DNA-Based Chemical Reaction Networks for Biosensing Applications

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The nature of biosensing is a biochemical reaction. DNA-based chemical reaction networks (DNA-CRNs) as a powerful programming language for describing behaviors of chemical reactions have shown great potential in designs and applications of biosensors. Due to their programmability, modularity, and versatility of the DNA strand, the performance of different detection strategies can be improved mainly by the rational design of DNA-CRNs. Herein, an overview of the fundamental theory and biosensing processes of DNA-CRNs is provided. Various detection strategies of DNA-CRNs are introduced, either in a simple low-order reaction model or in a complicated high-order reaction type, in combination with some typical cases for the different detection purposes. In addition, an overview of the recent development of DNA-CRNs for monitoring the cell microenvironment is presented, which is of significance to uncover some specific cell behaviors and functions. Finally, the roles of DNA-CRNs in the rational design of high-performance biosensors are summarized by pointing out the remaining challenges that impede the precise biosensing using DNA-CRNs in complicated biological environments.

1. Introduction

In recent years, DNA has been recognized as versatile building blocks for the construction of appealing DNA-CRNs as its assembly can be controlled by the precise but straightforward Watson–Crick base pairing. In a simple concept, DNA-CRNs refer to a finite set of DNA molecules, participating in crossing–interacting chemical reactions, which can affect the dynamics of the CRNs process either directly or indirectly. As a powerful mathematical language to describe and analyze chemical reactions of DNA systems, DNA-CRNs can display a variety of reaction network behaviors, either in equilibrium state or in a nonequilibrium state. The concept of equilibrium state used in this Review is to describe a closed system, where the concentrations of reactant DNAs and product DNAs no longer change with time. While the nonequilibrium state was used to describe an open system, where the concentrations of reactant DNAs and product DNAs still can be changed with the dynamic change of environment, e.g., pH or temperature. However, the design and control of reaction network behavior are not easy due to nonlinearities and uncertainties in CRNs. Therefore, by rational design of DNA complexes, DNA-CRNs can be an effective tool for the implementation of arbitrarily complex chemical reaction kinetics. Moreover, DNA-CRNs, as reaction modules, play a crucial role in biomedical applications by programming behaviors of complex networks, such as computing systems based on Boolean logic, gene regulatory networks, neural networks, signaling cascades in metabolic networks, and biosensor networks.

Due to their modularity, programmability, versatility, and inherent biocompatibility, DNA-CRNs have shown great potential in the field of biosensing. The recognition process of biosensing is a simple biochemical reaction. Based on the specific recognition of probes to their targets under different reaction conditions, a variety of flexible signal transformation strategies have been designed, which could greatly improve the biosensor performances, such as the sensitivity, specificity, throughput, and efficiency. Along with the advances in DNA nanotechnology, a large number of functional DNAs as recognition elements have been reported, such as aptamers and DNAzymes, which can be used for effective target recognition and signal conversion. Therefore, DNA-CRNs have become an important research direction and are full of great potential in the development of spatiotemporally controllable detection tools for biosensing applications. In this Review, we will first give a brief introduction of the fundamentals of DNA-CRNs, and then provide a comprehensive overview about the latest progress of DNA-CRNs in biosensing applications. Finally, the perspectives of DNA-CRNs and their unsolved problems that need to be addressed in the future direction are pointed out.

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2. Fundamentals of DNA-CRNs in Biosensor Design

2.1. Basics of DNA-CRNs

The construction of DNA-CRNs usually requires a rational design of building blocks that can not only rapidly respond to a single chemical entity, but also enable the function of the network as a whole. The fundamental mechanism that enables DNA-CRNs to be rationally programmed is determined by the sequence, length, and secondary structure of DNA strands. Therefore, one chemical reaction corresponds to one specific behavior of DNA-CRNs, which endows DNA-CRNs a powerful programming language for engineering chemical reactions with desired dynamic behavior.\(^{(14)}\)

