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Applications of Catalytic Hairpin Assembly Reaction in Biosensing

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Nucleic acids are considered as perfect programmable materials for cascade signal amplification and not merely as genetic information carriers. Among them, catalytic hairpin assembly (CHA), an enzyme-free, high-efficiency, and isothermal amplification method, is a typical example. A typical CHA reaction is initiated by single-stranded analytes, and substrate hairpins are successively opened, resulting in thermodynamically stable duplexes. CHA circuits, which were first proposed in 2008, present dozens of systems today. Through in-depth research on mechanisms, the CHA circuits have been continuously enriched with diverse reaction systems and improved analytical performance. After a short time, the CHA reaction can realize exponential amplification under isothermal conditions. Under certain conditions, the CHA reaction can even achieve 600 000-fold signal amplification. Owing to its promising versatility, CHA is able to be applied for analysis of various markers in vitro and in living cells. Also, CHA is integrated with nanomaterials and other molecular biotechnologies to produce diverse readouts. Herein, the varied CHA mechanisms, hairpin designs, and reaction conditions are introduced in detail. Additionally, biosensors based on CHA are presented. Finally, challenges and the outlook of CHA development are considered.

1. Introduction

Nucleic acids, which are well-known genetic information carriers, play an essential role in transcription and translation.[1] Pioneering work by Seeman led to a new line of "structural DNA nanotechnology" research based on the highly specific Watson–Crick base pairing capability, which confers powerful properties to nucleic acids that include desirable biocompatibility, high predictability, and nanoscale controllability.[2,3] Nucleic acids are considered as one of the most programmable materials for the "bottom-to-up" construction of 2D and 3D nanostructures of various sizes and shapes,[4] such as junctions, lattices, double crossovers, and DNA origami.[5] In the past few decades, nucleic acids have been investigated in "dynamic DNA nanotechnology" instead of depending on the simple phosphate-sugar backbone. Dynamic DNA nanotechnology includes the dynamic displacement and movement of nanostructures stimulated with the transitions of nucleic acids,[6] such as DNA tweezers,[7,8] DNA walkers,[9,10] DNA dendrimers,[11] and DNA circuits.[12] Among them, toehold-mediated strand displacement (TMSD), which is powered by the free energy of strand displacement, is a typical example. TMSD is triggered by a complementary single-stranded domain called the toehold. Nucleic acid strands with a certain degree of complementarity hybridize with each other, displacing one or more prehybridized nucleic acid strands. The kinetics of TMSD are largely dependent on the sequence and length of the toehold. In theory, the rate of the strand-displacement reaction of TMSD can be quantitatively controlled over 10^6-fold.[13,14]

Among TMSD reactions, catalytic hairpin assembly (CHA), was originally introduced by Yin et al.[15] and engineered for multiple analytical applications by Li et al.,[16] is an enzyme-free, high-efficiency, and isothermal amplification method. A typical CHA reaction is executed using two complementary DNA strands prepared as stable hairpin structures. Thus, spontaneous interaction between the two DNA strands is kinetically blocked, as the complementary domains are caged within the hairpin stems. With single-stranded nucleic acids, the two hairpins are opened successively because of the toehold nature, and the most thermodynamically favorable DNA duplexes are quickly formed.[17]

Normally, the isothermal nucleic acid amplification technologies including enzyme-assisted amplification method and

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enzyme-free amplification method are easy to realize high sensitivity. But the enzyme-assisted amplification method, such as rolling circle amplification (RCA), strand displacement amplification (SDA), loop-mediated isothermal amplification (LAMP), has the shortcomings of high cost, storage difficulty, and complicated reaction conditions. In comparison to other enzyme-free amplification method, including hybridization chain reaction (HCR), DNAzyme, and entropy driven circuit (EDC), CHA endows with higher catalytic efficiency, lower background signals, simpler and more stable reaction system.[2,18,19] As a simple, yet versatile isothermal nucleic acid amplification strategy, CHA has emerged as a satisfactory alternative for biomarker determination. Studies show that CHA can be adapted to multiple analytical formats, such as fluorescent,[20] electrochemical,[21] colorimetric,[22] surface plasmon resonance (SPR),[23] and electrophoretic methods.[24] Studies also indicate that CHA can be easily and rationally integrated with other isothermal amplification reactions under various conditions (different temperatures, buffers, and enzymes) to improve the sensitivity and specificity of assays.[25] Although the entity of CHA is nucleic acid strand displacement, the detection targets are not confined to nucleic acids. Many other analytes including metal ions,[26] proteins,[27] enzymes,[28] and even cancer cells[29] have been measured based on CHA.

Numerous studies related to CHA have been published over the past decades. In this review, we provide a map of the CHA landscape. First, we describe the mechanism, development, kinetics characterization of CHA, and the analytical techniques for CHA products. Furthermore, we summarize the biosensing applications of CHA. Finally, the challenges and outlook of CHA development are considered.

2. Characterization and Development of CHA

2.1. Mechanism of Typical CHA

CHA, a programmable DNA circuit, was first proposed by Yin et al. in 2008.[15] These authors attempted to engineer nucleic acid sequences to execute dynamic functions. As shown in **Figure 1**A, CHA is achieved using two partially complementary DNA hairpins (H1 and H2) and one single-stranded oligonucleotide (I) in three steps. Both hairpins are composed of three continuous domains, and each domain contains a toehold. First, I initiates the allosteric transition of H1 by specifically interacting with the exposed toehold H1. The intermediate product H1/I complex is thus formed. Similarly, the subsequently exposed toehold of H1 initiates another assembly reaction with H2. H2 is thus opened, and the unstable complex H1/I/H2 is generated. Then, the newly released H2 triggers branch migration, and I is dissociated from H1. Such circular DNA assembly–assembly–disassembly reactions yield 100-fold amplified H1/H2 duplexes within a short time. Nonspecific reactions between hairpins are prevented because the complementary sequences are trapped within a secondary structure. Because of the inherent modular and scalable characteristics, the two main features of CHA on signal amplification are enzyme-free and isothermal. Using the same principle, four kinds of circuits were designed as follows: catalytic formation of branched junctions (three-arm and four-arm), autocatalytic duplexes formation by cross-catalytic circuits, nucleated dendritic growth of a binary molecular "tree," and autonomous locomotion of the bipedal walker.

The kinetic analysis data reported by Yin indicate that 3.5% of A and B spontaneously hybridize without an initiator.[15] The high background values would greatly counteract the diagnostic applications of CHA. Various factors may contribute to the leakage, including the impurities of reactants and toehold-independent reactions. The key reaction in CHA is

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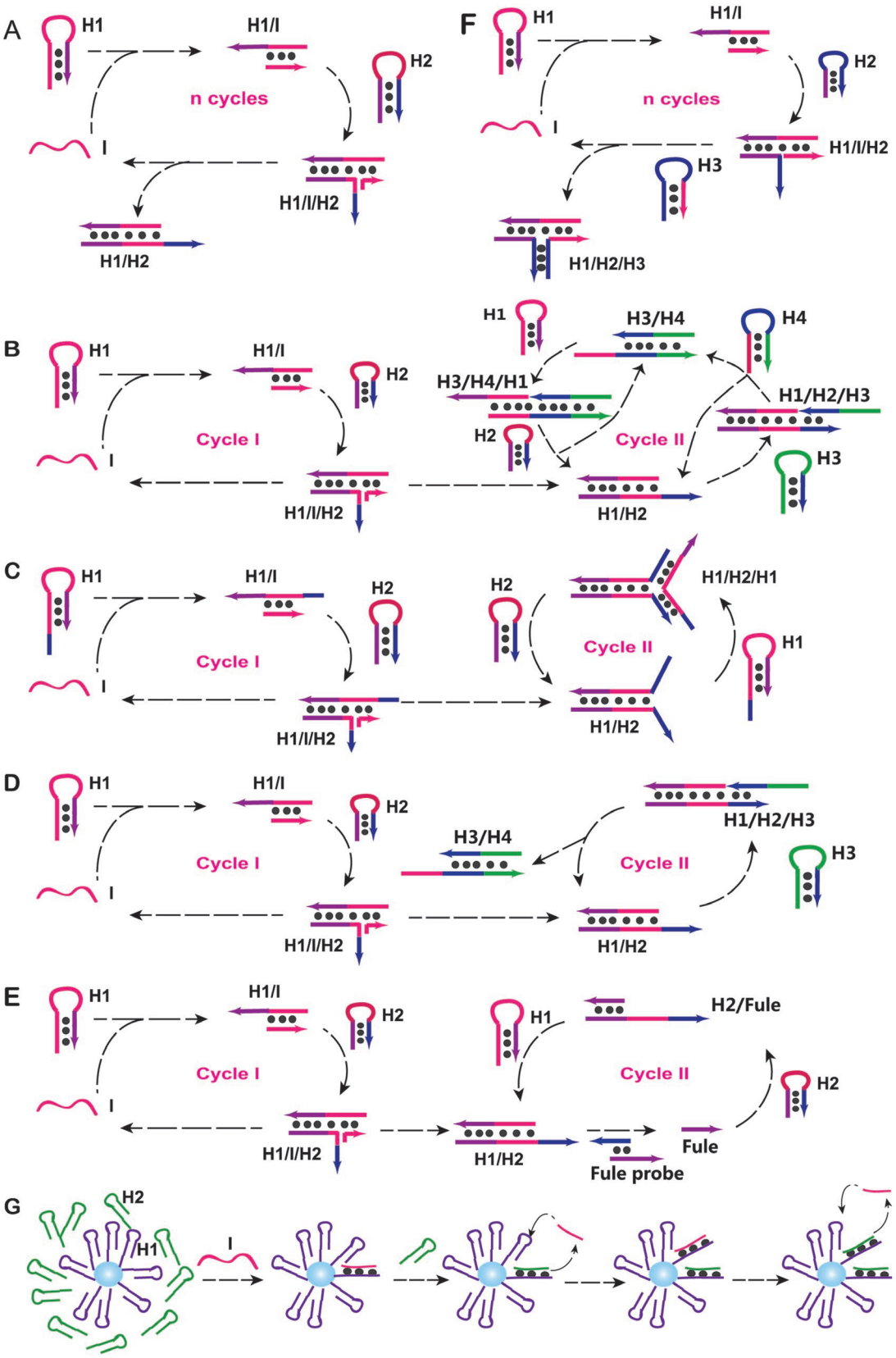

