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Review

Recent advances in nucleic acid biosensing for the detection of pathogenic microorganisms and disease biomarkers

Zuanguang Chen[1,2,*], Zhixian Liang[2,3], Yiting Huang[1] and Yifei Sun[1]

[1] School of Health Medicine, Guangzhou Huashang College, Guangzhou 511300, China
[2] School of Pharmaceutical Sciences, Sun Yat-Sen University, Guangzhou 510006, China
[3] National Engineering Research Center for Healthcare Devices & Guangdong Provincial Key Laboratory of Medical Electronic Instruments and Materials, Institute of Biological and Medical Engineering, Guangdong Academy of Sciences, Guangzhou 510316, PR China

* **Correspondence:** Email: chenzg@mail.sysu.edu.cn; Tel: +8613286803228.

Abstract: Nucleic acid testing (NAT) is widely used in disease screening and diagnosis due to its high sensitivity and strong specificity. In addition, whole blood circulation analysis has become a promising non-invasive strategy for cancer diagnosis and surveillance. With the growing testing demand and the advancement of new amplification technologies, nucleic acid detection methods are evolving toward simplicity, speed, and cost-effectiveness. As the gold standard in nucleic acid detection, real-time fluorescence quantitative PCR (qPCR) relies on expensive fluorescence reading equipment and professional operators and is not suitable for the rapid diagnosis of infectious diseases and other diseases. Biosensors have attracted the attention of scientists due to their advantages of rapidity, reliability, and cost-effectiveness. They have been widely applied in medical diagnosis, including point-of-care testing, forensic science, and biomedical research. This paper reviews the recent research progress of NAT methods for pathogenic microorganisms and disease markers and points out future prospects of point-of-care testing, with great significance in improving health care and disease surveillance in resource-constrained areas.

Keywords: nucleic acid detection; pathogenic microorganisms; disease markers; biosensors

1. Introduction

The 2019 coronavirus pandemic (COVID-19) has underscored the critical need for a rapid, cost-effective, and reliable large-scale screening method to effectively control and prevent disease spread [1]. However, current diagnostic tools, such as over-the-counter (OTC) antigen detection kits approved by the Food and Drug Administration (FDA), face significant limitations. These rapid home tests, while convenient, exhibit low sensitivity and may produce false negatives in individuals with early or asymptomatic infections [2]. Similarly, serological antibody immunoassays can identify the presence of antibodies produced in response to infection, but they cannot reliably distinguish between active and resolved infections, resulting in diagnostic uncertainty. Consequently, rapid antigen or antibody testing is primarily suitable for presumptive screening, with nucleic acid testing remaining essential for confirmation of positive results.

Nucleic acids, as fundamental biological molecules, are ubiquitously present in all cellular organisms and play a pivotal role in genetic inheritance, mutation, and metabolic processes [3]. Therefore, nucleic acids are important biomarkers for evaluating biological processes and pathogenesis.

Since Watson and Crick proposed the double helix structure of DNA in 1953, modern molecular biology has undergone significant advancements through nucleic acid research. This foundational discovery, which was validated through X-ray diffraction data and model construction, laid the groundwork for understanding genetic mechanisms and molecular interactions. With the in-depth study of gene structure and function, nucleic acid research has become a cornerstone in the diagnosis of genetic diseases in life sciences, gaining increasing attention due to its inherent sequence variability and specificity [4]. Nucleic acid testing (NAT) focuses on specific nucleic acid fragments as detection targets and encompasses multiple stages, including sample processing, nucleic acid extraction, amplification, and product detection. In clinical and biological analysis contexts, nucleic acids are extracted from samples to serve as templates for amplification, providing essential data for disease diagnosis and other analytical applications [5]. Compared with traditional cell detection and immune detection methods, nucleic acid detection offers superior sensitivity, specificity, and speed, positioning it as one of the most valuable analytical techniques in modern diagnostics. It has been widely applied in diverse fields, such as the diagnosis of metabolic diseases (e.g., cancer) and infectious diseases (e.g., HIV and HBV), as well as food safety assurance (e.g., genetically modified organisms and foodborne pathogens).

This article aims to summarize common methods of nucleic acid detection, including polymerase chain reaction (PCR), next-generation sequencing (NGS), and biosensing technology, review their progress, and focus on the potential applications of nucleic acid biosensors in the detection of pathogenic microorganisms and disease markers (Figure 1). Building on this foundation, the second chapter systematically reviews the principles and advancements of nucleic acid detection methods, including traditional techniques like dig, as well as emerging isothermal amplification strategies (e.g., LAMP, RPA, RCA), highlighting their sensitivity, specificity, and applicability in clinical diagnostics and environmental monitoring. The third chapter delves into the design and application of nucleic acid biosensors for pathogen detection, emphasizing their roles in rapid, point-of-care diagnostics for viral and bacterial infections. Finally, the fourth chapter synthesizes the current state of the field, addresses challenges such as signal amplification and overcoming interference substances, and proposes future directions for miniaturized, AI-driven, biosensor systems.

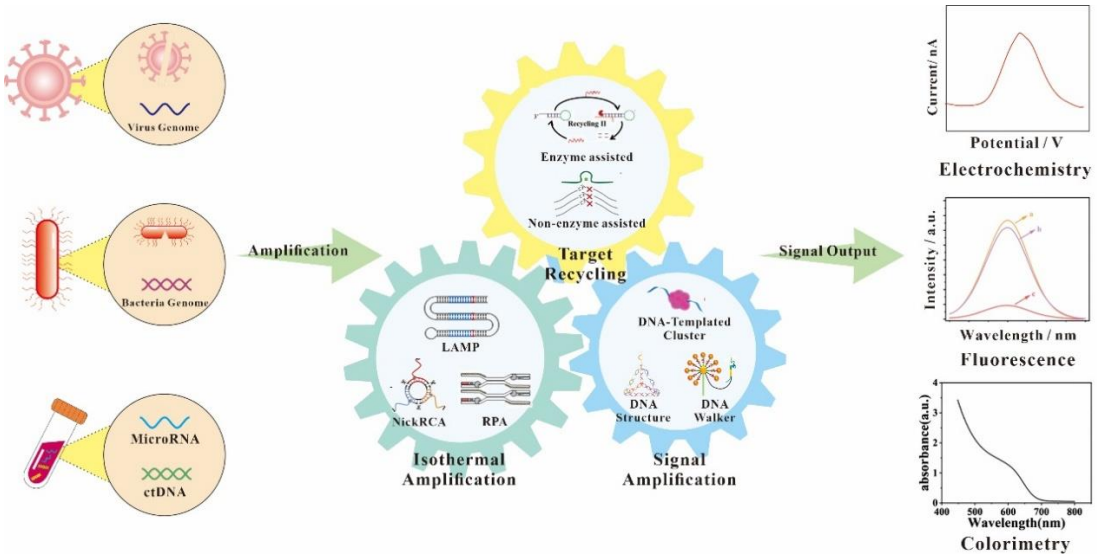

Figure 1. Schematic diagram of nucleic acid biosensors for pathogenic microorganisms and disease biomarkers.

2. Methods of nucleic acid detection

2.1. PCR

To date, the gold standard for nucleic acid detection remains polymerase chain reaction (PCR), a method of enzymatic amplification that isolates DNA strands containing gene fragments, marks their positions with primers, and utilizes DNA polymerase to extend new double strands, thereby generating a large number of DNA fragments [6]. PCR is widely used to amplify trace amounts of nucleic acid targets in order to provide sufficient specimens for laboratory studies. However, traditional PCR methods are time-consuming, labor-intensive, and require specialized equipment and trained operators, making the development of fast and economical alternatives particularly important.