As reaction modules, DNA-CRNs provided the basis for developing biosensors. As shown in Figure 1, the input of specific DNA strands that can selectively interact with target molecules, such as DNAs, RNAs, and proteins, activates the formation of DNA-CRNs, which finally gives rise to enough output signals for detection.\(^{(14)}\) By rational design of DNA-CRNs based on the thermodynamic and kinetic processes of reaction behavior, the target molecules can be detected both quantitatively and qualitatively. The reaction mechanisms of the DNA-based CRNs for biosensing mainly include DNA strand displacement reactions, pH-triggered i-motif reactions,\(^{(15)}\) target-triggered G-quadruplex reactions,\(^{(16)}\) aptamer-based recognition reactions,\(^{(17)}\) and enzyme catalytic reactions.\(^{(18)}\) Due to the different characters of the specific target as well as the versatile molecules involved in the reaction, the design of each DNA-CRNs varies from one to another. According to the classical theories of chemical kinetics, the chemical reaction rate in a specific reaction with A and B as the reactants can be expressed as:

\[
\text{rate} = k[A]^a[B]^b
\]

where, \(k\) is the constant of the reaction rate, \([A]\) and \([B]\) are the concentrations for reactants A and B, respectively, and the order of reaction equals to \(a + b\).

![Diagram of DNA-CRN biosensor](image)

Figure 1. Modular organization of DNA-CRNs for biosensor.

As for the rational design of DNA-CRNs, information of the reaction order allows us to understand factors that may influence the outputs of a specific reaction, such as the throughput, specificity, and sensitivity toward the quality of biosensing. Therefore, we organized the following discussions with respect to the different orders of DNA-CRNs. By means of the classical theory of chemical reaction dynamics, we classified the DNA-CRNs into two categories: the low-order DNA-based chemical reaction (DNA-CR) and the high-order DNA-CR. A low-order DNA-CR is defined when \(a + b \leq 2\), whereas a high-order DNA-CR is defined when \(a + b \geq 3\). In the biosensor design, the low-order DNA-CRs usually occurs in a relatively simple condition with just one or two DNA specie(s) taking part in the reaction, similar like the traditional coupling or decoupling reactions. While high-order DNA-CRs always involve multiple types of DNA molecules \((\geq 3)\) or other species that synergistically take part in the reactions, such as the signal amplification by the self-propagating cascade reactions. Understanding the order of the DNA-CRs is an important step before the rational design of biosensors because one can easily design the sequences and identify the quantity of input DNA molecules based on the complexity of the chemical environment.

2.2. Biosensing Processes of DNA-CRNs

The aforementioned model describes the principle of DNA-CRNs biosensors with the module system. We then propose the modular implement as the formal description of modular
programming. As shown in Figure 2, the main procedure that the DNA-CRNs are compiled down to DNA molecules can be divided into the following steps: first, a suitable DNA-CRNs model was constructed to encode all of the biosensor behaviors. Second, the established DNA-CRNs model is compiled as domain specification. Third, the domain specification is translated to the nucleotide sequences by the professional software, e.g., NUPACK. Finally, the DNA molecules synthesized based on the modeled sequence can further take part in chemical reactions to behave as a biosensor. [19]

Comparing with traditional detection strategies, DNA-CRNs possess some advantages for biosensing: 1) The directionality and programmability of DNA molecules led the excellent regulation performance of the DNA-CRNs biosensor, such as the specificity, sensitivity, stability, throughput, and fiducial interval range; 2) The excellent biocompatibility considering that DNA is a naturally biocompatible material; 3) the application of DNA-CRNs is capable of being expanded into a greater area because the sequence and the length of DNA molecules can be precisely adjusted to adapt to different purposes of target detection, including biomolecules, metal ions, and cells. It should be noted however, DNA-CRNs also have some shortcomings in the real applications: 1) the cost of DNA materials is relatively high comparing to traditional materials, such as polymers or metals, in the fabrication of biosensors; 2) the successful construction of DNA-CRNs always needs a mild and DNase/RNase-free buffer condition and the buffer pH and temperature should be well controlled as well; 3) additional equipments, such as electrical and optical appliances, are needed to support the detection of signal outputs. Increasing studies have focused on the strategies to overcome these shortcomings and a comprehensive overview of the recent progresses on DNA-CRNs is shown in the following sections.