Figure 1. Mechanisms of diverse CHA systems. A) Typical CHA circuit.[16] B) Cross-CHA.[15] C) Self-replicating CHA.[36] D) Two-layer CHA.[37] E) Dual CHA.[38] F) Branched CHA.[15] G) Localized CHA circuit.[42]

the design and preparation of DNA sequences with functional domains and specific hairpin structures. The DNA sequences are designed according to the target structures, and the DNA strands form the prescribed structures through self-assembly. Undesirable DNA sequences and inappropriate reaction conditions result in unsatisfactory or unstable secondary structures. Even if the hairpins are perfectly designed and formed, the hairpin stems can "breathe" inadvertently, revealing the toehold domains that initiate the strand displacement reactions. In the kinetics curves, a fast signal increase is observed until the impure reactants are depleted in cases in which leakage is induced by impurities. A steady signal increase is observed until the substrates are used up in cases of toehold-independent leakage.[16]

2.2. Diversification of CHA

Regarding the analytical applications of CHA, the effective control of background signals is the foremost problem. Many researchers have attempted to solve this problem. Li et al. dissected the DNA sequence designed by Yin, and improved the CHA circuit by optimizing the domain organization and sequence (Figure 1B).[16] First, they found that several "clamp" domains designed to avoid uncatalyzed reactions in the original hairpins play a weak role in reducing the background. Therefore, they simplified the hairpins sequences by eliminating these domains. Because of dimerization, the hairpin loops were optimized without complementary sequences to the overhangs. Second, they hypothesized that proper lengths of the domains guarantee the sensitivity and specificity of CHA regarding kinetics and thermodynamic considerations. This view is widely held, as many researchers have determined the signal-to-noise ratio of CHA circuits to select the optimal toehold lengths.[30–32] The simplified CHA circuit was introduced to construct various biosensors. Nuclease contamination is common in the laboratory, and most nucleases require Mg^{2+} or other ions to function. In addition, nonspecific reactions are largely prevented in the absence of Mg^{2+}. For this reason, researchers identified an improved CHA reaction buffer named TNaK buffer (20×10^{-3} M Tris, pH 7.5; 140×10^{-3} M NaCl; 5×10^{-3} M KCl), which led to the CHA circuit achieving nearly a zero background (<0.5 M^{-1} s^{-1}) and a good turnover rate (>1 min^{-1}). Meanwhile, CHA was adapted to determine nucleic acid and non-nucleic acid analytes through multiple analytical readouts. This work has significantly improved the performance of the CHA reaction and greatly promoted biosensor research based on CHA.

Ellington et al.[33] considered blocking the inadvertent binding reaction or its continuations driven strand exchange reactions to prevent uncatalyzed strand exchange reactions. They considered this as a simple way to introduce mismatched base-pairs for developing mismatched CHA (MismatCHA) circuits. According to the source of leakage, both original hairpins introduce single or double mismatches into different domains (ends of helices or adjacent to the loop) to perform MismatCHA reactions. The collected fluorescent signal showed that the mismatched variants decrease the background, and CHA reactions are compromised to varying degrees. Generally, MismatCHA reactions yield higher signal-to-background ratios than the typical CHA reactions. In addition, various biosensors have been developed based on MismatCHA.[34] These strategies achieve higher sensitivity, a broader linear range, and better specificity and reproducibility than matched CHA. Simultaneously, these methods possess excellent performance for the analysis of cell extracts and serum specimens.

Several double and multiple CHA reactions have been developed for practical applications. Earlier in this article, we mentioned that Yin developed cross-catalytic hairpin assembly (cross-CHA) (Figure 1B).[15] Four hairpin species (H1, H2, H3, and H4) are involved in this system. The initiator I triggers the first CHA reaction to produce the duplex H1/H2. Then, the overhangs of B function as the catalyst that triggers the second CHA reaction to form the duplex H3/H4. In turn, the overhangs of H4 bind with H1 to circularly trigger the first CHA reaction. Hence, in the presence of I, the exponentially autocatalytic reaction between H1/H2 and H3/H4 is executed in cycles. The system not only shows that autocatalysis and exponential system kinetics are driven by the free energy of base-pair formation, but also pave the way for enzyme-free, cascade, and isothermal biosensors. Cross-CHA based biosensors for DNA have achieved significantly improved sensitivity and selectivity. Yue and co-workers established cross-CHA based assay. The control experiments clearly show that the sensitivity of cross-CHA (10×10^{-12} M) is improved 100-fold in comparison with that of traditional CHA (1×10^{-9} M). Different initiators with one-base mismatched, completely mismatched, and perfectly complementary were respectively introduced into the cross-CHA system. The signal of complementary initiator was significantly increased, whereas the signals of both mismatched initiators is negligible.[35] Interestingly, two hairpin substrates have also been engineered to complete double CHA reactions.[36] This system is named self-replicating catalyzed hairpin assembly (SRCHA). In this system, the complementary domains are still blocked in the stems of hairpins, but the two contrived split initiator sequences are designed in two hairpins. Two substrates can coexist stably. However, in the presence of catalytes, the CHA reaction is triggered, and duplexes are formed. Thus, the two split initiator sequences are close and trigger the CHA reaction (Figure 1C). The spontaneous, self-catalyzed, and cyclic CHA circuit is achieved. Based on cascade signal amplification, SRCHA has been applied for the determination of nucleic acids and other small molecules. Ellington et al. researched multilayer CHA for further improving amplification (Figure 1D).[19,37] Compared with the cross-catalytic hairpin assembly, the stage at which the duplex of the second CHA circularly triggers the first CHA reaction does not exist in two-layer CHA. They focused on the origins, outcomes, and characteristics of leakage in multiple CHA reactions, and finally achieved high quality two-layer CHA circuits that exhibit the lowest leakage and yield 7000-fold signal amplification. Thereafter, they successfully devised a four-layer CHA reaction that showed a record-high 600 000-fold signal amplification. However, the turnover rate of the second CHA is relatively slow. The overall four-layer CHA reaction takes as long as 19 h. Recently, Zhang et al. demonstrated dual CHA aiming to establish an electrochemical cytosensor with improved analytical performance (Figure 1E).[38] The introduced fuel probe is key for dual CHA because it not only hybridizes with the capture probe to decrease the background owing to

"breathe", but also efficiently initiates the second CHA. The cytosensor has a detection limit of 30 cells mL^{-1} within 1.5 h. As expected, the sensor also performs well in real samples with spiked cancer cells. This work has provided a platform with a wide detection range, high sensitivity, high selectivity, and low matrix effect for diagnostic applications.

The concept of catalytic formation of branched junctions was established by Yin et al..[15] Catalysts circularly initiate the CHA reactions between different amounts of metastable hairpins in succession to form branched junctions with different numbers of arms (Figure 1F). The rational design of sticky ends can bring branched DNA junctions into full play. In addition to the initial signal amplification, the sensing platforms based on branched DNA junctions show significantly improved sensitivity because the ends of every arm can be engineered to form DNAzymes,[39] transduce other isothermal amplification methods,[27] and bind with nanoparticles[40] to further amplify signals. With additional hairpins, the process and final structure are more complicated. Hence, the sequence design should be approached with caution.

Recently, another interesting CHA system called localized catalytic hairpin assembly (LCHA) has been described.[41,42] LCHA is performed in a compact space rather than common homogeneous solution because the successive CHA reactants are confined in biocompatible mediums, such as microparticles, gold electrodes and DNA nanowires. That is achieved via toehold-mediated strand exchange and proximity effect (Figure 1G). Initially, the researchers were surprised at that the LCHA is incredibly robust in varied conditions and verified the potentials of practical application. Thanks to the local high concentration of substrates, several advantages have been proved in subsequent LCHA based assays. The sensitivity of LCHA is obviously increased for the completion time for LCHA was 5 times shorter than that of CHA, whereas the signal of CHA was 1.5 times higher than that of CHA.[43]

The above CHA circuits are finished with DNA. However, genetically encoded and transcribed RNA circuits are alternatives for reagent production and in vivo function. For this reason, a CHA circuit with RNA substrates was developed by Bhadra and Ellington[44] Theoretically, RNA possesses the same base-paring properties as DNA. The results demonstrated that the CHA circuit with RNA substrate functions with similar kinetics and sensitivities. It's interesting that the unpurified RNA hairpins transcribed in vitro achieved the CHA reaction as well. Based on this, an intracellular RNA imaging method with genetically encoded RNA circuit was reported.[45]

We stated the importance of nucleic acid sequence design in CHA circuits. For this purpose, the NUPACK (www.nupack.org), OligoAnalyzer 3.1 (www.Idtdna.com), and Primer Primer 5 software provide great convenience.[46] On one hand, the initial number of nucleotides simulated in diluted solution can be obtained. On the other hand, these softwares can analyze the structures and parameters of nucleic acid sequences. Thus, unintended substrates or undesired structures can be precluded.