In recent years, microfluidic technologies have revolutionized on-chip PCR devices. Digital droplet PCR has emerged as a highly sensitive and quantitative method, capable of absolute quantification of target nucleic acids without the need for a standard curve. This technique partitions samples into thousands of nanoliter-sized droplets, enabling precise detection of rare targets and minimizing contamination [7]. Therefore, droplet-based microfluidic technology has attracted widespread attention, especially digital PCR technology, which has the advantages of high throughput and high sensitivity, and is directly used in the analysis of genetic material without the need for DNA purification [8]. For instance, Yin developed a multiplexed digital PCR chip that can simultaneously detect six target genes by pre-introducing specific probes and primers, addressing the challenges of long duration and high cost in multi-target detection (Figure 2). In addition, these samples can be divided into multiple parts and amplified separately in each part to achieve multiplexed detection, playing an important role in the field of disease diagnosis [9]. During droplet generation, bubble formation due to air ingress can cause droplet aggregation, a common issue in microfluidic systems [10]. Jiang proposed a fountain-like structure for a ddPCR chip, which eliminates bubbles before amplification, enhancing sample throughput and enabling processing of up to 48 samples at once. This design holds

significant potential for high-throughput clinical diagnostics and personalized medicine [11].

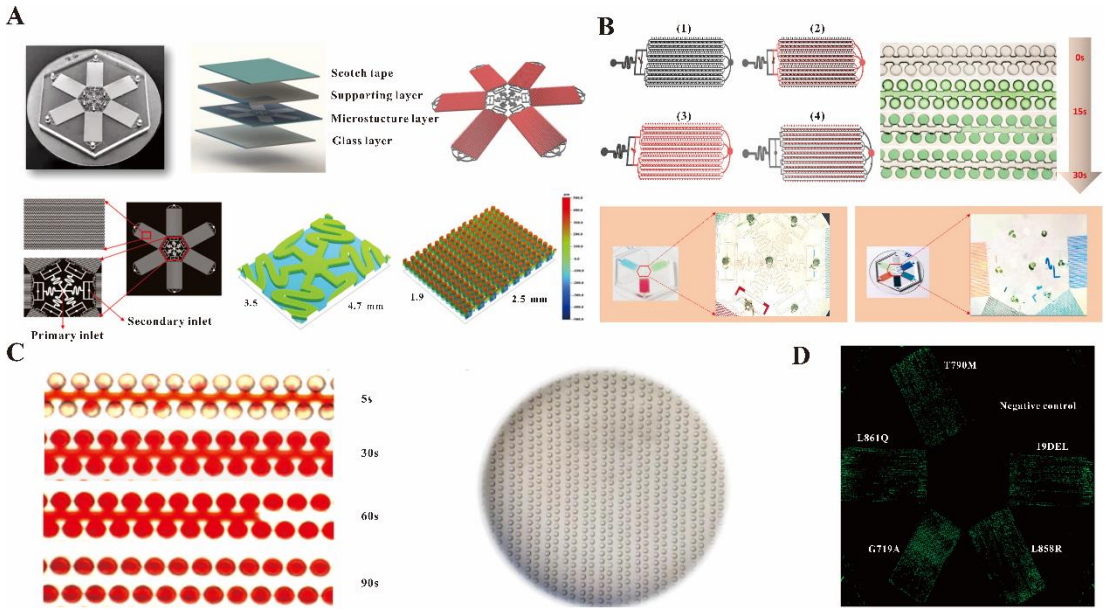

Figure 2. Multiplex digital PCR is used for disease diagnosis. (A) Design and structure of the multiplex digital PCR chip. (B) Pre-introduction of a reaction mix into each detection area. (C) Photographs of the self-priming process and the microchamber in the chip after the self-priming process. (D) Detection of a 5-plex using this multiplex digital PCR chip [9].

The integration of PCR into microfluidic platforms has further advanced molecular diagnostics. Continuous-flow PCR and droplet-based systems have reduced reaction times and enabled parallelization, while digital PCR has demonstrated superior performance in complex samples compared to quantitative PCR. These innovations align with the broader trend of developing portable, point-of-care devices for rapid nucleic acid detection, particularly in scenarios requiring high sensitivity and specificity, such as infectious disease diagnostics.

2.2. NGS

NGS represents a significant advancement in DNA sequencing technologies, enabling the simultaneous sequencing of thousands to millions of DNA fragments, thereby revolutionizing DNA sequencing methodologies and expanding researchers' understanding of genomic structure, function, and evolution. Unlike traditional Sanger sequencing, which sequences DNA fragments one at a time, NGS facilitates parallel sequencing of multiple fragments, offering higher throughput and efficiency [12].

Several widely adopted second-generation sequencing platforms have emerged, each employing distinct technological principles. The Roche 454 sequencing method, for instance, utilizes pyrosequencing technology, which detects the release of pyrophosphate during nucleotide incorporation into DNA templates, generating light signals for sequence determination [13]. In contrast, the Ion Torrent platform employs semiconductor-based detection, measuring pH changes resulting from hydrogen ion release during DNA synthesis [14]. Meanwhile, the Illumina sequencing platform leverages reversible dye terminators, where fluorescently labeled nucleotides are incorporated into

DNA strands, and the emitted fluorescence is captured to identify base sequences [15]. These technologies have facilitated breakthroughs in whole-genome sequencing, transcriptome analysis, and targeted sequencing, advancing studies on gene variants, disease mechanisms, and personalized healthcare. For example, Illumina's sequencing-by-synthesis approach, combined with high-density flow cell clustering, allows for gigabase-scale data generation per run, while Ion Torrent's semiconductor detection offers rapid and cost-effective solutions for targeted applications. Roche 454, though initially dominant, faced challenges with homopolymer detection and was eventually superseded by newer platforms like Illumina and Oxford Nanopore, which provide longer reads and improved accuracy.

The widespread adoption of NGS has transformed genomics, enabling cost-effective, high-throughput sequencing that supports large-scale population studies and precision medicine initiatives. Despite limitations such as PCR bias and repetitive region sequencing, NGS remains a cornerstone of modern genetic research, driving innovations in cancer diagnostics, infectious disease surveillance, and evolutionary biology.

2.3. Nucleic acid detection by biosensing

In the rapidly evolving field of nucleic acid detection technologies, the emergence of biosensors has catalyzed a transformative technological revolution. Biosensors, representing the convergence of life sciences and information science, exhibit high sensitivity to biological substances and possess the capability to transduce analytes into measurable acoustic, optical, and electrical signals. Nucleic acid-based biosensors offer numerous advantages for genetic testing, including rapid analysis times, streamlined preprocessing protocols, and minimal sample requirements. Currently, three primary strategies have been identified to enhance the sensitivity of nucleic acid-based biosensors: nucleic acid amplification (not discussed in the previous section on PCR), target recycling, and signal amplification. These strategies collectively encompass the design principles of most nucleic acid biosensors.

2.3.1. Isothermal amplification

Isothermal amplification (IA) is a technique that enables the streamlined exponential amplification of target DNA or RNA at a constant temperature, offering a simplified and accelerated alternative to traditional PCR methods. Unlike conventional PCR, which requires thermal cycling, IA eliminates the need for temperature fluctuations, significantly reducing reaction time and complexity. Herein, we review different isothermal amplification methods, showing their potential in the realization of biosensors with reasonable cost-effectiveness, robustness, sensitivity, and specificity (Table 1).