3. Biosensing Applications Based on Low-Order DNA-CRNs

According to the principle of chemical reaction dynamics, lower-order DNA-based reaction kinetics can be categorized as zero, first, and second orders. [20] In detail, the zero-order kinetics occurs when the endless constant amount of reactants takes part in the reaction per unit time and therefore the reaction rate is independent of reactant concentration. While for the first-order kinetics, the rate of DNA-based chemical reaction is proportional to the concentration of the single reactant. For the second-order kinetics, the rate of the DNA-based chemical reaction is determined by the concentration of two reactants involved or the square of the concentration of one chemical reactant. [21] Similar to the classical theory of reaction dynamics, the detection strategy based on lower-order kinetics of DNA-based chemical reaction can be subcategorized into two major types: reversible reaction and irreversible reaction. Compared with the high-order reaction models which will be discussed in Section 4, the low-order DNA-CRNs offers a clear and straightforward model for understanding the biosensing strategy. [19]

3.1. The Low-Order Reversible Reaction

Figure 3a shows topologies of a low-order reversible reaction. The low-order reversible reaction involves two species, A and B, which are combined to form a product AB. [23] On the contrary to combination reactions, the decomposition reaction involves the chemical species AB that can decompose into two products, A and B. While in some complicated situations that need to sense the dynamic and changeable environment (nonequilibrium state), the reversible reaction is considered in the biosensor design. In low-order reversible reaction for biosensing, the signal readout of most cases are almost instantaneous, suitable for real-time biosensing. The electrochemical methods are featured by their rapidity, sensitivity and high temporal resolution, suitable for capturing the signal of low-order reversible reaction. For example, Xia and coworkers developed an electrochemical DNA sensor (E-DNA) based on the target-driven DNA triplex formation which could generate an electrochemical output, thus supporting the continuous, real-time measurement of melamine in flowing milk, [24] and HIV-diagnostic antibodies in human serum. [25] Moreover, structure-switchable aptamers have demonstrated promising performances in the real-time detection of cytokines in vivo, [26] which is typically low abundances in physiological conditions and thus the aptasensing shows great value in biology and medicine. [27] Through decoration of graphene/methylene-blue/Au nanocomposites with ferrocene-labeled aptamer, Liu and coworkers successfully realized the real-time detection of vascular endothelial growth factor in serum in a wide linear range (2–500 pg mL$^{-1}$) with a much lower detection limit.
(0.1 pg mL$^{-1}$).\[^{28}\] Electrochemical aptasensors can also be well designed to achieve the continuous monitoring of interferon-gamma both ex vivo\[^{29}\] and in inflammatory mice.\[^{30}\]

As one typical example of reversible DNA-CRNs, DNA nanoswitch can undergo a reversible structural change upon addition or removal of external stimuli such as analytes,\[^{26}\] enzymes,\[^{31}\] metal ions,\[^{32}\] or pH values.\[^{33}\] As an example, Figure 3b shows a pH-dependant DNA nanoswitch for monitoring dynamical changes of intracellular pH. The shifting process of intracellular pH was nonequilibrium behavior. Briefly, the cytosine-rich fragment as pH-sensitive DNA reaction modules, could fold to form the i-motif structure in the appropriate pH values, the process of which was reversible and pH sensitive.\[^{22}\] Krishnan and coworkers used a similar strategy to construct a DNA nanoswitch for monitoring dynamical changes of intracellular pH in the process of endosome maturation.\[^{13}\] This work is of great importance in understanding the dynamic events of cellular metabolism. Significantly, they successfully applied the DNA nanoswitch system to spatially and temporally map the pH changes in living cells with high efficiency.\[^{13}\]

Moreover, the reaction directionality of low-order reversible reaction can be finely controlled by the rational design of DNA-CRNs modules. As a traditional but effective DNA-CRNs model, DNA strand displacement reaction can regulate the reaction direction, which has great potential in the design of autonomous biosensors for probing the dynamics of biochemical reaction. As strand displacement reactions are DNA sequence-dependent and sensitive to base mismatches, a variety of dynamic DNA-CRNs modules with high specificity can be designed.\[^{34}\] As a representative of DNA strand displacement reaction, toehold-mediated strand displacement reaction (TSDR) modules play critical roles in the construction of diverse, dynamic DNA biosensors.\[^{35}\] In TSDR modules, the invading strand binds to the overhanging complementary domain (toehold) of a double-stranded DNA (dsDNA) and then triggers the strand displacement reaction through branch migration, which leads to the release of the prehybridized strand. For example, Li and coworkers applied TSDR modules to build reversible DNA nanoswitch for the dynamic detection of ATP.\[^{36}\] Furthermore, the directional regulation of DNA strand displacement can be applied to the detection of multiple biomolecules. For instance, Yuan and coworkers constructed a direction-controlled biosensor using TSDR modules, which acts as a bidirectional DNA walking machine driven by a dual microRNA (microRNA-21 and microRNA-155) (Figure 3c).\[^{12}\] The direction-controlled biosensor was realized ultrasensitive determination of miRNA-21 (1.51 fM) and miRNA-155 (1.67 fM). Interestingly, the reaction rate of the TSDR model can also be regulated by the rational design of the length of a toehold. The reaction rate is a valuable research content in the biosensing application. Tan and coworkers constructed a biosensor based on the TSDR model on the live cell membrane to study the rapid transient membrane lipid encounters, which is essential to understand the mechanism of cellular communication and cell signaling networks.\[^{10}\]