2.3. Analytical Techniques of CHA

The diverse CHA circuits bode well for developing biosensors for a variety of analytes. Versatile CHA circuits are adopted for practical applications in several ways. The CHA circuits can be performed alone first, and then coupled with DNAzymes,[30] nanoparticles,[47] and biotins[48] to generate signals. In addition, CHA circuits can be used for mediating other isothermal amplification methods to achieve cascade signal amplification.[49]

The CHA system needs to be confirmed before use. Gel electrophoresis techniques, including agarose gel electrophoresis (AGE) and polyacrylamide gel electrophoresis (PAGE), are the commonly used methods. The positions, amounts, and brightness values of bands enable assessments of the purity of the substrates, the presence of nonspecific reactions, catalyzed products, and the reaction efficiency.[50] Even analytes with single base mismatched can be distinguished with CHA gel assay.[51] In addition, the morphology of CHA products produced with novel CHA systems, such as branched junctions,[52,53] train-shaped nanostructures,[54] nanotetrads[55] and dendrimers,[56] is normally imaged directly using atomic force microscopy (AFM). The AFM pictures clearly illustrate the shape and size of the formed DNA nanostructure.

Multiple analytical readouts applied for CHA based bioanalysis methods are illustrated in **Figure 2**, including fluorescent,[57] chemiluminescent,[35] colorimetric,[58] electrochemical,[59,60] and SPR[23] methods. The optical method is the most satisfactory and widely used technique for the detection of biomarkers. CHA based optical biosensors comprise fluorescence, colorimetry, chemiluminescence, SPR, and strip sensor techniques. With respect to fluorescence, the strategy is divided into four types as follows. 1) The fluorescent reporters (fluorophore, quantum dot, etc.) are labeled onto the hairpins, which is the most simple and direct method. Additionally, the fluorophores are separated from the quencher or nano materials (graphene oxide, Au) through fluorescence quenching ability to release signals.[61–63] This strategy has been employed to develop robust and user-friendly strip biosensor for visual, rapid and sensitive detection of microRNAs (miRNA).[64] 2) CHA duplex products specifically hybridize with the DNA reporter complex to produce fluorescence. The method decreases the background signals.[65] 3) A strategy in which DNAzyme (G-quadruplex, ion-dependent DNAzyme) located in the hairpins catalyzes fluorescence substrates[66] or hydrolyzes the fluorophore labeled strands is popular.[67] 4) For simple biosensors, researchers have introduced materials (such as 2-AP) with optical properties that change with DNA structures into the hairpin substrates.[68,69] These CHA based fluorescent methods are not confined to quantification. Intracellular imaging and dynamic monitoring have also been reported. CHA based colorimetric determination methods possess attractive properties, as they are enzyme-free, label-free, cost-effective, simple, and practical. Initially, these biosensors are obtained through nanoparticles[70] or G-quadruplex/hemin DNAzyme[71] with peroxidase-like catalytic activity. Hence, the H_2O_2-mediated oxidation of substrates involving 3,3′,5,5′-tetramethylbenzidine (TMB) and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) is catalyzed to achieve naked-eye visualization. Then, DNA-associated Au nanoparticle aggregation[72,73] with different colors promotes CHA based colorimetric detection approaches. Point-of-care testing (POCT) diagnostics are particularly important in emergency circumstances or in low-resource settings. Versatile CHA circuits have even been employed to detect biomarkers using

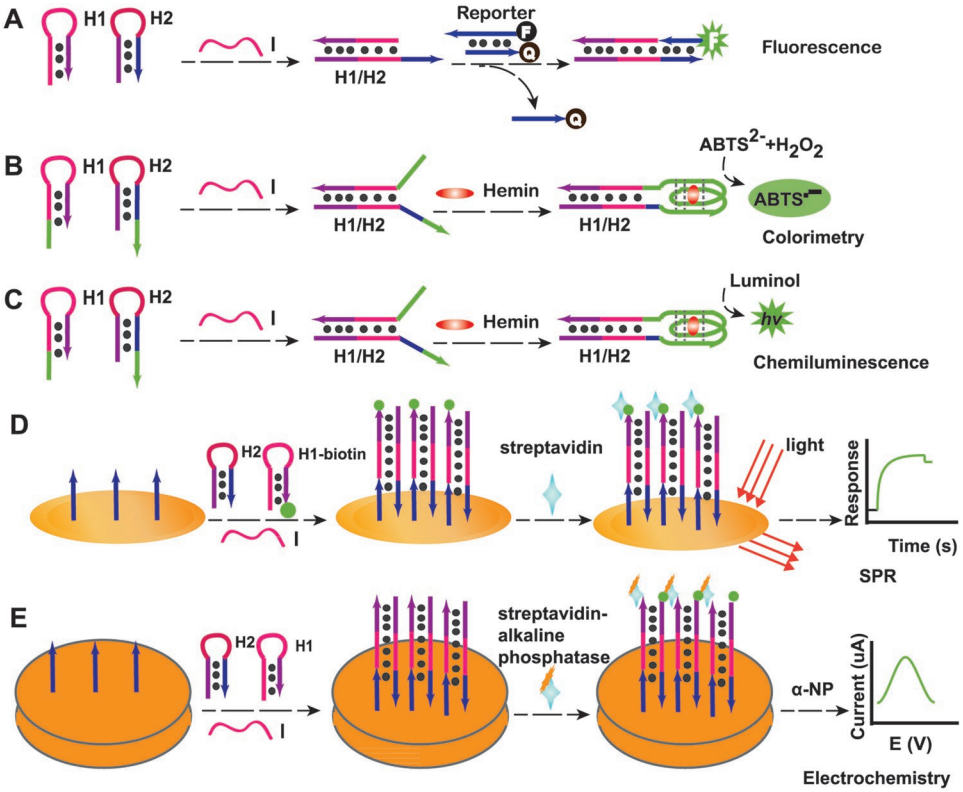

Figure 2. Various readouts applied for CHA circuits including A) fluorescence,[57] B) colorimetry,[58] C) chemiluminescence,[35] D) surface plasmon resonance,[23] and E) electrochemistry.[60]

the strip biosensing platform. Besides, chemiluminescence and SPR have immense potential for quantitatively analyzing the CHA system.

Electrochemical methods are also popular for developing CHA based biosensors because of their simplicity and sensitivity. Electrochemical signals (current, differential pulse voltammetry) of reactants possess electrocatalytic capability for H_2O_2 or electron transfer. Electrochemical biosensors based on CHA are classified into four types: 1) hairpins synthesized with electroactive indicators (toluidine blue, methylene blue, streptavidin-alkaline phosphatase); 2) horseradish peroxidases[74] or peroxidase mimics (G-quadruplex/hemin complex, PdNPs@Fe-metal organic frameworks microcrystals)[75,76] formed in the resultant products that generate signals; 3) modified nanoparticles (Au nanoparticles, Fe_3O_4/CeO_2@Au magnetic nanoparticles, Ag nanoclusters, MoS_2 nanoflowers, Au@Pt nanospheres) covalently bound to CHA products to magnify currents;[77–80] and 4) electrochemical indicators (Pb nanoparticles, methylene blue) intercalated into the backbone of CHA products to produce signal changes.[81]

3. CHA Based Biosensors for Various Targets

Because of their multiple advantages such as high efficiency, easy operation, and great versatility, CHA circuits are broadly applied in bioanalysis. This technique not only specifically recognizes the targets, but also transduces signals with 100-fold amplification. Owing to the innate character of the CHA reaction, CHA is naturally designed for quantitative determination of nucleic acids. Because of the significance of biomarkers and the application of antibodies, aptamers, and DNAzymes, CHA offers promising detection alternatives for a wide spectrum of biomarkers. In this section, we summarize CHA based biosensing methods for nucleic acids, proteins and enzymes, ions, cancer cells, and intracellular imaging.

3.1. Detection of Nucleic Acids

Nucleic acids are widely accepted vital biomarkers for clinical diagnosis. CHA based nucleic acid sensors have been developed for DNA, messenger RNA (mRNA), and miRNA. And a comparison of different CHA strategies for detecting nucleic acids is listed in **Table 1**.

3.1.1. Detection of DNA

According to the mechanism of CHA, single-stranded DNA triggers the cascade assembly and disassembly reaction with two hairpins. Thus, CHA-mediated DNA sensors constitute the most practical use of CHA. Most CHA systems have been successfully adapted for establishing DNA analysis strategies, such as cross-CHA, SRCHA, and branched DNA junction.

DNA biosensors based on CHA are artificially divided into four categories according to the principles of the detection

Table 1. CHA based assays for nucleic acids.