Among the various IA techniques, loop-mediated isothermal amplification (LAMP), developed in 2000, stands out for its robust performance. LAMP utilizes four to six primers designed to recognize six distinct regions of the target gene, along with Bst DNA polymerase with strand-displacement activity, to achieve rapid amplification within 30−60 min at 60–65 °C. The reaction generates stem-loop DNA structures and produces a visible magnesium pyrophosphate precipitate, enabling visual detection without electrophoresis. LAMP has demonstrated high specificity and sensitivity, with a detection limit (LOD) as low as 1 copy/μL of template, making it suitable for field applications and point-of-care testing (POCT) [16]. During the COVID-19 epidemic, Lalli developed a rapid

colorimetric detection method using reverse transcription loop-mediated isothermal amplification (RT-LAMP) technology and optimized human saliva samples to achieve 90% accuracy without the need for RNA purification steps [17].

In addition, two isothermal amplification techniques, recombinase polymerase amplification (RPA) and rolling circle amplification (RCA), were employed in NAT. RPA leverages a protein–DNA complex formed by the binding of recombinase proteins and primers to identify homologous sequences in double-stranded DNA (dsDNA). This initiates a strand exchange reaction, followed by DNA synthesis and exponential amplification of the target. Operating at 37–42 °C, RPA achieves detectable amplification within 10 min, enabling rapid detection of nucleic acids. Its advantages include high specificity, tolerance to impure samples, and compatibility with microfluidic devices for point-of-care (POC) applications. For instance, Xu coupled RPA with CRISPR-Cas12a and multiplex RPA, creating a microfluidic device (named MiCaR) to detect 30 nucleic acid targets simultaneously, achieving a sensitivity of 97.8% and specificity of 98.1% at a LOD of 0.26 aM in 40 min (Figure 3A) [18]. Similarly, Ying applied RPA to amplify the spore wall protein gene of *Bacillus hepaticus* in shrimp, completing the detection process within 30 min without cross-reactivity, with a LOD of 7 copies per reaction [19]. Li developed a rapid, low-cost, and user-friendly method for the early diagnosis of *M. pneumoniae*-CRAFT (CRISPR-based rapid assay device for field testing). The CRAFT analysis device accomplishes the entire process, namely sample pretreatment, pathogen lysis, DNA extraction, RPA, CRISPR/Cas12a reaction, signal acquisition, and result interpretation. This platform was able to detect as low as 10 copies of DNA, holding significant potential for POCT of *M. pneumoniae*, particularly in resource-limited settings [20].

RCA, on the other hand, amplifies circular DNA templates using a strand-displacing DNA polymerase and a single primer, generating long single-stranded DNA (ssDNA) with tandem repeats. It operates at 37–60 °C and is particularly suitable for detecting pathogens like HPV and influenza due to its high sensitivity and specificity. Fang et al. demonstrated RCA's utility in HIV and let-7a detection, achieving a LOD of 8.3 pM and 89.5% specificity [21]. Taking advantage of highly efficient primer generation RCA-PER/Cas12a amplification and inherent high selectivity, Ju et al. developed a fluorescence biosensing strategy for sensitive detection of miR-141 based on cascade amplification RCA-PER and Cas12a (Figure 3B). This method can detect as low as 94 fM [22]. Both RPA and RCA offer advantages over traditional PCR, including simplified protocols, reduced equipment requirements, and suitability for low-resource settings. However, challenges such as primer design complexity and nonspecific amplification remain areas for improvement.

Table 1. Comparison of the principles and advantages of different isothermal amplification methods.

Technology	Principle	Advantage
LAMP	Using 4–6 specific primers, designed for 6 regions of the target gene, the strand displacement activity of Bst DNA polymerase is relied on to conduct isothermal amplification at 60–65 ℃, achieving a nucleic acid amplification of $10^{\wedge 9}$ to $10^{\wedge 10}$ within 15–60 min.	Short reaction time (15–60 minutes) High sensitivity and specificity No complex equipment required, suitable for on-site testing Products can be observed by the naked eye Can be combined with reverse transcription to detect RNA
RPA	Relying on recombinase, ssDNA binding protein (SSB), and strand displacement DNA polymerase, isothermal amplification is carried out at 37–42 ℃, and detectable levels of products can be obtained within 10 min.	Low-temperature operation (without the need for a high-temperature denaturation step) High sensitivity and specificity Suitable for amplification of small DNA fragments Enables rapid multiplex detection
RCA	Using circular DNA as the template, dNTPs are catalyzed by phi29 DNA polymerase to be converted into ssDNA, resulting in long-chain products (>20 kb) with a sensitivity reaching the single-copy level.	High amplification efficiency (single molecule detection) The product is ssDNA, making it easy to detect Applicable to circular DNA templates
HDA	Simulates the process of DNA replication in the body. Uses helicase to unwind the dsDNA at 64 ℃ and then uses single-strand binding protein to stabilize the single strand, achieving exponential growth of the target sequence.	Short reaction time (approximately 100 min) High sensitivity (single-copy detection limit) No need for high-temperature denaturation step Simpler than LAMP (only requires one primer pair)
NESA	Based on the recognition sites of restriction endonucleases and the chain-replacement activity of DNA polymerase, isothermal amplification was carried out at 37 ℃	Short reaction time (approximately 30 min) High sensitivity and specificity No special equipment required
MCDA	By designing multiple sets of specific primers (such as 10 primers covering multiple regions of the target sequence), the chain replacement reaction is utilized to achieve exponential amplification of the target DNA	Short reaction time (12–40 min) High sensitivity and specificity Applicable to the detection of multiple pathogens
NASBA	By using reverse transcriptase, T7 RNA polymerase, and RNase H for amplification at 42 ℃, this method is suitable for the detection of RNA viruses.	Suitable for RNA detection High sensitivity and flexibility The product is single-stranded RNA, unaffected by DNA interference

