadruplex conformations upon proton or metal ions stimulation. The arrows of the strands indicate the direction of oligonucleotides from 5' to 3'. C-tracts and G-tracts are colored green and red respectively. Please note only one topology out of many is adopted in this figure for clarity (i.e., 5'E intercalation topology for i-motif, antiparallel chair-type topology for G-quadruplex), but the strategies shown here can be readily applied to other topologies. In strategy b, different bimolecular quadruplex conformations found in DNA nanostructures have been included: i-motif of symmetric 2 + 2 strand stoichiometry [49], G-quadruplex of symmetric 2 + 2 strand stoichiometry [50], G-quadruplex of asymmetric 1 + 3 strand stoichiometry [29], short duplex assisted bimolecular i-motif [51, 52], short duplex assisted bimolecular G-quadruplex [53] and small hairpin assisted bimolecular G-quadruplex [28].

Taking advantage of the high fidelity and programmability of duplex base pairing, a large number of DNA nanostructures have been fabricated artificially in the past decades. They are readily implementable in various application scenes, exhibiting superior properties in the following aspects. Firstly, DNA nanostructures are inherently biocompatible and little cytotoxicity has been observed for them [54]. Secondly, they are resistant against nuclease degradation, allowing them to retain the intact structure in biological media. For instance, DNA tetrahedron remained intact after 4 h incubation in 50% non-inactivated fetal bovine serum, showing superior enzymatic resistance compared with linear duplex which was almost completely degraded within only 2 h incubation [54]. Similarly, increased stability was seen for SNA

as compared to the linear form, because the high surface-negative charge of the nanoparticles resulted in high local salt concentrations which retarded enzymatic hydrolysis [55]. Thirdly, as for applications in living cells, favorable cellular entry efficiency was observed for artificial DNA nanostructures. Turberfield's group found DNA tetrahedron could enter cultured mammalian cells without the aid of a transfection reagent [56]. Its structural integrity within cells was remained substantially intact up to at least 48 h. Mirkin's group found the cellular uptake of SNA was significantly higher than that of the linear counterpart in the absence of additional transfection reagents, as a result of enhanced binding of class A scavenger receptors on the cell surface [57]. Lastly, they exhibit persistence length 4–6 orders of magnitude longer than that of single-stranded (ss) and double-stranded (ds) DNA, giving rise to high mechanical stiffness and thermal stability [58]. This mechanical rigidity allows artificial DNA nanostructures to present in the exact predesigned shapes, which made possible the spatial organization of chemical entities on the DNA nanostructure scaffold with near-atomic precision [59, 60].

3 DNA nanostructure-scaffolded sensors with DNA quadruplex as recognition probe

The folding of DNA i-motif and G-quadruplex rely on protonation and metal-chelating respectively, which endows them with the ability to detect protons and metal ions. Cells and organelles strictly maintain appropriate proton and metal ion gradients to function properly. Aberrant pH alterations are associated with apoptosis and cell proliferation, leading to the development of cancer [61]. Among the metal ions existing in human body, potassium ions (K^+) play a significant role in multiple biological processes including nerve transmission, heartbeat, enzyme activation, as well as renal function [62]. Deviation from potassium ion balance in body fluids results in diseases such as stroke, hypokalemia, and high blood pressure. Therefore, detecting and visualizing protons and K^+ in biological media will be very useful in dissecting and interfering these processes. However, the intrinsic weaknesses of ssDNA and DNA helix, such as liability to enzymatic degradation and low cellular uptake efficiency [20], hamper the bio-sensing applications of DNA quadruplexes in biologically relevant environments. This obstacle can be overcome by structural DNA nanotechnology, which represents a source of stable structural skeletons capable of resistance to enzymatic degradation as well as internalization by various cell lines. DNA nanostructures could act as carriers to transport quadruplex-forming DNA strands into cells or even specific organelles [16, 20]. This provides a means to reveal the role of certain intracellular analytes in disease progression and will contribute to the development of new diagnostic tools.