Target	Method	Output	Reaction time	Detection limit	Cost	Ref.
DNA	Pure CHA	Electrochemistry	2.5 h	0.39×10^{-15} M	Low	[83]
DNA	Pure CHA	Fluorescence	1.5 h	19×10^{-12} M	Middle	[84]
DNA	SRCHA	Fluorescence	15 min	58×10^{-12} M	Middle	[85]
HIV DNA	CHA-DNAzyme	Colorimetry	19 h	10^{-17} M	Low	[86]
DNA	CHA-DNAzyme	Fluorescence	5 h	0.5×10^{-12} M	Low	[87]
DNA	CHA-nanoparticle	Colorimetry	2 h	57.4×10^{-12} M	Middle	[88]
DNA	CHA-nanoparticle	Colorimetry	1.5 h	9×10^{-12} M	Low	[89]
P53 DNA	CHA-SDA-DNAzyme	Chemiluminescence	4 h	0.85×10^{-15} M	High	[90]
miR-122	CHA-HCR-DNAzyme	Chemiluminescence	2.5 h	0.72×10^{-12} M	Middle	[92]
miR-21	Pure CHA	Electrochemistry	30 min	0.6×10^{-12} M	Low	[93]
miR-141	CHA-DNAzyme	Colorimetry	5 h	0.5×10^{-12} M	Low	[94]
miR-21	CHA-nanoparticle	Fluorescence	1 h	37×10^{-15} M	Low	[95]
miR-21	CHA-HCR	Fluorescence	5 h	2×10^{-12} M	Middle	[96]
miR-21	Pure CHA	Electrochemistry	20 min	30×10^{-15} M	Low	[97]
miR-21	DNAzyme and Nuclease-assisted CHA	Colorimetry	100 min	9.2×10^{-15} M	Low	[98]
miR-21	CHA gel assay	Electrophoresis	1.5 h	10×10^{-15} M	Low	[51]
Three miRNAs	microscopy	Fluorescence	15 min	10^{-17} M	High	[101]
mRNA	RNA CHA	Fluorescence	2 h	2.5×10^{-9} M	Low	[45]

system, including pure CHA-mediated DNA biosensors, CHA-DNAzyme-induced DNA biosensors, CHA-nanoparticle-mediated DNA biosensors, and multiple isothermal amplification technique-mediated DNA biosensors.

Initially, bare CHA products hybridize with a fluorophore-quencher reporter and release fluorescence. The fluorescence intensity is proportional to the concentration of the DNA targets. In addition, the signals can be dynamically monitored.[16] Many researchers have attempted to construct a DNA sensing strategy by labeling reporters (fluorophore, electroactive indicator) onto the hairpins.[82–84] The corresponding signals can be collected by following the progress of the system reaction. The analysis of the signals allows the estimation of DNA targets' amounts (**Figure 3**A).[85]

In addition, G-quadruplex/hemin is greatly favored to be combined with nucleic acid circuits. G-quadruplex/hemin is easy to control and efficiently catalyzes H_2O_2-mediated oxidation. The G-quadruplex is separated into two hairpins with negligible noncatalyzed signals. With target DNA, G-quadruplex fragments are close enough and exhibit peroxidase-like catalytic activity. These enzyme-free and economical methods are available for POCT.[86] Interestingly, programing Mg^{2+}-dependent DNAzyme in the CHA hairpins was attempted for DNA determination. DNAzyme subunits in an inactive state were introduced into the two hairpins separately. Duplex formation led the two subunits to be in close proximity. The active Mg^{2+}-dependent DNAzyme recurrently cleaves the substrate nucleic acid strands, leading to

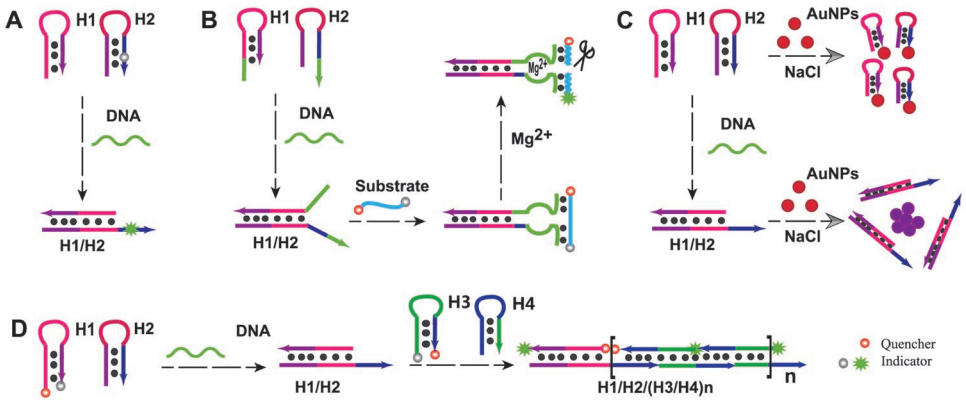

Figure 3. DNA biosensors based on CHA. A) Pure CHA-mediated DNA biosensor.[85] B) CHA-DNAzyme-induced DNA biosensor.[87] C) CHA-nanoparticle-mediated DNA biosensor.[89] D) Multiple isothermal amplification techniques mediated DNA biosensor.[96]

the production of fluorescence. And detection limit as low as 0.5×10^{-12} M was achieved, indicating the efficacy of this method (Figure 3B).[87]

At the same time, an increasing number of nanoparticles with good biocompatibility and multifunctionality have been developed, and they have shown good results in bioassays. Graphene/gold nanoparticles (graphene/Au-NPs) possess peroxidase-like catalytic activity at the interface. Furthermore, graphene/Au-NPs present differential catalytic activity when DNA with different structures is absorbed onto the surface. For the CHA system, hairpins tend to bind to graphene/Au-NPs and inhibit their enzyme catalytic activity, whereas CHA products have weak binding affinity to graphene/Au-NPs. As a result, in the presence of target DNA, the catalytic activity of graphene/Au-NPs is recovered with the formation of CHA duplex products, which catalyze the oxidation of TMB to show different colors. This strategy is effective for the quantitative and semiquantitative detection of DNA.[88] Because of their inherent optical properties, Au-NPs have received considerable attention for use as colorimetric biosensors. SsDNA and dsDNA present different adsorption behaviors on unmodified Au-NPs. Through van der Waals forces, ssDNA is easily adsorbed onto the Au-NPs, which restrains salt-induced Au-NP aggregation. Electrostatic repulsion between the phosphate backbone and negatively charged Au-NPs has no effect on salt-induced Au-NP aggregation. Inspired by the differential adsorption behaviors and characteristics of CHA, a signal amplified but enzyme-free Au-NP-based colorimetric DNA assay has been successfully constructed. In the absence of target DNA, hairpins are adsorbed onto the Au-NPs, and the solution appears pale purple. In the presence of the target, DNA duplexes are formed, and the color of the solution changes from pale purple to blue. This assay has a low detection limit of 9×10^{-12} M, and well distinguishes target DNA from several kinds of nonmatched DNA in saliva (Figure 3C).[89]

Many DNA nanotechnologies have emerged for the amplification of nucleic acids, such as the HCR, LAMP, RCA, DNA walker, etc. These technologies have proven to be satisfactory options for both amplifying and transducing signals. In the same way, these methods are able to be conveniently combined to improve the performance of tests. In the early stage, CHA was applied for monitoring RCA and SDA reactions.[90] First stage reaction products of RCA or SDA initiate the CHA circuit for dual amplification. This kind of cascade amplification system improved the specificity and increased the sensitivity by ≈10 000-fold over comparable real-time methods. Under isothermal conditions, RCA and SDA amplify the DNA targets with polymerase and nicking enzymes. These enzymes may require a complex environment. Then, HCR, another typical TMSD circuit, is coupled with CHA for bioassay applications. Generally, the CHA products are used to trigger HCR. This system generates long nicked duplexes, providing additional output pathways (Figure 3D).[91] Moreover, the system enables intracellular nucleic acid imaging.[92]

3.1.2. Detection of miRNA

miRNAs play an important role in posttranscriptional regulation and serve as circulating biomarkers with great potential for diagnosis, therapy, and monitoring. miRNAs are 18–23 nt, which remains challenging for determination with traditional techniques, while it is great for initiating the CHA reaction. miRNA sensors based on CHA account for the largest portion of CHA based biosensors. The miRNAs and DNA share similar nucleic acid properties, although miRNAs exist in body fluids in low abundance. Generally, the amplification strategies for CHA based miRNA biosensors are the same as those for DNA biosensors: 1) bare CHA circuits;[93] 2) CHA coupled with DNAzyme;[94] 3) CHA combined with nanoparticles;[95] and 4) the integration of CHA and other isothermal amplification methods.[96] Based on these signal amplification approaches, CHA circuits are triggered in two ways: miRNAs function directly to initiate the reaction, or miRNAs can also be converted into specific DNA sequences involved in hairpin hybridization. The principles of miRNA-initiated miRNA detection methods are analogous to those of DNA detection methods. A detailed description of these methods is therefore not necessary here. We will focus on introducing different methods for transforming miRNA into DNA.

To construct miRNA detection module and overcome the miRNA limitations of instability and degradation, miRNAs were equivalently converted into DNA strands. The original idea was to design a molecular beacon (MB) as a transducer containing the target miRNA complementary domain in the loop and the CHA initiator domain in the stem.[17] Once the target miRNA binds to the MB, the exposed initiator begins the subsequent amplification reaction. Coupling with the strand displacement reaction is another approach (**Figure 4**A).[97] The miRNA hybridizes to the double-stranded DNA probe and displaces the translator DNA, resulting in hairpins assembly and disassembly.