3.2. The Low-Order Irreversible Reaction

The low-order reversible reaction as a traditional strategy for biosensing has some intrinsic weaknesses, such as low parallel detection capability. The low-order irreversible reaction was developed to overcome these weaknesses. Figure 4a shows the topologies of a low-order irreversible reaction. The reaction directionality of low-order irreversible reaction is constant.

The low-order irreversible reaction modules can realize the detection of various target molecules by regulating the recognition element of DNA-CRNs modules. For example, Xu and coworkers proposed a distinct intense chiroplasmonic response based on irreversible reaction modules of DNA-CRNs for real-time measurement of microRNA in living cells.\[^{37}\] In their work, side facets of the gold nanorods (AuNRs) were functionalized...
with two different single-stranded DNA (ssDNA) (DNA1 or DNA2) and backfilled with the cell-penetrating peptide (derived from the transactivator of transcription of human immunodeficiency virus) to facilitate the transport of AuNR-DNA across cell membranes. The intracellular mammalian microRNA-21 could hybridize with the DNA1 and DNA2, leading to forming AuNR-DNA into side-by-side (SBS) dimers, which produced a distinct intense chiroplasmonic response (Figure 4b). Along with the advances in DNA nanotechnology, a large number of functional DNAs as recognition elements have been reported, such as aptamers and DNAzymes, which can be used for effective target recognition and signal conversion. As shown in Figure 4c, Pei and coworkers fabricated a series of multiple detection strategies by regulating aptamer fragment of recognition site in DNA scaffolds. The detection strategies based on combination reaction modules achieved specific detection of various targets, including biomolecules (ATP, thrombin, and cocaine)[31] and heavy metal ions (Ag⁺, Pb²⁺, and Hg²⁺).[32] The self-folding character of some specific ssDNA that may form reversible secondary structure can be used to design target biomolecules or ions-triggered DNA-CRNs to enhance their detection specificity. Tan and coworkers also designed a universal DNA triplex switches based on low-order irreversible reaction.[38] As shown in Figure 4d, the DNA triplex switch consists of a dual-labeled oligonucleotide (with a pyrene modified at both ends), which formed a stem-loop structure containing a target-specific aptamer sequence. Initially, the dual-labeled oligonucleotide in the DNA triplex exhibited an expanded state. The aptamer/target complex was formed in the presence of the target, which caused the aggregation of pyrene, leading to the fluorescence signal readout. By designing a suitable recognition element of the DNA-CRNs module, various targets could be detected. Besides, the DNA molecules can be designed into DNAzyme as an output signal element of DNA-CRNs modules. Willner and coworkers, for instance, designed a DNAzyme for high-efficient detection of DNA and telomerase activity.[39] In their systems, a DNAzyme fragment (guanine-rich sequence) was designed as biocatalytic beacons for signal output. Hence, the low-order irreversible reaction as DNA-CRNs modules shows a powerful parallel processing capability in biosensing.

4. Biosensing Applications Based on High-Order DNA-CRNs

As mentioned in Section 2, the high-order DNA-CRNs refers to the event with more than three reactants taking part in the reaction. In biosensing, improving the sensitivity requires finding pathways going from linear to nonlinear processes. The high-order DNA-CRNs can lead to reaction amplification beyond linearity. Figure 5a and 6a shows the topologies of high-order DNA-CRNs. Due to the rapid development of versatile amplification mechanisms, the high-order DNA-CRNs, to some extent, should be semantically designated to signal amplification reaction (SAR). Because SAR relies on the cascade reactions, just a small amount of target molecule input could initiate sufficient signaling output with high sensitivity. In general, SARs can be processed in an enzyme-dependent or enzyme-free manner have become a powerful technique in biosensing. A detailed