A straightforward approach to transport DNA quadruplex recognition probes into cells is to use SNA as a transporter. The commonly used SNA bears a Au core which possesses a well-established Au-thiol bond chemistry, allowing easy anchoring of oligonucleotides of any base compositions. The quadruplex-forming strands can be labeled with Fluorescence resonance energy transfer (FRET) pairs at both terminals, and then hybridized to their complementary oligonucleotides immobilized on the gold nanoparticles (Au NPs) via Watson-Crick base-pairing. In this way, SNA was turned into a "carrier" molecule that holds the recognition probe "cargo". The dense shell of radially oriented oligonucleotides of SNA ensures the high loading capacity of the quadruplex-forming strands. Before target-binding, the FRET donor and acceptor moieties are separated from each other, showing low FRET efficiency, while in the presence of the

target, the recognition probe folds into quadruplex conformation, which brings the FRET pairs into close proximity and generates high FRET efficiency. In an example, SNA was employed to deliver dual-fluorophore-labeled i-motif-forming sequences into living cells by firstly loading a multitude of C-rich sequences on the SNA *in vitro* [20]. When the SNA was taken up into acidic organelles, the i-motif-forming strands folded into quadruplex structures and were unloaded from the SNA, bringing the donor and acceptor close and emitting FRET signal.

One type of nanocomposites, gold nanoshells (Au NS), exhibited interesting photothermal properties and thus has been fabricated recently into SNAs for temporally controllable sensing (Fig. 3(a)) [21]. Specifically, a thin layer of Au (10 nm) was coated on the surface of spherical silica core to afford the AuNS. Onto the surface of the AuNS was conjugated with dsDNA composed of dual-labeled G-quadruplex-forming strands and their complementary strands (DSAP in Fig. 3(a)). After the as-prepared nanoparticles were internalized by cells, near-infrared ray (NIR) was applied on the cells. Then the nanoparticles converted light into heat, which increased local temperature and triggered the dehybridization of G-rich strands from the nanoparticles. They folded into G-quadruplexes in the presence of endogenous K^+ , and thereby turned FRET on. Because NIR is an external stimulus that can be applied on demand, this type of sensing can be implemented at desired time at target site. Additionally, the strands that can form G-quadruplex were tightly hybridized by their complementary strands prior to laser illumination, which inhibited G-rich strands to bind K^+ in the transport route, and thereby prevented undesired response and enhanced sensing specificity.

Another type of SNA termed protein spherical nucleic acids (ProSNAs), consisting of a protein core instead of Au nanoparticle, has been fabricated more recently into an intracellular pH sensor by covalently anchoring i-motif-forming sequences to the protein core (Fig. 3(b)) [16]. Notably, thiazole orange molecules were inserted in the ss oligonucleotides prior to internalization and pH measurement. It exhibits low fluorescence in ssDNA but generates enhanced fluorescence in folded i-motif

because of restricted rotation about the methine bridge between stacked base pairs. Hence, when cells were incubated in acidic buffers with this type of ProSNAs, the structural change of i-motif-forming sequences from unfolded to folded states led to increased fluorescence of the cells.

In addition to SNA, other forms of nanostructures (DNA triangular prism, DNA tetrahedron, DNA origami, etc.) can also be readily constructed into biosensors with DNA quadruplex recognition probes [23, 25]. In a study, an adenosine triphosphate (ATP) aptamer-forming G-rich strand was transported into cells by a DNA triangular prism bearing C-rich strands [24]. The C-rich edges folded into intramolecular i-motif, thereby releasing the G-rich strand which generated FRET signal by capturing ATP. FRET signal could thus only be observed in the coexistence of low pH and ATP, resembling an AND logic gate. More recently, our group constructed a series of highly responsive pH sensors by using DNA tetrahedron to pre-organize the folding of dimeric i-motifs (Fig. 3(c)) [22]. Weak positive cooperativity was found for i-motif structures of bimolecular conformation, which prevents them to be adopted in fabricating highly responsive pH sensors, since high sensitivity requires strong positive ligand-binding cooperativity for multivalent receptors. Enhancement in cooperativity is accomplished by anchoring the i-motif-forming strands at the DNA tetrahedron vertex due to DNA tetrahedron's preorganization effect. Using this mechanism, we obtained highly responsive pH sensors with the minimum responsive width of 0.2 pH units and sequential transition midpoints, which was confirmed in intracellular pH sensing. This mechanism holds potential in performance improvement of other bimolecular nucleic acid structures, such as bipartite DNA aptamers and split G-quadruplexes, which could facilitate the creation of stimuli-responsive molecular devices that exhibit programmed behaviors, enabling precision control in applications where accurately-defined responsiveness is critical, such as biosensor systems with finely-tuneable sensitivity, or the dynamic regulation of molecular machines.