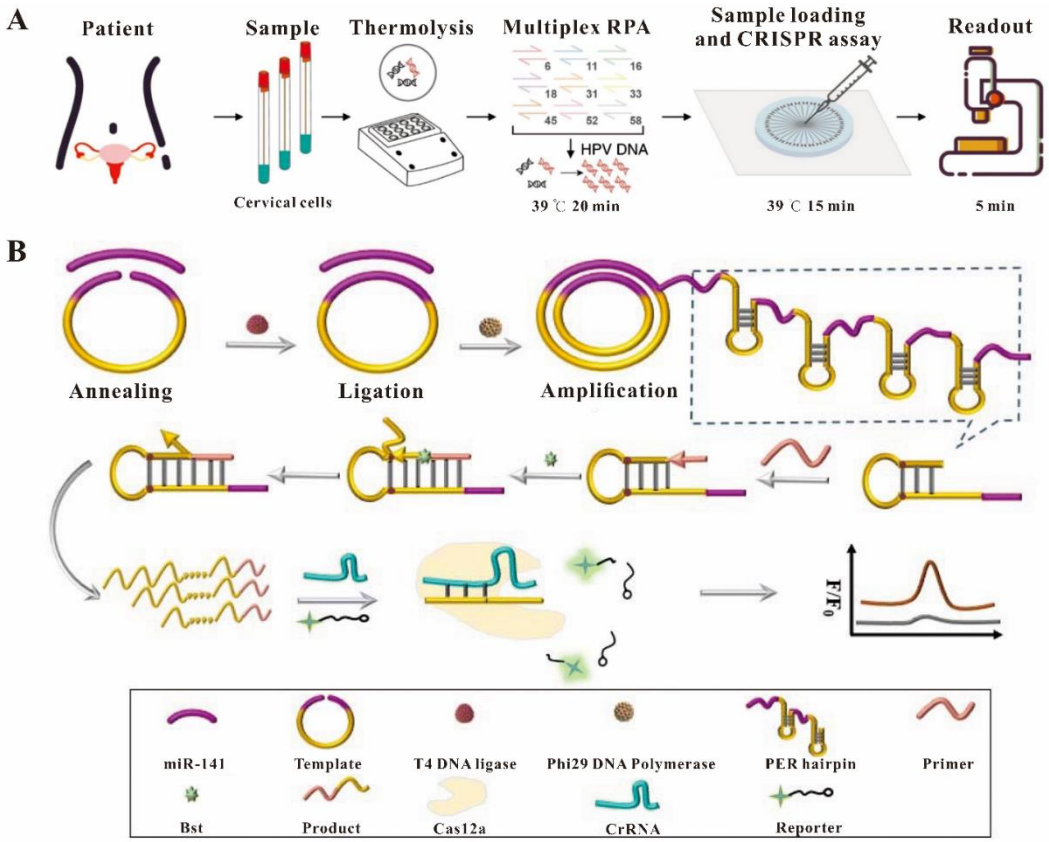

Figure 3. (A) Scheme of the MiCaR-based HPV subtyping strategy [18]. (B) Fluorescence biosensing strategy for sensitive detection of miR-141 based on cascade amplification RCA-PER and Cas12a [22].

Other isothermal amplification techniques have been introduced into the construction of biosensors to generate large quantities of copies at minimum time and constant temperature, such as helicase-dependent amplification (HDA) [23], endonuclease-assisted amplification (NESA) [24], multiple cross-displacement amplification (MCDA) [25], and nucleic acid sequence-based amplification (NASBA) [26]. Despite their impressive performance, further optimization is needed to balance detection efficiency with integration, cost, and scalability.

2.3.2. Target recycling

The strategy based on nucleic acid cycling leverages base pairing interactions to overcome the traditional limitation of the signal-to-background ratio (1: 1), converting it to a 1 to n format to enable effective signal accumulation. In this approach, the same target nucleic acid is repeatedly bound to the signal tag without increasing its copy number, thereby generating amplified signal output [27]. This technology distinguishes itself through its programmability, simplicity, speed, and low cost. The nucleic acid recycling strategy primarily employs enzyme-assisted reactions, specifically using nucleases to identify and digest DNA complex structures, thereby releasing target nucleic acids. This release mechanism facilitates signal conversion within the sensor, promotes the binding of target

nucleic acids to additional substrates, triggers the recognition signal output process in a single cycle, achieves signal amplification, and enhances sensitivity.

Wang et al. designed an electrochemiluminescence (ECL) sensor for sensitive miRNA-499 detection based on dual-specificity nuclease DSN and toehold-mediated strand displacement (TMSD). The DSN-mediated cyclic reaction converts unstable miR-499 into stable DNA strands, while TMSD simplifies the experimental process and enhances sensitivity. The sensor exhibits a linear response range of 100 aM to 100 pM with a detection LOD of 69.99 aM [28].

Tian et al. integrated exonuclease III (Exo III)-assisted nucleic acid cycling with dynamic DNA nanomachines (DNAzyme/AuNPs) to develop an electrochemical biosensor for hepatitis B virus (HBV) DNA detection (Figure 4A). The sensor initiates the Exo III reaction by targeting the complementary strand of DNAzyme on gold nanoparticles, activating multivalent DNA nanomachines to cleave ssDNA modified with the signal molecule ferrocene (Fc) on the sensor surface. This process induces current changes, demonstrating innovative coordination between Exo III-mediated signal amplification and the spatial confinement effect of multivalent DNAzyme nanomachines, significantly improving reaction and amplification efficiency. The method achieves rapid operation (within 80 min) and a low detection LOD of 45 aM [29].

Unlike Exo III, Lambda exonuclease (λ-Exo) promotes the transformation of dsDNA to ssDNA by catalyzing the gradual degradation of the 5' phosphorylated strand in the 5'–3' direction in dsDNA. Liu et al. constructed an electrochemical biosensor (λ-eLCR) based on λ-Exo and ligase chain reaction (LCR) for high-sensitivity CYP2C19*2 allelic genotyping in zero background (Figure 4B). Specifically, during the LCR ligation process, the presence of the target DNA triggers the production of the linked dsDNA product, activating the λ-Exo cleavage reaction to obtain the linked methylene blue (MB)-labeled ssDNA probe. The product then hybridizes with a moderately dense capture probe on the gold electrode, causing the MB mark to approach the surface of the gold electrode, thereby generating an electrical signal. This sensor successfully distinguishes CYP2C19*2/*2, CYP2C19*1/*2 from CYP2C19*1/*1 in human whole blood samples [30]. Two years later, the same team added RecJf exonuclease (λ-RecJf exo) to enhance the degradation ability of the target complex and constructed a second-generation λ-eLCR, which was improved by 3 orders of magnitude from the previous generation λ-eLCR and could detect as low as 0.01% of EGFR-L858R mutations, with the LOD as low as 0.8 fM. This provides a powerful alternative to the diagnosis and personalized medical treatment of point mutation–related diseases [31].

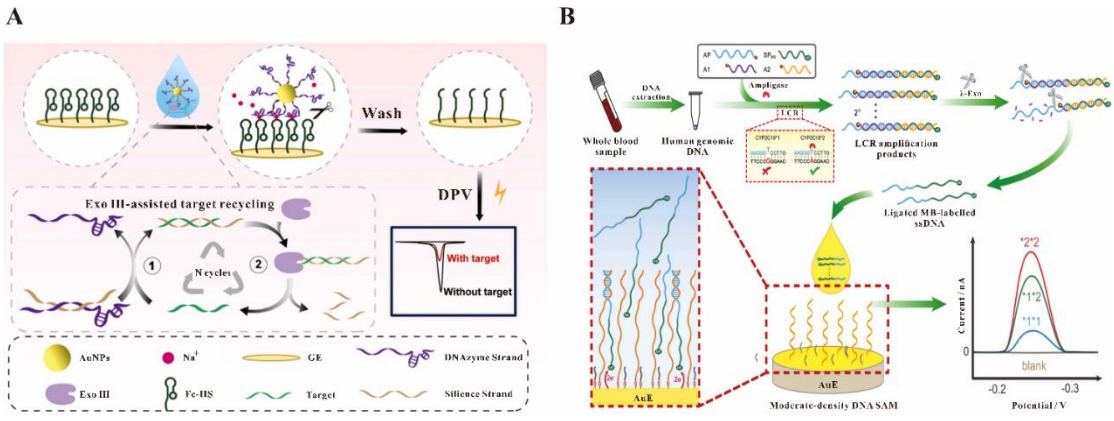

Figure 4. Nucleic acid biosensors based on (A) Exo III [29] and (B) λ-Exo [30].

In addition to enzyme-assisted target cycling reactions, catalytic hairpin assembly (CHA) and toehold-mediated strand displacement (TMSD) are two classic enzyme-free nucleic acid cycling mechanisms that significantly enhance the performance of enzyme-free biosensors [32]. TMSD operates on the principle of strand displacement amplification (SDA), utilizing ternary DNA complexes and fuel strands to replace the double-stranded complex formed by the template and target (invading chain) with a more stable duplex, thereby releasing the target to initiate new SDRs. Notably, TMSD initiates from short-chain precursors, with the reaction driven by configurational entropy reduction, classifying it as an entropy-driven chain displacement process [33].