Figure 4. a) Network motif of low-order irreversible reaction for biosensing. b) Schematic representation of target miRNA-driven formation of AuNR chiral SBS dimers in the live cell. Reproduced with permission.[37] Copyright 2018, Wiley-VCH. c) Construction of a series of scaffold-supported aptamers-assisted DNA switches to detect various targets, including ATP, cocaine, and thrombin. Reproduced with permission.[31] Copyright 2017, American Chemical Society. d) Triplex-assisted DNA switch to detect the various targets, including α-thrombin, ATP, and L-argininamide. Reproduced with permission.[38] Copyright 2011, American Chemical Society.
comparison among different signaling amplification methods of DNA-CRNs in the biosensor designs is shown in Table 1. In the following section, several strategies on how to design a specific SAR module to achieve highly sensitive biosensing will be discussed.

4.1. Enzyme-Dependent SARs

Polymerase chain reaction (PCR) module is the most common SAR with an enzyme in biochemical analysis and has been widely used in biomedical researches and in vitro disease diagnostics. However, it requires thermocycling. Due to the rapid development of molecular biotechnology, a variety of isothermal amplification methods using specific enzymes have been developed, including restriction enzyme-dependent amplification,[40,45,79,76] helicase-dependent amplification (HDA),[44,46,77,78] and rolling circle amplification (RCA).[48–51] The restriction enzyme-dependent amplification is based on degenerated primers that anneal with sequences of restriction sites highly and randomly distributed along the genome, which allows amplification of products consistently and efficiently with high quality. For example, Pei and coworkers developed a cascade signal amplification strategy based on exonuclease III (Exo III)-catalyzed DNA walker to detect single-base mismatches of DNA and bacteria.[40] The basis of the Exo III-catalyzed DNA walkers is through enzyme-catalyzed DNA-CRNs. As shown in Figure 5b, the movement of a stochastic ssDNA walker on the 3D spherical nucleic acids (SNA) track was via an Exo III-catalyzed burnt-bridge mechanism. The 3D SNA track consists of AuNPs functionalized with a dual-labeled oligonucleotide (with fluorescein [FAM] and thiol groups modified at the 3’ and 5’, respectively). The DNA walker was hybridized on the surface of SNA via base-pairing. Then, the DNA walker moves autonomously, and the dual-labeled oligonucleotide of SNA continually

Figure 5. a) Network motif of enzymatic amplification based on high-order DNA-CRNs. b) The working principle of the Exo III-catalyzed DNA walkers. Reproduced with permission.[40] Copyright 2017, Wiley-VCH. c) Fundamentals of HDA modules. d) Scheme of HDA module for microRNA assay. e) Schematic illustration of the RCA module for in situ amplified monitoring of microRNA accompanying catalytic hairpin assembly with the DNAzyme formation. Reproduced with permission.[41] Copyright 2014, Royal Society of Chemistry.
undergo hydrolysis via Exo III-catalyzed, leading to the release of FAM from the gold nanoparticle. The Exo III-powered DNA walker provides a new strategy to cascade signal amplification for ultrasensitive detection of single-base mismatches and bacteria. Wang and coworkers also developed a new track (gold nanocages@graphene nanoribbons) to achieve signal amplification based on exonuclease III-catalyzed DNA walker. Similarly, Ju and coworkers developed a cascade signal amplifier based on restriction enzyme-catalyzed DNA walker. The DNA walker for electrochemical detection of thrombin under nicking endonuclease Nb. BbvCl. The same group recently reported another system based on a DNAzyme-dependent signal amplification DNA walker for ultrasensitive detection of nucleic acid through a pixel counting strategy. 

HDA, which utilizes a DNA helicase to generate single-stranded templates for primer hybridization and subsequent primer extension by a DNA polymerase, has recently become an important enzyme-mediated SAR in biosensor design. An isothermal amplification method, which does not require thermocycling, can be easily achieved by the combined use of helicase and the polymerase. Comparing with other isothermal DNA amplification methods, HDA has a simple reaction scheme and can be easily performed at one temperature for the entire process. As shown in Figure 5c, the DNA helicase could separate dsDNA and generate single-stranded templates for primer hybridization and the following extension with the help of a DNA polymerase. It can provide much faster detection than PCR. Recently, HDA was exploited as a signal amplifier to detect intracellular low-abundance miRNAs. As shown in Figure 5d, the target microRNA could selectively bind to the 3′-end of the ssDNA probe to form a DNA–microRNA heteroduplex. Subsequently, the forward primer displaced the microRNA.