The dynamic conformational changes of DNA i-motif have been utilized in the design of pH sensors working inside

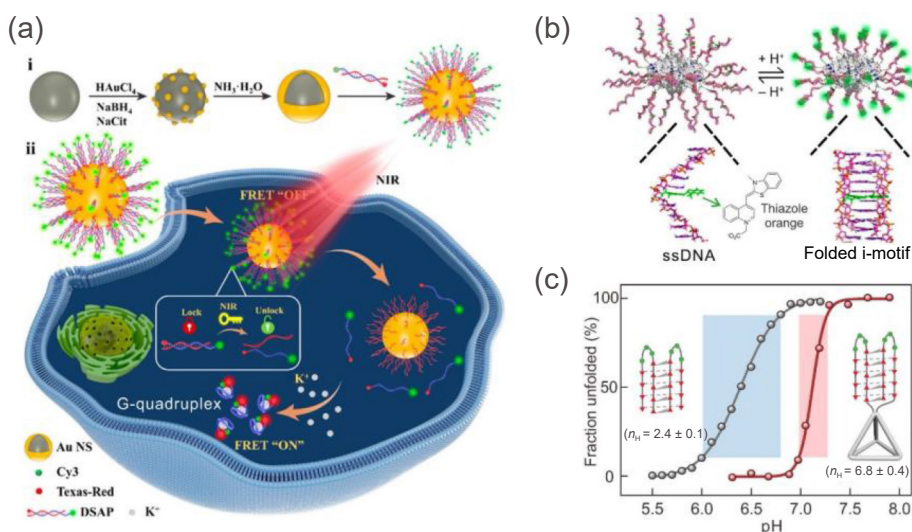


Figure 3 DNA nanostructure-scaffolded sensors with DNA quadruplexes as recognition probes. (a) Intracellular K^+ sensing based on SNAs fabricated by AuNS with photothermal property. Dual-stranded-aptamer precursor (DSAP) denotes DNA duplex formed by G-quadruplex-forming strand and its partly complementary strand. The G-rich strand (colored blue) was labeled with fluorophores at its two ends (Cy3 as FRET donor and Texas-Red as acceptor). Reproduced with permission from Ref. [21], © The American Chemical Society 2020. (b) Schematic of protein spherical nucleic acids bearing i-motif-forming strands and thiazole orange intercalators. The formation of the i-motif structure significantly enhanced the fluorescence of thiazole orange. Reproduced with permission from Ref. [16], © The American Chemical Society 2020. (c) Typical pH denaturation curves for a dimeric i-motif before (cooperativity coefficient $n_H = 2.4 \pm 0.1$) and after pre-organized by tetrahedral DNA ($n_H = 6.8 \pm 0.4$). The Hill coefficient n_H is a quantitative estimation for cooperativity, and strong cooperativity is reflected by a large n_H value. The transition width was narrowed from 0.81 (blue) to 0.29 pH units (red). The color coding of residues in the structure scheme is as follows: red for cytosine, and green for thymine. Reproduced with permission from Ref. [22], © The Royal Society of Chemistry 2021.

cells [22, 63] and on cell surfaces [64, 65], in order to study cell physiological or pathological processes. Fluorescence imaging methods are often used in these designs due to their high spatial and temporal sensitivity, as well as their ability in generating signal output in cellular milieu in real time. For instance, a fluorophore-labeled i-motif strand has been grafted on a DNA duplex, which could be internalized by cells into lysosomes [63]. Fluorescence variations induced by i-motif conformational changes indicated the identity of different subpopulations of lysosome, which could enable the monitoring of disease progression. In another study, an i-motif sequence has been incorporated in the middle of a DNA tweezer nanostructure, which was further anchored on cell surface via cholesterol-cell membrane interaction [64]. With i-motif as a response element, it could respond to extracellular pH quickly and reversibly, and performed real-time imaging with excellent spatiotemporal resolution.

4 DNA nanostructure-scaffolded sensors with DNA quadruplex as signal probe

In addition to act as recognition probe for K^+ in a biosensor, G-quadruplex is able to be employed as signal probe by complexing with hemin [Fe(III)-protoporphyrin IX], which represents one kind of artificial enzyme mimicking horseradish (termed here heme-DNAzyme). Using hydrogen peroxide as oxidant, the incorporated hemin can be readily activated toward oxidative catalysis of various reducing substrates, such as ABTS, dimethylbenzidine and amplex red [32, 66]. This enabled heme-DNAzyme to serve as a convenient catalytic label for biosensors, producing signals in many different ways (chromogenic [28], fluorescent [67], chemiluminescent [30], electrochemical [26, 68], etc.).