Researchers have attempted to convert miRNAs into DNA to improve sensitivity. This was achieved using four strategies as follows. 1) The first was by introducing sequence-dependent enzymes, such as duplex-specific nuclease (DSN) (Figure 4B),[98] exonuclease III (Exo III),[60] and Klenow fragment-Nb.BbvCI,[25] as the MB/miRNA complex can produce numerous translator DNAs. Because DSN tends to hydrolyze DNA duplexes with more than 10 bp or DNA in DNA/RNA hybrids, Exo III has a strong preference for catalyzing blunt or recessed 3′-termini of double-stranded DNA, the Klenow fragment possesses polymerase activity, and Nb.BbvCI recognizes and cleaves the specific domain. 2) Numerous DNA probe-modified magnetic NPs and Au NPs have been used for transforming miRNAs.[99] The miRNAs hybridized with these DNAs to form a "sandwich." After magnetic separation and dissolution of Au NPs, large amounts of translators are released. 3) The combination of magnetic NPs and DSN accelerates the collection of translator DNA.[100] DNA probes modified on magnetic NPs and miRNAs are hybridized to form duplexes. Then, DSN cleaves the duplexes and releases the translator DNAs, which are separated by magnetic fields. 4) A multicomponent nucleic acid enzyme (MNAzyme) can be adapted and formed in the presence of target miRNAs.[34] Hence, hairpin substrates are digested to produce translator DNA. The transformation of miRNA to DNA facilitates the collection of low-abundance target miRNAs and circularly results in DNA. These techniques can be easily extended to multiple miRNA determination without redesigning the CHA circuits.

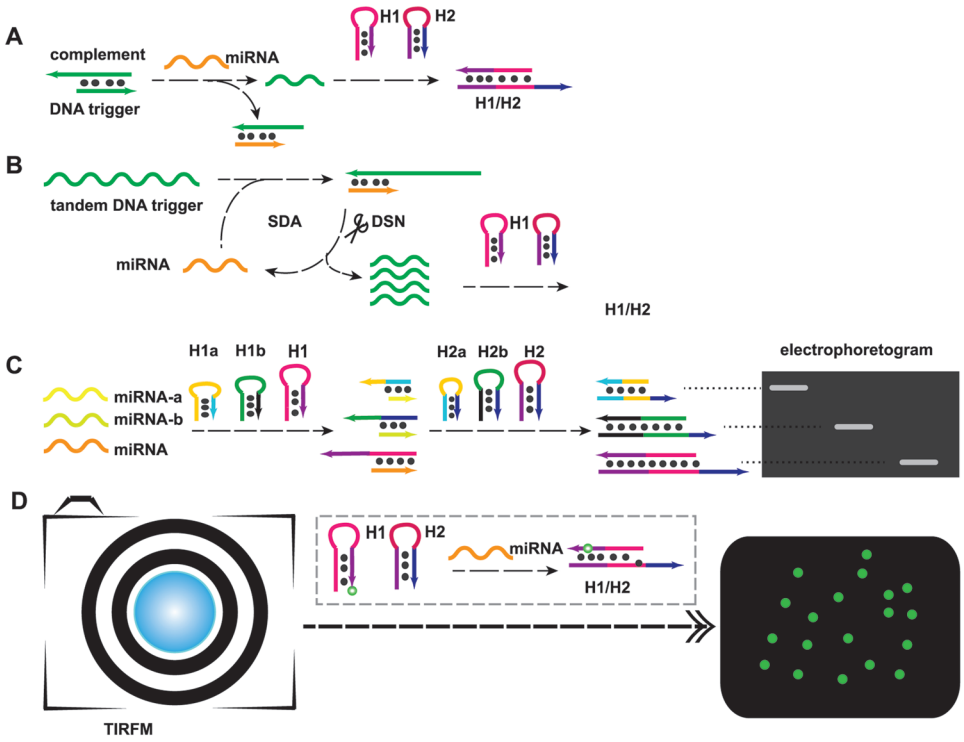

Figure 4. CHA based miRNA detection assays. A) Equivalent transformation from miRNA to DNA triggers mediated miRNA biosensor.[97] B) Numerous DNA triggers converted from miRNA mediated miRNA biosensor.[98] C) Gel assay mediated multiple miRNA biosensor.[51] D) Total internal reflection fluorescence microscopy mediated miRNA biosensor.[101]

In addition, several unique methods have appeared for miRNA quantification. Zhang and co-workers[51] designed three pairs of hairpin substrates with different lengths for three miRNA species to perform CHA reactions (Figure 4C). The products of each CHA reaction migrate to different levels in gel electrophoresis. The experimental results revealed that the CHA gel assay can effectively and simultaneously determine these miRNAs at the femtomolar level, and exhibits high discrimination capability for miRNA family members and base-mismatched miRNAs. Also, the assay can be applied for analyzing miRNAs extracted from cell lysates. Li et al used total internal reflection fluorescence microscopy (TIRFM) to measure the products generated after 15 min of CHA amplification (Figure 4D).[101] This method was verified using three miRNAs. Moreover, it was applied for directly counting miRNAs in the serum of 29 volunteers, 18 patients with prostate cancer, and 23 patients with breast cancer. Notably, these two assays provide powerful and simple tools for multiple miRNA analysis for practical application.

3.1.3. Detection of mRNA

mRNA transcribed from target genes carries genetic information and guides protein synthesis. Differential mRNA expression levels provide vital information for medical diagnosis and therapy. However, the quantification and visualization of exceedingly low concentrations of mRNA remain challenging. In light of its enzyme-free nature, extraordinary signal amplification properties, and isothermal conditions, CHA is widely accepted for mRNA determination, especially for intracellular imaging. Initially, pure CHA was used for imaging manganese superoxide dismutase (MnSOD) mRNA in MDA-MB-231 cells.[102] In consideration of the thermostability and nuclease resistance, four locked nucleic acid (LNA) nucleotides are incorporated into the fluorophore-labeled reporter. The amplification effectiveness in buffer is verified first. The CHA system is transfected into the cells using lipofectamine. Significant fluorescence is observed for CHA with the LNA-modified reporter, whereas a negligible signal is detected for CHA with a regular DNA reporter. To further confirm the sensitivity of the CHA system, the strategy was used to detect target mRNA in MDA-MB-231 cells treated with MnSOD, a mRNA inhibitor. The CHA reaction with the LNA-modified reporter was capable of specifically imaging mRNA in living cells even with low expression levels. Furthermore, LNA-modified CHA was coupled with Au nanorods (AuNRs) to fabricate a mRNA imaging platform. AuNRs were functionalized with DNA duplexes labeled with fluorophore, and the fluorescence was quenched by AuNRs.[103] The introduction of target mRNA initiated the CHA reaction after addition of the DNA transfection reagent. The formed duplexes hybridized with fluorophore-labeled DNA, and a restoration of intracellular fluorescence signal was observed. The system showed low cytotoxicity, and endowed high sensitivity and specificity.

Most DNA-based CHA circuits are limited by problems associated with biological delivery and degradation. However, RNA molecules have inherent advantages such as genetic encoding

and transcription. Genetically encoded CHA is therefore used for RNA detection.[45] Hairpins conjugated to part of the fluorescent aptamer and target RNA sequences were separately cloned into expression vectors. Two kinds of vectors were transformed into the same cell. After cellular transcription, the CHA system is ready to perform, and the fluorescent aptamer enables function. To avoid redesigning the entire RNA circuit each time, a modular system for imaging different RNA targets was developed. A genetically encoded RNA-based molecular beacon probe was engineered for various RNA detection and imaging applications.

3.2. Detection of Proteins and Enzyme Activity

Proteins do not merely supply the right nutrients to the body, but are also directly involved in physiological and pathological life activities. The levels of proteins are closely associated with states and stages of diseases. Accurate proteins analysis plays an important role in improving survival rates and treating pain. Therefore, it is crucial to measure proteins with high sensitivity, significant specificity, and desirable simplicity. Although traditional immunoassays have achieved great success in protein detection, they have certain limitations, such as complicated operations, expensive regents and equipment, and unstable enzymes. Great efforts have been made to adapt enzyme-free nucleic acid isothermal amplification techniques for proteins analysis. Among these strategies, CHA is widely accepted for detecting proteins including antigens, enzymes, and telomerase. And a comparison of different CHA strategies for detecting proteins and enzyme activity is listed in **Table 2**.

3.2.1. Detection of Proteins

CHA is specifically initiated by single-stranded nucleic acids, and affinity ligands including antibodies (Abs), aptamers, and biotin are required for recognition and transduction of proteins into nucleic acid information. Abs are the most commonly used recognition elements for proteins. Carcinoembryonic antigen (CEA) was used as an example to illustrate Ab-mediated CHA circuits for protein detection. First, Abs are modified at the ends of two single-stranded DNA molecules (DNA1 and DNA2) separately. After the introduction of CEA, the formed sandwich complexes DNA1-Ab-CEA-Ab-DNA2 hybridize with hairpin substrates to initiate and accelerate the CHA reaction. Subsequently, positively charged Pd NPs are absorbed onto the negative phosphate backbone of the duplex products. Then, Pd NPs with high catalytic activity produce a significant electrochemical signal. This method exhibits excellent analytical performance even in serum samples.[104] For an ultrasensitive electrochemical immunoassay of CEA, the multi-layer cascade amplification strategy (CHA-enzyme digestion-RCA) was adopted (**Figure 5**A).[105] The hybridization of DNA1-Ab-CEA-Ab-DNA2 complexes with hairpins leads to degradation by exonuclease III and the release of transducer DNA. The transducer DNA binds with another hairpin to perform CHA and RCA. Finally, the electroactive indicators combine with single-stranded DNA to yield signals. This analytical method dramatically improves the analytical performance and can detect CEA even at the fg mL^{-1} level. These strategies hold great potential for the determination of other biomarkers.