Building on this foundation, Liu et al. integrated miR-21-induced bipedal DNA walkers as the initiation chain for TMSD, coupling them with amino-modified perylene-3,4,9,10-tetracarboxydianhydride/lumino nanocomposites to construct an ECL sensor. This system achieved ultra-sensitive miR-21 detection with a LOD as low as 33 aM (Figure 5A) [34]. Similarly, Bai et al. incorporated double-footprint and bulge-loop (DT/BULGE) probe designs into the TMSD ternary complex, leveraging their specificity to create a co-chain displacement circuit. This innovation enabled precise single-nucleotide polymorphism (SNP) analysis, achieving low-abundance detection of cancer-related genes (EGFR T790M, EGFR L858R, KRAS G12D) at 0.05%–0.1% mutation rates, offering a robust platform for disease diagnosis [35].

CHA is another typical enzyme-free chain displacement reaction strategy, originally designed by Pierce et al. to assemble two complementary hairpin structures only upon target miRNA binding. Unlike traditional hybridization chain reactions (HCR), CHA employs a foot-mediated mechanism where the first hairpin's opening catalyzes the assembly of the second, enabling continuous target recycling and exponential signal amplification. Recent advancements include localized CHA (LCHA), which immobilizes hairpins on nanomaterials (e.g., DNA cubes, nanowires, DNA tetrahedrons) to enhance substrate concentration and sensor sensitivity [36]. For instance, Hou et al. anchored CHA hairpins on dendritic macromolecules, yielding a DOCO-CHA platform with 16.4 pM LOD and 96.3%–99.3% serum recovery for miR-21 detection [37]. Based on the triple amplification effect of domino-type localized CHA (DT-LCHA), Li et al. constructed a new electrochemical sensor for the detection of exosome-derived miR-1246 (Figure 5B). Taking advantage of the local high concentration, the embeddability of signal molecules, and the integrity of DNA nanowires, the sensor is able to detect miR-1246 at concentrations as low as 24.55 aM within 20 min [38].

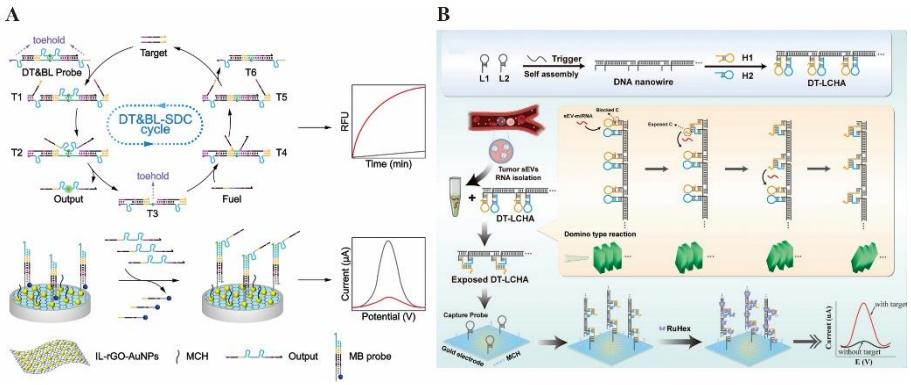

Figure 5. (A) ECL biosensor for miR-21 detection based on classic TMSD [34]. (B) A sensitive and rapid electrochemical biosensor for sEV-miRNA detection based on domino-type LCHA [38].

2.3.3. Signal amplification

The first two strategies enhance the intrinsic signal of the nucleic acid itself, while the third strategy leverages alternative signals to amplify the existing nucleic acid signals. Signal amplification strategies, particularly those based on nanotechnology, represent a primary focus in nanotechnology applications. The rapid advancement of nanotechnology and materials science has introduced numerous novel nanomaterials, offering unprecedented opportunities for biosensing applications.

Due to their exceptional physical, chemical, electronic, optical, and mechanical properties, nanomaterials effectively complement various nucleic acid amplification experiments by converting and amplifying trace nucleic acid signals into detectable forms. Currently, nanomaterial-based signal amplification biosensors are widely employed in NAT [39], exemplified by nanoenzymes, fluorescence-emitting nanoclusters/quantum dots, and photomagnetic composite materials. For instance, Yu et al. developed a novel photothermal sensor for miR-21 and miR-155 detection by integrating DNA amplification with cerium dioxide@gold nanoparticles (CeO_2@Au). This sensor utilizes a pen thermometer to translate biomolecular amplification and computational interactions into temperature readings, achieving a LOD of less than 10 pM, which provides a promising avenue for lung cancer diagnosis [40]. Similarly, Tsai et al. incorporated polycytosine AT-TA sequences into PCR primers, enabling the formation of copper nanoclusters (DNA–CuNCs) from DNA templates during amplification (Figure 6A). This approach eliminates the need for traditional PCR purification and electrophoresis, saving time and labor. The DNA–CuNCs exhibit a large Stokes shift, effectively reducing background interference. Applied to tuberculosis (MTB) IS6110 detection, this method achieved a LOD of 0.12 fM with a fluorescence signal reading time of less than 3 min [41]. Bai et al. synthesized a composite material (Au-nano-C_{60}/NGS) using fullerene (C_{60}), nitrogen-doped graphene nanosheets (NGS), and AuNPs as an electrical signal label for amplification (Figure 6B). The nanocomposite demonstrated excellent electrical conductivity and redox activity, yielding a LOD of 3.0 aM for target nucleic acid detection [42].

Similarly, the rapid advancement of nanotechnology has continuously driven the innovation and refinement of DNA nanomaterials, offering novel solutions for NAT. Taking advantage of the precise base-pairing specificity of DNA and its ability to self-assemble into unique three-dimensional structures, DNA nanomaterials can selectively recognize and bind target nucleic acids at the single-nucleotide level. This capability effectively eliminates background interference in complex samples while enabling highly sensitive signal amplification [43]. DNA nanostructures, including tetrahedrons, cubes, DNA tweezers, DNA walkers, and nanowires, have demonstrated broad applicability in drug delivery and molecular diagnostics. Among these, signal amplification strategies based on DNA tetrahedrons and DNA walkers have garnered significant attention for biosensor development.

DNA tetrahedrons are assembled by annealing four partially complementary DNA strands, forming rigid, enzyme-resistant nanostructures with excellent cellular permeability. To address the sensitivity limitations of electrochemical biosensors caused by electrode surface congestion, Fan's team pioneered the immobilization of DNA tetrahedrons on electrode surfaces, achieving ultrasensitive nucleic acid detection. Compared to single-stranded probes, DNA tetrahedrons facilitate stronger target binding, thereby enhancing detection sensitivity and reproducibility [44]. Through sequence modification and end-functionalization, researchers have engineered DNA tetrahedrons with diverse functionalities. For instance, Tian et al. utilized a DNA tetrahedron as a scaffold to anchor biotin at three anchor points, forming a stable sandwich structure with a covalent organic framework and

circular RNA (Figure 7A). By coupling this with horseradish peroxidase–catalyzed H_2O_2–TMB reaction, they achieved femtomolar-level detection of circular RNA (60 fM) [45] .