An important goal in biosensor construction is the intricate control over signaling transduction, which can be hardly achieved in a signal probe consisted of quadruplex structure *per se*. More

control capacities have been introduced by static or dynamic DNA nanostructures in recent years. Static DNA nanostructures can precisely define the spatial position and orientation of chemical entities in three dimensional (3D) microenvironment at nanometer accuracy, which can be readily turned into a power of regulating signal transduction abilities. Taking tetrahedral DNA as an example, the oligonucleotides in its edge are positioned at a fixed angle around the helical turns relative to the tetrahedron cage. Turberfield group examined the orientation of oligonucleotides in the 20-base-pair double helical edge of a DNA tetrahedron, and found the 8th nucleotide faces toward the central cavity and the 13th nucleotide faces outward, displaying an approximately 180 rotations (Fig. 4(a)) [69]. By taking advantage of the differences in microenvironment at different positions along the edge, the property of functional units anchored on the tetrahedron can be regulated. For instance, it was found that the enzymatic activity of heme-DNAzyme which covalently attached on the tetrahedral DNA varied depending on whether it was placed inside or outside of the tetrahedral scaffold [70]. When this DNA-tetrahedron-scaffolded DNAzyme was anchored on electrode surface, its electrocatalytic efficiency was regulated simply by placing the G-quadruplex at different positions (top, side or bottom) and orientations (in or out) on the tetrahedron (Fig. 4(b)) [26]. The heme-DNAzyme generally exhibited higher catalytic efficiency when it was placed in the cage than outside of the cage, as a result of enhanced stability and more uniform distribution of G-quadruplex-hemin complex inside the DNA tetrahedral framework. The catalytic efficiency was gradually increased when its position on the surface-anchored tetrahedron was changed from the top to the side, and further to the bottom, which represented a means of modulating the distance between the enzyme active centre and the gold electrode, with shorter distance favouring faster charge transfer and thus giving higher catalytic efficiency.

On the other hand, dynamic DNA nanostructures, in which the structural components can move in response to external stimuli,

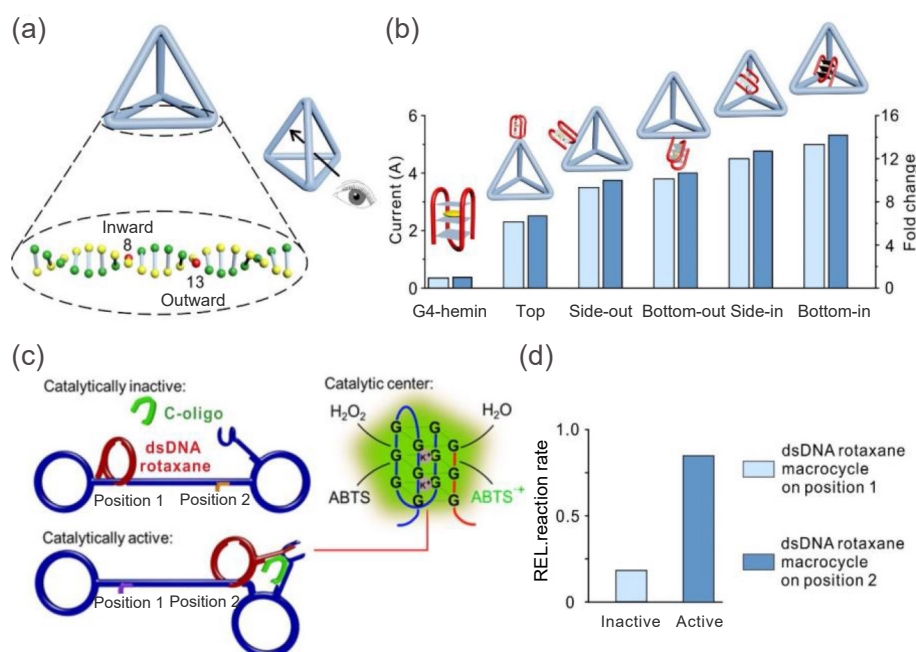


Figure 4 Sensing ability regulation of DNA nanostructure-scaffolded sensors with DNA quadruplex as signal probe. (a) When the 20-base-pair double helical edge of a DNA tetrahedron fabricated by Turberfield group [69] is viewed from the direction shown in the figure, the 8th nucleotide faces inward and the 13th nucleotide outward. (b) Electrocatalytic activities of DNA tetrahedron-scaffolded electrochemical sensor varied by altering the position and/or orientation of heme-DNAzyme. Reproduced with permission from Ref. [26]. © The American Chemical Society 2019. (c) and (d) The oxidative catalytic activity of a split heme-DNAzyme was fine-tuned by addition of release oligos which alters the spatial position of dsDNA rotaxane along the axle of the dumbbell DNA nanostructure. Reproduced with permission from Ref. [29], © The American Chemical Society 2017.