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**Table 1.** Comparison among different signal amplification strategies of DNA-CRNs in biosensor design.

| Amplification methods                  | Features                                                                 | Advantages                                                                 | Disadvantages                                                                 | Refs   |
|----------------------------------------|--------------------------------------------------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------------|--------|
| Enzyme-dependent signal amplification  | REDA[4] Consistent amplification with high quality                        | Rapidness; high specificity; high sensitivity                             | Requires unstable and expensive enzymes; sensitive to environmental conditions; time consuming | [44–47]|
|                                        | HDA[5] No need of thermocycling; simple reaction scheme                  |                                                                           |                                                                                  | [48–53]|
|                                        | RCA[6] Can produce a long ssDNA                                           |                                                                           |                                                                                  | [41–43,54–63]|
| Enzyme-free signal amplification       | HCR[7] A type of TMSD[8]; enables control over the displacement kinetics | Enzymes-free; isothermal conditions; simple protocols; high amplification efficiency | Requires careful design of hairpin structures; needs signal readout techniques to improve the sensitivity | [64–74]|

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REDA: restriction enzyme-dependent amplification. HAD: helicase-dependent amplification. RCA: rolling circle amplification. HCR: Hybridization chain reaction. TMSD: toehold-mediated strand displacement.
and was extended by DNA polymerase to form a dsDNA, followed by the amplification of the ssDNA probe through HDA. Compared with other DNA amplification-based microRNA assays, the HDA module was much more sensitive and much faster, and could exhibit a broader dynamical range.\(^{44}\) In addition, the HDA module could also be developed for the detection of HIV-1 DNA,\(^{46}\) telomerase,\(^{77}\) transcription factor,\(^{78}\) and pathogens.\(^{97}\)

As another efficient and straightforward amplification modules, RCA has been used as a promising tool to generate large-scale DNA templates for the construction of various detection platforms. RCA uses a single DNA primer on a short circular ssDNA template to generate a long ssDNA by DNA polymerases (e.g., F29 DNA polymerase).\(^{58}\) Compared with other signal amplification modules, the RCA module can produce a long ssDNA with tens to hundreds of repetitive sequence units elicited from the short circular ssDNA template.\(^{48}\) Therefore, RCA as a signal amplifier has been widely exploited to detect a variety of targets, including nucleic acids,\(^{47-69}\) DNA methylation,\(^{52}\) proteins,\(^{50,53}\) cytokines,\(^{51,59}\) and tumor cells.\(^{60}\)

To avoid the tedious primer design, Tang and coworkers reported a novel RCA module for the sensitive screening of microRNA.\(^{41}\) Figure 5e shows the sensing strategy by coupling the RCA module with catalytic hairpin assembly for the formation of a DNAzyme. DNAzyme could act as the peroxidase mimics to catalyze the TMB/H\(_2\)O\(_2\) to generated signals that were detectable by the ultraviolet (UV)–visible (vis) absorption spectroscopy. The detection limit of the RCA module for microRNA assay was as low as 1.53 fM.\(^{41}\)

Taken together, the enzyme-dependent SARs have the advantages of rapidness, high specificity, and high sensitivity. Importantly, these signal amplifiers could be applied as a universal signal amplification module to detect different kinds of targets (such as nucleic acids, proteins, metal ions, small biomolecules, and so on) through the rational design of target-triggered network structure responses.

### 4.2. Enzyme-Free SARs

To date, enzyme-dependent SARs based on high-order DNA-CRNs that rely on enzymes or DNAzyme. Therefore, the reactions are usually sensitive to temperatures or whatever other environmental conditions that may influence the enzyme/DNAzyme activity. Some enzyme-free amplification modules have been developed by DNA strand displacement reaction based on DNA sequence-dependent to achieve signal amplification.\(^{42,43,54}\) For example, Xiang and coworkers reported an enzyme-free signal amplifier for the sensitive detection of microRNA by the mechanism of TSDR (Figure 6b).\(^{42}\) The platform was constructed from a gold disk electrode (AuE) covered by DNA duplex (two toehold-containing sensing probes), consisting of the thiolated template probe (SH-TP, the green sequence), assistant probe (AP, the purple sequence), and protection probe (PP, the violet sequence). After binding to the toehold region of the probe, the target miRNA-21 displaced AP through TSDR and exposes the secondary toehold region for subsequent hybridization with the methylene blue (MB)-modified DNA fuel strand, which further displaced both miRNA-21 and PP. This process was repeated over and over again, leading to an amplified current response for sensitive detection of miRNA-21 down to 1.4 fM.\(^{62}\) However, most existing enzyme-free SAR strategy is a lack of universality because they are designed to achieve their aims and therefore cannot others.