Aptamers, single-stranded oligonucleotides of 15–100 nt, bind to targets including proteins, metal ions, bacteria, small molecules, exosomes, and even cancer cells with high specificity and affinity. Aptamers originate from the systematic evolution of ligands by exponential enrichment (SELEX). In contrast to Abs, aptamers can be synthesized and controlled easily, and they show stability in storage, and are easy to separate. Essentially, aptamers are promising recognition elements for nucleic acid circuit-based protein biosensors. CHA based aptasensors have been successfully applied for proteins detection, including that of CEA, mucin-1, prostate specific antigen (PSA), thrombin, and fibronectin. According to the initiating modes of CHA, CHA based protein aptasensors can be divided into the following three categories: target-induced displacement of the initiator,[106,107] target-induced exposure of the initiator,[108] and target-induced joint binding-initiating.[109,110] Target-induced displacement of the initiator means that an initiator complementary to the aptamer is released concomitantly with aptamer–protein binding. As illustrated in Figure 5B, the complementary aptamer and initiator are modified on magnetic nanoparticles.[111] Upon the addition

Table 2. CHA based assays for proteins and enzyme activities.

Target	Method	Output	Reaction time	Detection limit	Cost	Ref.
CEA	Proximity induced CHA (Ab)	Electrochemistry	100 min	0.43×10^{-15} g mL^{-1}	Middle	[104]
CEA	Proximity induced CHA-Exo III-RCA(Ab)	Electrochemistry	2.5 h	4.2 fg mL^{-1}	High	[105]
Thrombin	Aptamer-proximity-CHA	Fluorescence	100 min	8.3×10^{-12} M	Low	[106]
CEA	CHA-HCR (aptamer)	Electrochemistry	5 h	70 ag mL^{-1}	Low	[107]
MUC-1	Enzyme-free target recycling and double-output amplification	Chemiluminescence	2 h	0.58 fg mL^{-1}	Low	[108]
Fibronectin	DNA base-stacking using CHA	Colorimetry	10 h	2.3×10^{-12} M	Low	[109]
PSA	CHA-aptamer	Electrochemistry	3 h	2.3 fg mL^{-1}	Low	[111]
CEA	CHA-HCR (aptamer)	Fluorescence	4 h	0.3 pg mL^{-1}	Low	[112]
Avidin	CHA-DNAzyme	Fluorescence	2 h	2×10^{-12} M	Low	[87]
RNase H	CHA	Fluorescence	10 min	0.037 U mL^{-1}	Low	[115]
Telomerase	CHA-Au	Colorimetry	70 min	15 cells	Low	[28]

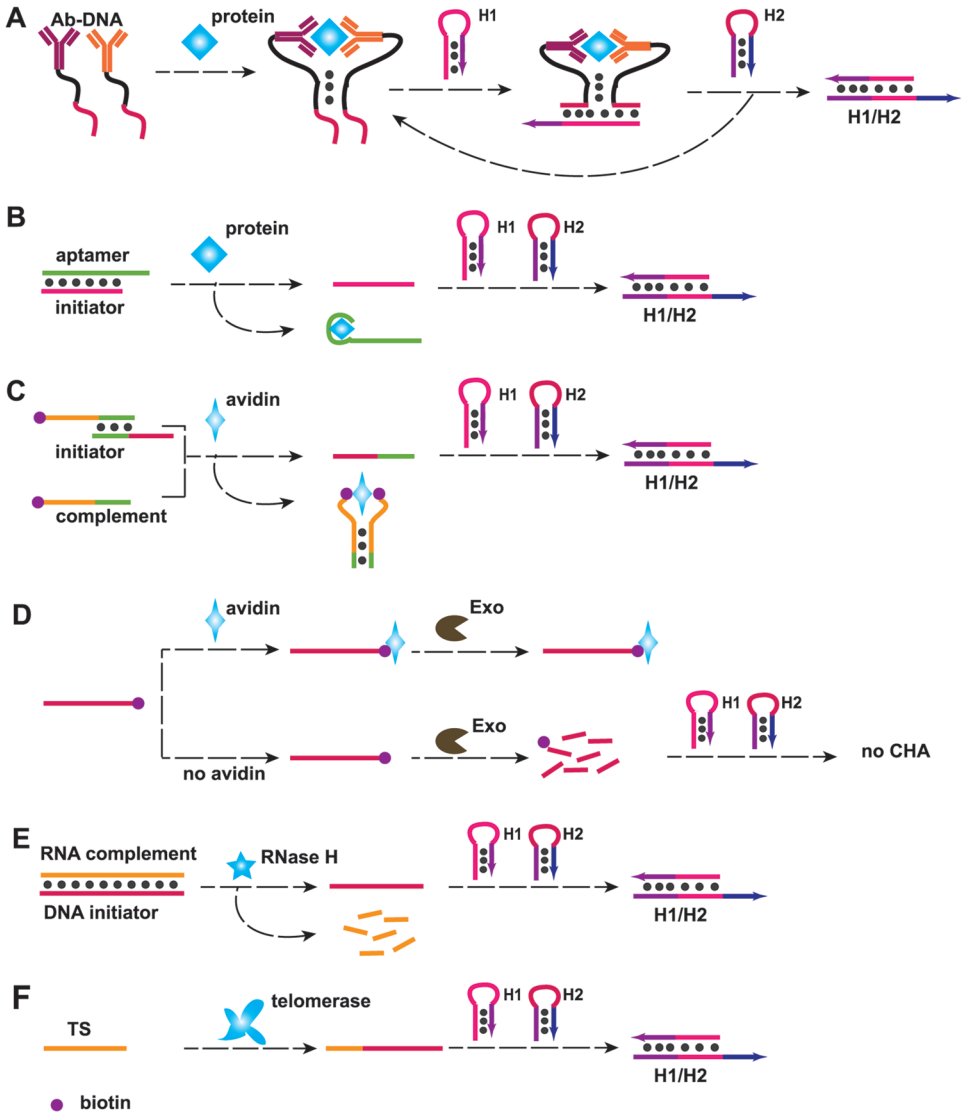

Figure 5. Protein and enzyme biosensors based on CHA. A) Ab-mediated protein biosensor.[105] B) Aptamer-mediated protein biosensor.[111] C) Biotin mediated avidin biosensor.[113] D) Avidin–biotin protection mediated avidin biosensor.[87] E) Enzyme activity detection based on CHA.[115] F) Telomerase assay based on CHA.[28]

of target proteins, the aptamer specifically binds to the target, resulting in the release of the initiator. Hence, CHA amplification and signal output can be observed. Target-induced exposure of the initiator consists of the exposure of the initiator caged in hairpins or duplexes to trigger hairpin hybridization following aptamer–protein binding. Then, the cascade signal amplification of CHA and HCR is successively performed. Finally, the G-quadruplex formed in the long duplex transmits the corresponding fluorescent signals.[112] Target-induced joint binding-initiating is achieved with CHA substrates containing aptamer sequence. The fluorophore labeled hairpins including the aptamer domain are modified on Au NPs to quench the fluorescence. Once the hairpins bind to thrombin, the structure is transformed into a single strand. The newly formed single strand then binds to another hairpin and releases thrombin. The above processes progress through cycles. The fluorescence is recovered because the massive fluorophores are far from the Au NPs.[31]

Beyond the abovementioned protein detection methods, avidin can be used as a CHA mediator with biotin. Avidin binds to biotin with extremely high affinity, and biotin can be easily conjugated to proteins or nucleic acids. Li et al. proposed an avidin detection system including partially complementary DNA duplex OT, single-stranded DNA C, and a pair of DNA hairpins as shown in Figure 5C.[113] Biotin is linked at the ends of O and C. Avidin strongly interacts with biotin, bringing C and OT into proximity, which accelerates the strand displacement between OT and C. Therefore, CHA is initiated by O for signal amplification. Meanwhile, avidin–biotin protects single-stranded nucleic acids from hydrolysis by Exo I. Liu et al. used this characteristic for developing a protein assay. Biotin is prepared at the end of a single-stranded nucleic acid that can be digested by Exo I without a following reaction. In the presence of avidin, the strand stably exists and triggers a subsequent CHA reaction for signal amplification (Figure 5D).[87]

3.2.2. Detection of Enzyme Activity

Enzymes are unique proteins present in body fluids, cells, and tissues. Enzymes enable the catalyzation of substrates with high specificity in many biological processes. Abnormal expression of enzymes results in dysfunction, and accurate detection of enzymes is important for the treatment of diseases. CHA based enzyme biosensors function in a different manner with other biomarkers. Generally, enzymes act on specific substrates, yielding single-stranded DNA to unfold the hairpins of the CHA system. Most enzymes hydrolyze the probes to release the initiators.[114] A CHA based enzyme activity assay is presented in Figure 5E.[115] In the absence of RNase H, RNA/DNA duplex probes are intact, and the partially complementary hairpins are stable in solution. The RNA in the RNA/DNA duplex is prone to hydrolysis by RNase H. Hence, RNase H catalyzes the digestion of the RNA and the release of the DNA. Thereby, the hairpins are successively opened to form duplexes, and signals accumulate. These strategies enable the analysis of enzyme activity at mU mL^{-1} concentrations. Telomerase elongates tandem 6 bp DNA repeats (TTAGGG) at the ends of specific telomerase substrates (TS). Telomerase can hybridize with probes resulting in displacement or migration following the free initiator. As shown in Figure 5F, the CHA based telomerase assay is simple and can be visualized.[28] TS primers are successively added with repeated sequences. Then, the hairpin probes containing the initiator domain in the stem are unfolded by the repeat sequence. The CHA reaction is triggered and the G-quadruplexes at the ends of the products produce signals. CHA based telomerase assays exhibit high specificity and sensitivity, and work well for intracellular telomerase analysis.