are able to regulate the formation and deformation of the DNA quadruplex signal probe, and thereby controlling signal transduction [27–29]. As shown in Fig. 4(c), a DNA nanostructure exhibiting mechanically interlocked configuration, known as DNA rotaxane, has been adopted to control the oxidative catalysis of heme-DNAzyme [29]. This resembles the allosteric catalysis regulation mechanism of natural enzymes, in which conformational change of the protein scaffold occurs at a site distant from the catalytic center. The red-colored rotaxane macrocycle can move on the axle and stall at defined positions (i.e., position 1 and position 2) due to hybridization to the axle. One-fourth of a G-quadruplex structure was anchored outwardly on the macrocycle and another three-fourth on one of the rotaxane terminals. When the macrocycle was at position 1, the two G-rich strands were distant from each other, which prevented the formation of G-quadruplex and resulted in neglectable catalytic activity (Fig. 4(d)). After the addition of competitive strands, the macrocycle could be moved from position 1 to position 2. With the help of a complementary strand (C-oligo), the G-quadruplex was stabilized, giving rise to high catalytic activity.

Fluorescence excitation and quenching based on molecular recognition is one of the most common methods to develop functional materials for analytic usage *in vitro* and/or *in vivo*. For this propose, a wide variety of fluorescent molecules were developed for label-free nucleic acids fluorescence probes [71, 72]. They are generally weakly fluorescent, but give rise to a significant increase in fluorescence when binding to a certain type of DNA. In contrast to the well-documented fluorescence ligands for DNA duplex and G-quadruplex, fewer molecules were investigated to bind to i-motif due to the less understanding of i-motif's biological roles and its generally low stability at physiological conditions [72]. In order to build light-up systems, many G-quadruplex ligands, such as a porphyrin TMPyP4, neomycin-perylene conjugate, thiazole orange, Thioflavin and 2,2-diethyl-9-methyl-selenacarboxyanine bromide (DMSB), were used to study the fluorescence properties with i-motif structure [73–77]. They showed fluorescence response upon the conformational change of i-motif but lower affinity for i-motif over G-quadruplexes [78–81]. A number of small molecular fluorescence ligands were found more recently for i-motif, including crystal violet [82], berberine [83], neutral red [84] and quinaldine red [85]. And different binding sites were proposed for them. For instance, based on molecular docking simulation, crystal violet was suggested to bind to the sequence (ACCCT)₄ through end-stacking at the terminus of i-motif structure [82]. Possible binding site for berberine was the groove of i-motif because it did not displace crystal violet in the competitive displacement assay [83]. Being able to bind to the groove of G-quadruplexes, DMSB, neutral red and quinaldine red were thought to located in the groove region of i-motif as well [77, 82–84]. Owing to these specific interactions with i-motif, they could be utilised as ligands to build fluorescent light-up pH sensors, with i-motif as a signal probe.

5 SNA-scaffolded signal amplifier bearing DNA quadruplex

In the case of loading G-rich oligonucleotides onto AuNPs, or in other words, synthesizing SNAs bearing G-rich oligonucleotides, the resulting G-rich SNAs will be endowed with catalytic ability once complexed with hemin, hence termed here heme-SNAzyme (Fig. 5(a)). Willner's group firstly invented the heme-SNAzyme as a signal amplifier to detect DNA analyte which was sandwiched by a capture DNA probe anchored on a glass plate and a reporter DNA extended from the SNA [86]. They found the sensor's

sensitivity was improved greater than 10-fold as compared with the use of a single heme-DNAzyme. A recent study showed the catalytic activity of one single heme-SNAzyme particle was about 100-fold that of heme DNAzyme, benefiting from the dense layer of G-quadruplex/hemin complexes conjugated onto individual AuNPs [30]. Additionally, a stronger nuclease resistance was seen for heme-SNAzyme as compared to heme DNAzyme [30], which is in accordance with the generally higher nuclease-resistant ability of SNAs than that of linear DNAs [55]. The improved catalytic activity, together with the intrinsic good stability and strong nuclease resistance of SNAs, made heme-SNAzyme a robust nanocatalyst, and a versatile signal reporter and amplifier.