Hybridization chain reaction (HCR), low cost, and straightforward amplification modules are highly efficient in the design of TSDR and thus has attracted more and more interest in the fields of bioimaging and biomedicine.\(^{63}\) As a highly efficient and universal amplification system, the operational process of the HCR module is a 1D dsDNA self-assembly process.\(^{62}\) As shown in Figure 6c, the HCR module involves a specific short ssDNA (initiator, target) that can trigger a cascade of hybridization reactions between the two hairpin DNA probes, the process of which can be repeated over and over again until all the hairpin DNA probes are exhausted. As a result, the products of HCR are a long-nicked double-stranded helix containing a large number of repeating units.\(^{54}\) In another study, Zuo and coworkers demonstrated an ultrasensitive electrochemical biosensor for microRNA detection by the HCR module (Figure 6d).\(^{43}\) The detection limit of microRNA by the HCR module was as low as 10 aM. The HCR module widens the range of biosensor applications, such as in situ chemical and biological sensing.\(^{54,63,64}\) For example, Wu et al. designed an electrostatic HCR module for the intracellular signal amplification and ultrasensitive fluorescence activation imaging of mRNA expression with a picomolar detection limit.\(^{63}\) Meanwhile, the HCR module is a probe-amplification technique, which can effectively reduce false-positive results (often occur in PCR).\(^{65}\) Importantly, the HCR module can also be combined with different types of targets, including proteins,\(^{79}\) small biomolecules,\(^{65}\) metal ions,\(^{66}\) nucleic acids,\(^{43}\) and tumor cells\(^{67}\) for various analytical readouts.

Taken together, the enzyme-free amplification modules showed high specificity and high sensitivity for biosensing. What’s more, compared with enzyme-dependent signal amplification modules, the enzyme-free amplification modules do not need unstable and expensive enzymes, and usually could be carried out at the room temperature, offering a good opportunity to broaden their application areas with low cost. While enzyme-free amplification modules of DNA-CRNs always involves the complicated design of DNA sequence and requires much longer amplification time.

### 5. Detection Strategies of DNA-CRNs for Monitoring of the Cellular Microenvironment

Monitoring of the cell microenvironment has significant for cell behavior and function in development, physiology, and pathophysiology.\(^{68}\) Especially, monitoring the behavior of biomolecules of interest outside the living cells is of great significance to fully reveal unidentified cellular activities, the information of which will further trigger specific disease-directed cell research and medications, e.g., identifying drug–target and constructing cell-based regenerative therapy. To this end, numerous approaches based on DNA-CRNs modules have been reported to detect specific targets in cellular microenvironment\(^{69-72}\) which fully demonstrates the excellent biocompatibility and extensibility of DNA-CRNs modules.
The extracellular pH affects the tumor cells adhesion, migration, and drug resistance. As shown in Figure 7a, Yang and coworkers developed a novel lipid–DNA-based cell-surface-anchored ratiometric fluorescent probe for monitoring extracellular pH change. One is pH sensitive, as pH indicator, and the other is pH insensitive, as an internal reference. Two lipid–DNA molecules are anchored on the cell membrane. The ratio of fluorescence intensity acts as the readout signal for monitoring extracellular pH change. To monitor metal ions in the cellular microenvironment in real-time, Tan and coworkers designed a diacyllipid–DNAzyme conjugate as metal ion-dependent DNA nanoswitch modules of DNA-CRNs for monitoring dynamical changes of extracellular metal ions. To monitor extracellular chemical transmitter dynamics, Sando and coworkers developed a fast-responsive tocopherol-labeled aptamer on the cell surface as a cell sensor for real-time monitoring of the release of adenine compounds, a chemical transmitter released from glia cells in the brain. Similarly, Nie and coworkers constructed a cell surface DNA-CRNs sensor by engineering DNA motifs and synthetic cofactors. The DNA-CRNs sensor for the ratiometric imaging of the cellular extrusion process of endogenous signaling molecules, including sulfur dioxide derivatives and nitric oxide