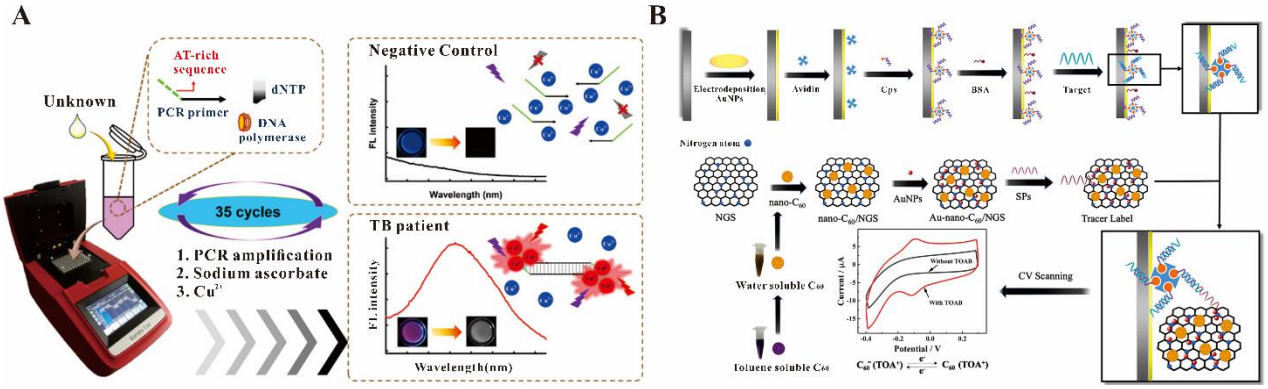

Figure 6. (A) Smartphone-assisted imaging analysis of the PCR-amplified product of the IS6110 sequence of TB using an AT-rich primer to simultaneously synthesize fluorescent dsDNA-CuNCs [41]. (B) Electrochemical biosensor for *Mycobacterium tuberculosis* IS6110 fragment detection using Au-nano-C_{60}/NGS [42].

Based on the spatial confinement effect, Jia et al. integrated specially designed hairpins into DNA tetrahedral nanostructures and developed an ECL sensor using a DNA tetrahedron-mediated three-dimensional DNA nanonetwork for the detection of two miRNAs and the identification of cancer cell subtypes (Figure 7B). Their ECL sensor, mediated by DNA tetrahedron-assisted catalytic hairpin assembly (DTCHA), exhibited faster kinetics and higher cyclic conversion efficiency than traditional CHA reactions. The three-dimensional DNA nanonetwork loaded with $Ru(phen)_3^{2+}$ significantly enhanced the ECL signal, enabling detection below the femtomolar threshold [46].

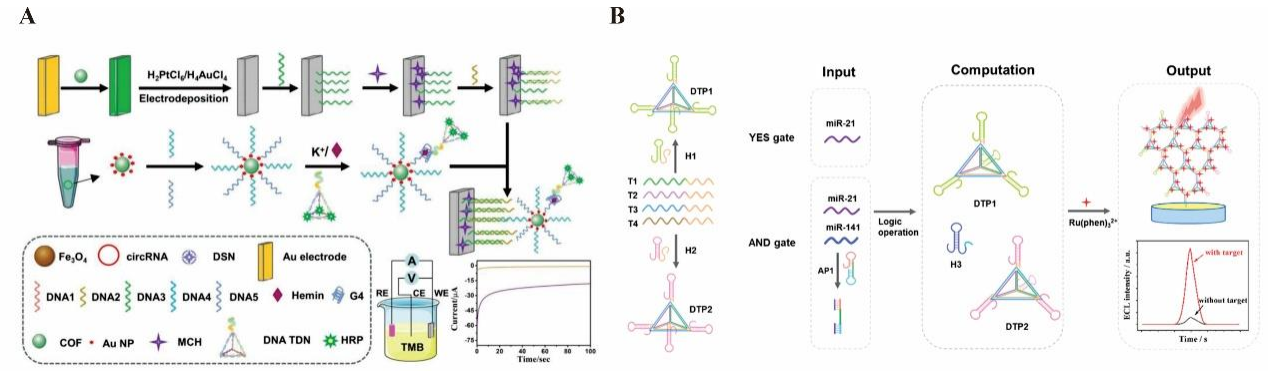

Figure 7. Electrochemical biosensors based on tetrahedral multi-anchor points (A) and spatial effects(B) are used for the detection of circular RNA and miRNA, respectively [45,46].

DNA walkers are dynamic DNA nanomachines capable of amplifying detection signals of individual DNA strands through stepwise progression driven by biomarkers [47]. Specifically, upon recognition of biomarkers, DNA walkers convert chemical energy into mechanical energy, enabling

autonomous motion along pre-assembled tracks. By performing repetitive stepping actions, DNA walkers generate amplified signals to characterize biomarker concentrations. Based on their powerful signal amplification capabilities, DNA walkers have emerged as a critical strategy for biosensors to detect low-concentration nucleic acid markers with high sensitivity. Driven by continuous toehold displacement, Miao et al. pioneered a DNA bipedal walker with dumbbell structural transformation for tumor circulating DNA (ctDNA) detection. This method initiates multiple reaction zones via DNA hairpin-mediated strand displacement reactions, facilitating the release of two reaction zones and transitioning into bipedal walkers for dual signal amplification (Figure 8A). The LOD for this approach is as low as 2.2 aM, demonstrating single-base resolution [48]. Beyond toehold-driven mechanisms, DNA walkers can also be propelled by enzymes or deoxyribozymes. For instance, Cui et al. utilized the aforementioned DNA bipedal walkers with the endonuclease Nb.BbvCI as the driving force to construct a photoelectrochemical sensor for ctDNA analysis. The LOD of this sensor was 0.31 fM, with a sample recovery rate of 96.8%–103.6% [49]. Similarly, Hou et al. designed a multifunctional DNA walker (MLDW) for ultrasensitive electrochemical biosensors targeting miRNAs. In this system, target miRNA competes to release loop template primers via strand displacement, subsequently activating RCA under T4 ligase and polymerase action to generate numerous MLDWs. These walkers traverse the electrode surface, cleave specific RNA phosphodiester chains, and release signal molecules, inducing electrochemical signal changes for sensitive miR-21 detection (Figure 8B). Benefiting from its multi-functional domains, small steric hindrance, and large reaction space, the MLDW-based sensor reduces detection time by fourfold and achieves an LOD of 36 aM, offering a novel perspective for nucleic acid signal amplification [50].

In addition, with the emergence of signal amplification technology over the past decade, the CRISPR/Cas system (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein) has emerged as a prominent tool in in vitro diagnostics. This technology stands out due to its highly compatible integration with isothermal amplification methods, such as LAMP and RPA. As a third-generation genome editing tool, the CRISPR/Cas system has demonstrated broad application prospects in gene imaging and biosensing, owing to its ability to precisely recognize and cleave dsDNA [51]. Unlike traditional restriction enzymes, RNA-guided cleavage of Cas effector proteins allows for flexible target adaptation by simply redesigning spacer sequences, without requiring modifications to the Cas proteins themselves.

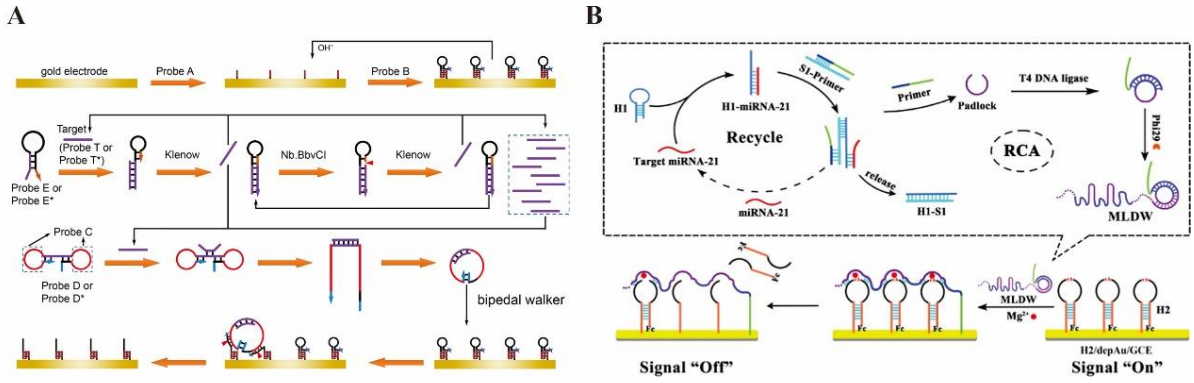

Figure 8. Toehold displacement-based (A) and enzyme-driven (B) DNA walker for biosensor construction [48,50].