In a recent study, an electrochemiluminescence sensing platform was built for detecting circulating miRNAs, in which probe hairpin immobilized on the electrode was opened when hybridized with target miRNA, triggering cascade HCR amplification and producing a long dsDNA chain with many sticky linkers to capture heme-SNAzymes onto the electrode (Fig. 5(b)). The large amount of heme-SNAzymes catalyzed H₂O₂ into oxygen-free radicals that further excited luminol and gave off distinct luminescence signal, which endowed this sensor with exceedingly high sensitivity (Fig. 5(c)) [31].

The signal amplification ability of heme-SNAzyme has also been proved to be valuable in point-of-care disease diagnosis [30, 32]. Comparing with spectrometric methods run on sophisticated equipments, point-of-care testing at home or outdoors generally suffers from relatively lower sensitivity. As a means to tackle this challenge, the integration of heme-SNAzyme in point-of-care methodology will contribute to the development of ultrasensitive devices. For instance, an ultrasensitive chemiluminescence sensor has been constructed for imaging a miRNA related to acute myocardial infarction using a smartphone as a portable detector [30]. As shown in Fig. 5(d), in the presence of the target miRNA, the two hairpin structures assembled to afford a sticky dsDNA linker which captured the SNA onto the substrate. Further incubation with hemin and potassium ion led to the formation of SNAzyme, which catalyzed the release of superoxide radical anion from artemisinin, then oxidized luminol and emitted luminescence. This miRNA could be detected with a lowest detectable concentration of 10 pM in both tris-acetate buffer and 10% serum (Fig. 5(e)). The high peroxidase activity and strong nuclease resistance exhibited by SNAzyme have also played a part in other portable imaging platforms [32].

6 Summary and outlook

Throughout this review, we have presented a summary of recent design strategies employed to engineer biosensors using natural DNA quadruplexes and artificial DNA nanostructures. Biosensors generally consist of three components: recognition element, signal probe, and signal amplifier. The recognition element binds to the target, and the signal probe converts the biological recognition event to a signal which is further amplified to a detectable level that can be measured optically, electrochemically, calorimetrically, etc. DNA quadruplexes hold the advantage of being able to play a part in each of the three components of a biosensor, with DNA nanostructures acting as excellent support platforms. Regulation of sensing capability can also be realized by modifying the spatial location or orientation of DNA quadruplex sensing module within a 3D nanostructure scaffold.

The ability of harnessing the structural properties of natural nucleic acids modules is the masterkey to create new devices for biomedical use [87–91]. Current structural optimization and functionalization methods rely heavily on the use of nucleic acids helix [92–95], which however slowed the advance of biomolecule engineering due to the intrinsic weaknesses of DNA helix motif,