3.3. Detection of Small Molecules and Metal Ions

Nucleic acids and proteins are the most common biomarkers. In addition to these two kinds of biomarkers, small molecules such as ATP, adenosine, and antibiotic participate in various biological processes. Metal ions are closely associated with human health and the environmental security. The development of simple and reliable detection methods for these molecules is important. However, these metal ions cannot activate the CHA reaction. Because of the functional nucleic acids, many strategies based on CHA have been explored for these molecules.

Small molecules with simple structures fail to directly transduce targets into a signal output. The appearance of aptamers broadens the analysis techniques for small molecules. Usually, the initiator DNA is protected in probes involving the aptamer domain. The introduction of the target brings a free initiator. The most typical CHA based aptasensor for small molecules is presented in **Figure 6**A.[116] The initiator domain and aptamer domain are predesigned in the probe. The aptamer domain is released along with the conjugation between aptamer and target. With varied signal output platforms, the system signal

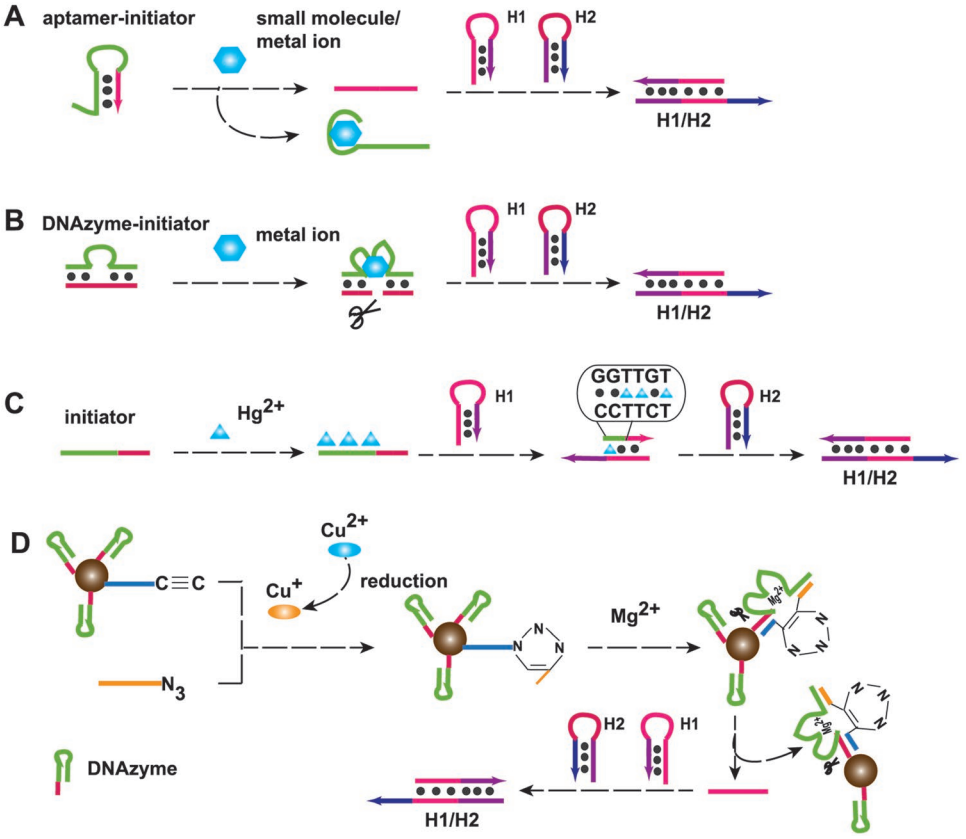

Figure 6. Small molecule and metal ion biosensors based on CHA. A) CHA-based aptasensor for small molecule detection.[116] B) DNAzymes assisted by target metal ions mediated metal ion detection.[124] C) Metallo-toehold induced Hg^{2+} detection.[125] D) CuAAC induced Cu^{2+} detection.[127]

can be delivered through indicators[32] or coupled with another signal amplification method recovering DNAzyme,[117,118] NPs,[119] and HCR-analogous isothermal amplification.[120,121] Li et al. extended the probe containing aptamers and metal ion-dependent DNAzyme for small molecules.[122] DNAzyme is generated with recognition between the aptamer and target. The target-aptamer-DNAzyme can circularly catalyze the substrate to produce accumulation of initiator DNA. Then, the CHA reaction proceeds. This strategy detects ATP as low as 0.6×10^{-9} M and well discriminates ATP from analogous molecules.

Metal ions also perform other roles. In terms of their roles as coenzymes, toehold assistants and mediums of click chemistry, assays for metal ions can be classified into three classes. Some DNAzymes function with metal ions, such as Pb^{2+}, Cu^{2+}, Zn^{2+}, and Na^{+} for cleaving the phosphodiester backbone at ribonucleotide sites on substrate chains.[123] Probes are arranged along the DNAzyme strand and the substrate chain containing initiator domain (Figure 6B).[124] The CHA reaction is activated because initiator DNA is released in the presence of metal ions. Through this strategy, different metal ions can be analyzed simultaneously.

Hg^{2+} can produce a bridge structure (thymine-Hg^{2+}-thymine) in the toehold region as a metallo-toehold. Thus, the rates of the DNA displacement reaction are controlled by Hg^{2+}. This property of Hg^{2+} is brought into full play for Hg^{2+} detection. Li et al. designed a reliable strategy for Hg^{2+} based on the metallo-toehold initiated branched CHA reaction.[125] The system functions using the initiator probe, substrate probe and hairpins, as shown in Figure 6C. When Hg^{2+} is introduced, the initiator probe forms thymine-thymine mismatched base pairs with H1 and activates the follow-up DNA hybridization and displacement. The split DNAzymes are closed alongside the generation of branched DNA junctions, and catalyze the substrate hairpins to generate output signals.

Cu^{+}-catalyzed azide-alkyne cycloaddition (CuAAC) is a typical example of click chemistry with high specificity and efficiency. Therefore, scientists have shown interest in developing a more practical assay for Cu^{2+}. Cu^{2+} can be reduced to Cu^{+} triggering CuAAC.[126] When azide and alkyne are modified onto DNA probes, CuAAC leads to structure switching. In this way, the initiator for CHA emerges. As shown in Figure 6D, CuAAC stimulates the activity of DNAzyme and the initiator is gained. The initiator circularly works toward the accumulation of CHA products and signals. This strategy opens the way for expanding Cu^{2+} assays.[127]

3.4. Detection of Cancer Cells

Cancer cells, derived from primary tumors, are significant and valuable for early cancer diagnosis and the discovery of minimal residual diseases. Cancer cells are also attractive circulating tumor markers. Because of their low concentrations and complicated components, developing accurate and simple cancer cell assays remains challenging. Specific constituents of cancer cells, such as DNA, mRNA, miRNA, and proteins, can provide information on cancer cells. Therefore, the amounts of cancer cells can be measured using the strategies described above. At the same time, cancer cells are intact biomarkers. The existence of cancer cells in blood or tissue specimens is the most direct evidence for cancer diseases. Hence, the quantification of intact cancer cells is important for practical applications. Researchers have found that aptamers and Abs can recognize and capture cancer cells with high specificity and strong affinity.

In previous work, we have constructed a CHA based cytosensor with an aptamer (**Figure 7**A). The system is accessible with the use of a probe and CHA hairpins. Mucin 1 is an overexpressed transmembrane protein in A549 lung cancer cells. Once mucin 1 binds to the aptamer strand of the probe, the initiator domain is exposed. Various CHA systems and analytical platforms can be applied. CHA based aptasensors for cancer cells exhibit high sensitivity down to 10 cell mL^{-1} and satisfactory selectivity between the target and interferent cells.[29] These cytosensors rely heavily on the development of aptamers.

A CHA based cytosensor with Ab recognition elements has been developed for SK-BR-3 breast cancer cells overexpressing HER-2 (Figure 7B).[128] In this system, the two single-stranded probes with split initiator sequences are partially complementary. HER-2-specific Abs are separately conjugated to the two probes through streptavidin–biotin interactions. The two Abs recognize the target cancer cells bringing the two probes into proximity. Then, the two probes hybridize, providing an initiator domain for CHA. This kind of cytosensor can distinguish targets from the isotype control.