Recent advancements have shown that Cas proteins from various families, such as Cas9, Cas12, and Cas13, exhibit high sensitivity and specificity for nucleic acid detection [52]. Notably, Cas12 and Cas13 not only possess the ability to cleave target nucleotides but also exhibit trans-cleavage activity against non-target ssDNA or RNA, a discovery that has expanded their utility in biosensing. Using this feature, researchers have developed a number of detection systems for nucleotides, such as SPARC [53], ID-CRISPR [54], HOLMES [55,56], SHERLOCK [57,58]. As the scientific community's understanding of the potential of Cas nucleases deepens, the potential of CRISPR/Cas systems in the field of biosensing has been demonstrated in the actual COVID-19 pandemic scenario [59,60]. In this context, it has become possible to develop new molecular, portable, and rapid detection sensing systems for early diagnosis of pathogenic microorganisms and diseases based on CRISPR signal amplification technology.

Finally, the three strategies can be combined to further enhance NAT sensitivity and improve the reliability of disease diagnosis. Sen et al. developed a LAMP-CRISPR integrated diagnostic platform for SARS-CoV-2 RNA detection, leveraging LAMP's simplicity and CRISPR's specificity. The CRISPR-associated protein Cas12a eliminates false-positive signals from LAMP amplification products, enabling highly selective and sensitive detection. This platform can accurately distinguish target pathogen sequences from genetically similar non-target sequences, achieving a LOD as low as 50 copies/μL [61].

Hu et al. designed a fluorescent nucleic acid sensor integrating RPA, multi-component nuclease (MNAzyme)-assisted nucleic acid circulation, and quantum dots. The sensor completes target nucleic acid amplification in 20 min through recombinase-assisted DNA melting. The target binds to a quantum dot fluorescent probe (QD-NB) to form a complex, which is subsequently cleaved by MNAzyme in the presence of Mg^{2+}. After cleavage, the intact target nucleic acid is released for the next cycle, while the quenching group in QD-NB is removed, producing strong green fluorescence observable under UV light. This multi-coordinated fluorescent sensor has been successfully applied to high-sensitivity detection of the *MTB* IS1081 gene, with an LOD of 3.3 Am [62]. A year later, Hu et al. introduced a novel, fast, sensitive, and low-cost fluorescence and visual detection platform for tuberculosis based on RPA-CHA-assisted dual-signal amplification strategy and quantum dot nanoprobes. The single-strand activation of the CHA reaction, triggered by DNA melting, facilitates the release of Ag_2^+ bound to cytosine in the substrate hairpin, leading to a cation exchange reaction that quenches the quantum dot fluorescence. This method achieves a low LOD (0.13 aM) and a short reaction time (90 min), and has simple operation and low reagent costs, offering significant potential for clinical tuberculosis screening and POCT development [63].

Finally, the performance characteristics of various NAT enhancement strategies, including sensitivity, specificity, cost, and operational complexity, have been systematically summarized (Table 2). Additionally, three representative NAT enhancement approaches and their corresponding LOD have been enumerated to facilitate a more intuitive comparison of their inter-strategy compatibility and amplification efficiency (Table 3).

3. Nucleic acid biosensors used in the detection of nucleic acid markers of pathogenic microorganisms

In recent years, the application of nucleic acid biosensors in the detection of pathogenic microorganisms has garnered widespread attention. Pathogenic microorganisms encompass bacteria,

viruses, fungi, and protozoa. Among them, the spread of bacteria and viruses has led to serious global public health challenges. Therefore, it is imperative to develop accurate and efficient detection methods. While traditional pathogenic microbial detection techniques, such as bacterial culture and immunoassays, are effective and accurate, they suffer from significant limitations, including time-consuming processes, low sensitivity, labor-intensive procedures, and high costs. These drawbacks render them unsuitable for rapid detection and analysis during disease outbreaks [64]. While traditional methods remain valuable in certain contexts, the advancement of nucleic acid and nanotechnology-driven biosensors represents a promising direction for addressing the urgent need for rapid, accessible, and cost-effective pathogen detection in clinical, environmental, and food safety applications.

Table 2. Comparison of different strategies for improving nucleic acid biosensors.

Technology	Sensitivity	Specificity	Cost	Operational complexity	Ref.
LAMP	High	High (depends on six-sequence recognition)	Medium (the primer design is complex)	High (requires 4 primers, risk of aerosol contamination)	[65]
RPA	High	High (enzyme system complex)	Medium (enzyme system complex)	Medium (requires product purification)	[20]
RCA	Medium	High (circular template specificity)	High (requires phi29 polymerase)	High (the size of the product affects nonspecific binding)	[66]
HDA	High	High (without a thermal denaturation step)	High (requires multiple enzymes)	Medium (requires two primers)	[23]
NESA	High	High (unidirectional amplification)	High (requires multiple enzymes)	Low (fast and simple)	[24]
MCDA	High	High (multiple region primer)	Medium (multiple primers)	High (requires multiple primers)	[25]
NASBA	High	High (requires helicase)	Medium (requires helicase)	High (requires denaturation initiation)	[26]
Enzyme-assisted target recycling	Medium	High (requires Exo III/Lambda Exo/DNAzyme)	High (requires Exo III/Lambda Exo/DNAzyme)	Medium (simple substrate)	[29]
Entropy-driven chain displacement (TMSD/CHA)	Medium	High (depends on the change in the entropy value of a single base)	Low (non-enzymatic)	Medium (simple operation but complex design)	[34,37]
Material-based biosensor	Medium	Medium (depends on molecular recognition elements)	Medium (material costs are controllable)	Medium (requires professional equipment)	[67]
DNA walker–based biosensor	Medium	High (single base recognition)	High (requires complex reaction system)	High (requires professional equipment, modified primers, and enzymes)	[50]
DNA nanostructure–based biosensor	Medium	High (single base recognition)	High (requires modifying group and complex synthesis)	High (requires precise assembly)	[44]
CRISPR/Cas-based biosensor	Low	Extremely high (single base mismatch recognition)	High (requires RNA synthesis and Cas protein)	Medium (simple system)	[57,58]

Table 3. Key platform data for the three strategies for enhancing NAT for nucleic acid detection.