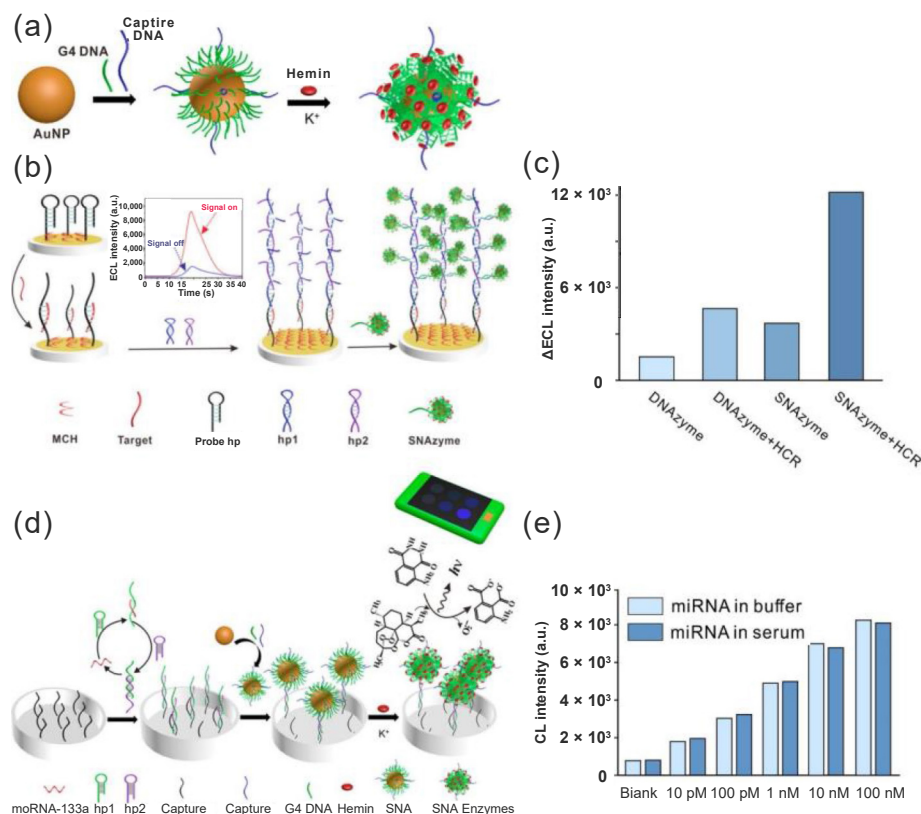


Figure 5 SNA-scaffolded signal amplifier and its applications. (a) Fabrication of heme-SNAzyme. Reproduced with permission from Ref. [30]. © The American Chemical Society 2019. (b) Ultrasensitive electrochemiluminescence (ECL) detection of a circulating microRNA using heme-SNAzyme as the signal amplifier. (c) Δ ECL intensity of the ECL reactions involving direct loading of DNAs, HCR-amplification-catalyzed loading of DNAs, direct loading of SNAs, and HCR-amplification-catalyzed loading of SNAs. Reproduced with permission from Ref. [31], © The American Chemical Society 2019. (d) Chemiluminescence detection of microRNA with a smartphone. (e) Chemiluminescence intensities of detecting miRNA in buffer and in 10% serum. Reproduced with permission from Ref. [30], © The American Chemical Society 2019.

such as weak rigidity, high thermosensitivity, liable to enzymatic degradation and difficult to manipulate or immobilize their spatial structures. In strong contrast, artificial DNA nanostructures provide a source of stable structural skeletons for repurposing natural nucleic acids modules, exhibiting superior properties such as inherent biocompatibility, high resistance to enzymes, favorable cellular entry efficiency, high mechanical rigidity, and spatial organization capacity of near-atomistic precision.

Up to date, one main obstacle of biosensors for clinical use is the trace amounts of analysts (e.g., nucleic acids) in clinical samples, which is difficult to be recognized by the sensor. Even if the recognition event between sensors and the low abundance targets occurs, the translation of the recognition event to a signal that can be readily detected is still a matter of concern. This clinical demand requires the biosensors to be satisfied with high signal amplification efficiency, where the heme-SNAzyme should play a role. From this perspective, the DNA quadruplex structures hold the potential to be incorporated in biosensors for the diagnosis of COVID-19, especially given the fact that consistency has been seen for tests in buffer solution and in clinical samples (e.g., serum).

The interweaving of DNA quadruplex and DNA nanostructures are likely to drive intriguing innovations in the field of biosensing, but several challenges lie ahead. For instance, the input/output response of a biosensor typically need to be optimized in order to obtain a relevant dose-response curve profile, but accurate control on the response sensitivity and specificity is far from straightforward to achieve [96]. Recent detailed studies on folding cooperativities of DNA quadruplexes will be a favourable way of approaching the need for biosensors with finely tuneable dynamic ranges and response

sensitivities [22, 97]. Moreover, when the DNA quadruplex biosensors go from laboratory to clinic, challenges in enhancing detection limit, accuracy and reproducibility are still need to be resolved [98]. *In vivo* sensing will also require a better characterization of the sensor's pharmacokinetic properties. In addition, approaches of increasing production yields and lowering costs are mandatory for large-scale production of DNA biosensors to meet commercialization need.

To summarize, the conformational polymorphism of natural DNA quadruplex and their combination with artificial DNA nanostructures of different shapes and compositions, hold great promises for constructing rapid, robust, and miniaturized highly sensitive biosensors for analysing real biological samples in the near future.

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Nos. 51972055, 81803480, and 21902101), the Shenzhen Science and Technology Research Project (No. JCYJ20180508152903208), the Shenzhen Pengcheng Scholar Program, and Shenzhen Bay Laboratory Open Fund (No. SZBL2020090501002).

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