3.5. Intracellular Analysis

Monitoring the markers in living cells provides first-hand and dynamic information, which contributes to disease surveillance and biological research. CHA, a nonenzymatic and isothermal

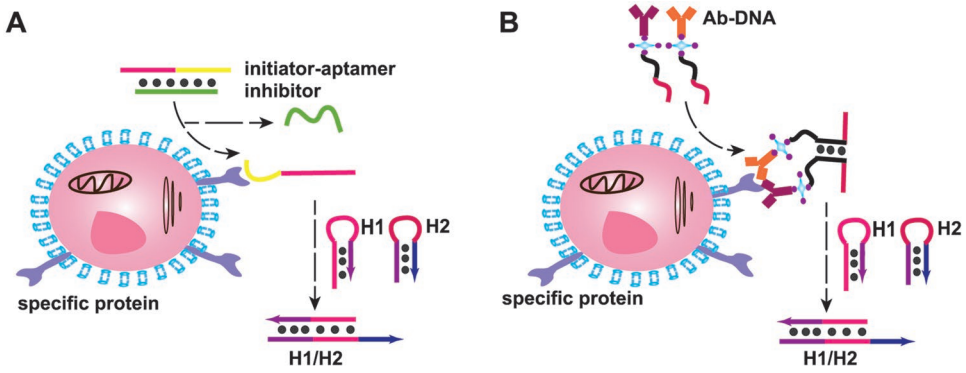

Figure 7. Cancer cell biosensors based on CHA. A) CHA-based cytosensor with aptamer.[29] B) CHA-based cytosensor with Ab.[128]

amplification technique, is valuable for biomarker analysis with high efficacy and promising versatility. The intracellular complexity and interference of endogenous detection still remain some problems. Transport and enzyme degradation are the most prominent problems. Great effort has been made to develop useful intracellular detection methods based on CHA.

The intracellular amplification circuits are almost the same as those used in fluorescent strategies in vitro and have been verified before intracellular determination. Biomarkers including mRNA, miRNA, metal ions, and telomerase, have been imaged with CHA based strategies. Scientists have employed modified nucleic acids[129] or NPs[130] to protect the system components.

Regarding delivery, four methods have been used for CHA systems: endocytosis, transfection reagent induced, nanomaterials mediated, and genetically encoded. Endocytosis is the most direct but risky method, as nucleic acids are prone to being digested by nucleases. The CHA system has been transported into living cells through endocytosis for miRNA-21 imaging.[131] Studies indicate that self-assembled DNA nanowires have cell membrane permeability. Based on this finding, Wei et al. proposed a nanowire bearing CHA substrates (**Figure 8**A). The DNA nanowire serves as a carrier. Then, cells overexpressing miRNA-21 are turned "on" with CHA amplification. The strategy skillfully delivers the CHA system without noxious transfection reagents and provides a satisfactory platform for biomarker imaging in living cells.[43]

In general, transfection reagents such as liposomes, cationic polypeptide G8, and H400 interact with nucleic acids, forming complexes and escaping nucleases. This is the most frequently used method for the delivery of CHA transcytosis. As shown in Figure 8B, the Na^+ detection system based on CHA-DNAzyme is successfully transported into living cells with the help of cationic polypeptide G8.[132] The strategy not only accurately quantifies endogenous Na^+, but also dynamically monitors the change of Na^+. Additionally, this work provides a general method for intracellular biomarker detection. As shown in Figure 8C, GO is the carrier of two sets of CHA substrates, moving them toward the cytoplasm.[26] Then, the CHA reaction is activated by the targets to amplify the signals. The detection limit is low to picomolar level, and strong fluorescence can be detected. This strategy may provide a new method by which to trace intracellular biomarkers.

In recent years, nanomaterials including Au NP, graphene oxide (GO) and carbon nitride nanosheet have been extensively used as biosensors. Nano materials possess good biocompatibility, little toxicity, and strong adsorption. These unique characteristics make nano materials suitable for intracellular biomarker imaging.

Karunanayake Mudiyanselage et al. not only validated CHA with RNA molecules, but also imaged the endogenous target RNA through genetically encoded and transcribed RNA CHA (Figure 8D).[45] The fluorescent RNA molecules Broccoli, which can activate the fluorescence of small molecule dyes, has been

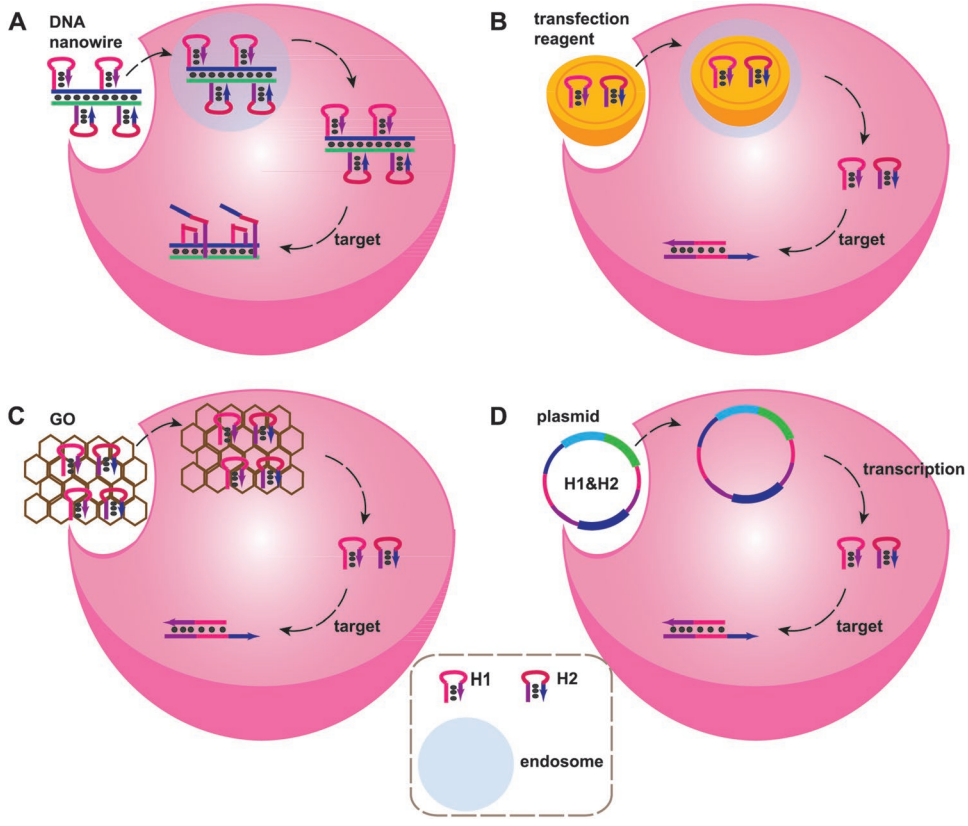

Figure 8. CHA based biomarker imaging in living cells. The CHA system is transported into cells through A) endocytosis,[43] B) transfection agent,[132] C) nano materials,[26] and D) genetically encoded and transcribed RNA CHA.[45]

divided into two parts at the two hairpins separately. First, the researchers designed and constructed the RNA assay in vitro. The strategy presents high sensitivity at concentrations as low as 0.5×10^{-9} M and an efficient and fast response to the target RNA. Then, the two hairpins are cloned into a dual expression vector, and the target sequence is cloned into another vector. The vectors are transformed into cells and induced for 2 h. Finally, the fluorescence signal is observed using a confocal fluorescence microscope. This strategy provided a brand-new method for cellular RNA determination.

4. Conclusions and Perspectives

In the past decade, tremendous achievements have been made regarding CHA based bioanalysis from origin to widespread application. Dozens of expanded CHA systems have been programmed with several nucleic acid strands. Through these efforts, the amplification efficacy was improved, and the background signal was controlled. CHA circuits function in the absence of enzymes under isothermal conditions, showing desirable amplification efficacy. For this reason, CHA circuits have been adapted for the specific and sensitive detection of various targets covering proteins, DNA, miRNA, mRNA, metal ions, small molecules, and cancer cells in vitro or in living cells. Because of its versatility, the CHA reaction can be coupled with NPs, other isothermal amplification techniques, and DNAzymes, achieving cascade signal amplification. Various signal outputs can be used for CHA based biosensors. CHA circuits with unique properties provide diverse biosensors with promising performance and good convenience.

To further extend the applications and make CHA suitable for practice, more efforts need to be devoted to the following points. 1) Various CHA systems possess their own advantages and work in different strategies. We may continue exploring the mechanism of CHA and design more interesting CHA systems to make CHA circuits more widely accessible. 2) Except for nucleic acid targets, most biomarkers require specific recognition and transducer elements. Hence, studies of specific, simple, and stable recognition elements (such as aptamers and Abs) are warranted. Thus, other emerging markers (for example, exosomes and rare cells) will be able to be easily analyzed in vitro or in living cells based on CHA. 3) CHA circuits, with excellent biocompatibility and perfect signal performance, are prone to being combined with novel nanomaterials (such as aggregation-induced emission molecules) and newly molecular biotechnology. For more novel and efficient CHA based assays, more contributions are needed to make full use of these materials. 4) The majority of biomarkers exist in blood or tissue in trace amounts. Absolute quantification technologies play an important role in practical applications and single-cell determination. CHA circuits, with the attractive advantages of high efficiency and constant temperature, are desirable choice for absolute quantification technologies (such as droplet microfluidics and nanoflow cytometry).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

biosensors, catalytic hairpin assembly (CHA), isothermal amplification, nucleic acid circuits, toehold mediated strand displacement

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