Figure 7. a) A novel lipid–DNA-based cell-surface-anchored ratiometric fluorescent probe for extracellular pH sensing. Reproduced with permission. Copyright 2014, American Chemical Society. b) A DNA-CRNs sensor for the ratiometric imaging of the cellular extrusion process of endogenous signaling molecules, including sulfur dioxide derivatives (top) and nitric oxide (bottom). Reproduced with permission. Copyright 2019, Wiley-VCH. c) Engineering aptamer on the cell surface as DNA switch modules for real-time probing of PDGF. Reproduced with permission. Copyright 2011, Springer Nature.
(Figure 7b). To monitor external proteins in real-time, as shown in Figure 7c, Karp and coworkers designed a simple engineering aptamer on the cell surface as DNA switch modules for real-time probing of platelet-derived growth factor (PDGF).[72] Further, Lin and coworkers developed a cell surface-anchored DNA probe for investigating the regulating effects of fluid shear stress on membrane protein internalization and the cell endocytosis process.[74]

In addition to the different detection strategies of DNA-CRNs demonstrated earlier for monitoring of the cellular microenvironment, DNA-CRNs also play essential roles in cell-based bio-computation, which has great potential in cancer diagnosis.[80]

When entering the complex biological environment, the DNA-CRNs face challenges that may impede the detection performance. These challenges include but not limit to the following aspects: 1) the possible degradation of framework by DNase/RNase present in the biological media, which could cause the inactivation of biosensors before getting to the targets; 2) nonspecific adsorption by serum proteins on the surface of DNA-CRNs, which may hide the sequence from recognition with the target molecules; 3) the possible dissociation or off-target DNA strand displacement by the endogenous biomolecules, such as proteins, nucleic acids, and physiological electrolyte. With the increasing demands of multifunctionality of DNA-CRNs, exploiting DNA-CRNs as theragnostics that can not only display highly efficient diagnostic function but also enable targeted delivery of therapeutics, show great potential in both disease diagnosis and cancer treatment.[81] However, to further expand the application of DNA-CRNs in vivo, all the aforementioned challenges should be carefully considered to achieve their functions.

To address these challenges, the structures of DNA-CRNs should be well designed, e.g., via chemical modification of DNA backbones to improve their stability against the degradation by DNases,[82] by means of machine learning-based predictive analysis tools,[83] to obtain the best structure information of DNA-CRNs to mitigate off-target effects and maximize their activities.

### 6. Conclusions and Future Perspectives

In summary, the development of accurate, sensitive, fast, and efficient methods for the qualitative and quantification of biological targets is of great significance in the biosensor design. Due to their predictability, cost effective, and chemically stable, DNA building blocks have shown great potential in the construction of DNA-CRNs, which have been widely exploited as a programmable language to engineer DNA circuits for designing high-performance biosensors.

In this Review, we focus on the regulating the performance of biosensing using different DNA-CRNs. Compared with other detection strategies, DNA-CRNs are more advantageous for biosensing because of the modularity of DNA-CRNs, which can serve as a programmable intermediate between inputs and outputs. In detail, 1) The directionality, programmability, and parallel processing capability of DNA base-pairing offers the biosensor with high specificity, high sensitivity, high stability, high throughput, and more extensive fiducial interval range; 2) The biocompatibility of DNA-CRNs are excellent because DNA is a naturally biocompatible material; 3) Because the sequence of DNA molecules can be easily tailored to adapt varying conditions, DNA-CRNs have demonstrated the promising extensibility in a variety of target detection. As described earlier, the DNA-CRNs can apply to the detection of targets in complicated biological environments, such as cellular microenvironment. It should be noted, however, there are still unsolved problems that impeded the precise biosensing using DNA-CRNs in complicated biological environments. These challenges include but not limited to the slow reaction kinetics, unintended hybridization, lack of robustness, and possible systematic errors due to the impurities in DNA strands. All in all, DNA-CRNs provide great opportunities for biosensor design, and exploiting DNA-CRNs to uncover more complex biological events of specific targets should be a foreseeable direction for this field.

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### Conflict of Interest

The authors declare no conflict of interest.

### Keywords

biosensors, chemical reaction networks, DNA-based chemical reaction networks

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