Strategy	Biosensing platform	Target	Amplification	LOD	Ref.
IA	opvCRISPR (one-pot visual RT-LAMP-CRISPR)	SARS-CoV-2 RNA	RT-LAMP, CRISPR/Cas12a	5 copies/reaction	[68]
	ULAMP-RCA-based platform	*Salmonella spp.* DNA	LAMP, RCA	100 copies/μL	[66]
	CPTMB (CRISPR/Cas12a-powered trimode biosensor)	MiRNA	RT-LAMP, RCA, CRISPR/Cas12a	20.75 fM	[69]
	CRAFT (CRISPR-based rapid assay device for field testing)	*M. pneumoniae* DNA	RPA, CRISPR/Cas12a	100 copies/μL	[20]
	CRISPR/Cas9-mediated lateral flow assay	SARS-CoV-2 dual-gene	RT-RPA, CRISPR/dCas9	100 copies/μL	[70]
	MiCaR (microfluidic device with CRISPR-Cas12a and multiplex recombinase polymerase amplification)	30 nucleic acids	RPA, microfluidic chip	0.26 aM	[18]
	RS-CRISPR (RCA-SDA-CRISPR-based platform)	MiRNA	RCA, SDR, CRISPR/Cas12a	57.8 fM	[71]
	LEHCP (λ-exo-assisted HDA-CRISPR/Cas 12a platform)	*Listeria monocytogenes*	HAD, Lambda Exo, CRISPR/Cas	11.5 CFU/mL	[72]
Target recycling	MOF-ERA platform	HBV-DNA	Exo-III, metal-organic framework (MOF)	97.2 pM	[73]
	SPOT (self-locked allosteric DNAzyme biosensor)	Virus RNA	DNAzyme	1.9 aM	[74]
	TTDC (TMSD-assisted TdT-DNAzyme-CuNCs platform)	MiRNA	TMSD, TdT extension, DNAzyme, DNA templated CuNCs	36 aM	[75]
	DTCHA (DNA tetrahedron-assisted catalytic hairpin assembly) based biosensor	MiRNA	CHA, DNA tetrahedron	0.63 fM	[46]
Signal amplification	3D DWCHA	ZIKA RNA	DNA walker/AuNPs, LCHA	20 pM	[76]
	SPARC (specific and precise mutation recognition with Cas12a/Cas13a)	HBV DNA	RPA, T7 exonuclease, CRISPR/Cas12a, CRISPR/Cas13a	1 aM	[53]
	ID-CRISPR (DNA hydrogel-based CRISPR/Cas12a detection platform)	EGFR L858R DNA	CRISPR/Cas12a, RCA reporter	5 aM	[54]
	TACAS (transcribing amplification Cas14a1-activated signal biosensor)	*E. coli* O157:H7 RNA	T7 RNA polymerase–assisted CRISPR/Cas14a1	1.52 CFU/mL	[77]

3.1. Bacteria

The application of biosensors in bacterial detection is primarily focused on prevalent pathogenic bacteria, such as *Staphylococcus aureus*, *E. coli*, *Mycobacterium tuberculosis* (*M. tuberculosis*/*MTB*), and *Salmonella* spp. When the target is bacterial nucleic acid, DNA/RNA must be extracted or released before detection.

3.1.1. Staphylococcus aureus

As an opportunistic pathogen, *Staphylococcus aureus* colonizes the human skin, mucous membranes, and animal tissues without causing disease in healthy individuals but exploiting immunocompromised hosts or breaches in barrier defenses to trigger severe infections [78]. This versatile bacterium produces a wide array of virulence factors, including coagulase, lipase, adhesins, and toxin families such as alpha-toxin, Panton-Valentine leukocidin (PVL), and superantigens, which enable it to invade tissues, evade immune responses, and establish systemic infections like skin abscesses, osteomyelitis, endocarditis, and toxic shock syndrome. Its remarkable environmental adaptability, mediated by sophisticated gene regulation systems and biofilm formation, enhances its persistence in diverse niches, while its ability to acquire multidrug resistance mechanisms poses persistent threats in both community and hospital settings. The dual role of *S. aureus* as a toxin producer and invasive pathogen underscores its significance in public health, necessitating stringent infection control measures and novel therapeutic strategies targeting its virulence mechanisms. Therefore, rapid and accurate diagnosis of *Staphylococcus aureus* is crucial.

Zhou and his colleagues developed a low-cost, simple, and sensitive lateral flow biosensor (CRA-LFB) based on functionalized quantum dots, RPA, and CRISPR techniques for *S. aureus* detection (Figure 9A). The CRA-LFB activates Cas12a/crRNA-mediated trans-cleavage via target DNA, cleaving biotin-modified DNA probes that lack complementarity with the capture probes immobilized on the test (T) line. This prevents the detection of T-line fluorescence signals on the lateral flow sensor. Under optimal conditions, the LOD of CRA-LFB for *S. aureus* genomic DNA is as low as 75 aM, and 5.4×10^2 CFU/mL was detected in pure cultures [79]. Wang et al. designed a novel signal amplification strategy integrating terminal deoxynucleotidyl transferase (TdT) and guanine/nanogold probe-rich (G-rich SNAs) into an RPA-CRISPR/Cas12a-based electrochemical biosensor for *S. aureus* DNA analysis (LOD 1.29 CFU/mL). This strategy comprises three key components: (1) The CRISPR/Cas12a system addresses false positives during RPA amplification, ensuring specificity; (2) the TdT-mediated template-free extension reduces nonspecific Cas12a cleavage; and (3) gold nanoprobes enhance stability and loading efficiency, improving sensor sensitivity (Figure 9B) [80]. Using the strong fluorescence emission characteristics of DNA-CuNCs, Sun et al. combined hybridization chain reaction (HCR) with Cas12a and DNA-CuNCs to construct a multifunctional, highly sensitive pathogen sensing platform (HTCas12a) for *S. aureus* detection. HCR amplifies low-concentration nucleic acids, and the amplified DNA containing a PAM sequence (TTTN) activates the Cas12a/crRNA complex, which cleaves thymine-rich ssDNA (T40), disrupting fluorescence signal generation from DNA-CuNCs. HTCas12a achieves high sensitivity (LOD 4.17 CFU/mL), reagent-free separation, and short detection time, offering a versatile platform for environmental monitoring, clinical diagnosis, and food safety [81].

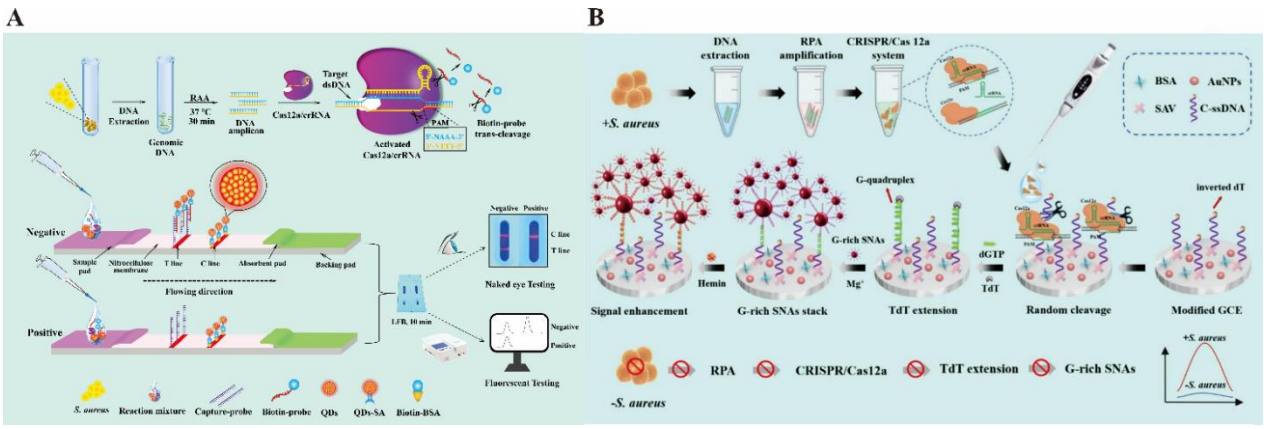

Figure 9. (A) CRA-LFB for *Staphylococcus* aureus detection [79]. (B) Electrochemical biosensor mediated by TdT and G-rich SNAs for *Staphylococcus aureus* detection [80].

3.1.2. Escherichia coli

One of the most common causes of severe food-borne illness, *E. coli* O157:H7 is a subgroup of enterohemorrhagic *E. coli* and is considered the most common highly pathogenic strain. It is responsible for approximately 2.8 million infections globally annually. This pathogen can cause serious diseases such as inflammation, diarrhea, hemolytic uremic syndrome (HUS), hemorrhagic colitis, and even death in vulnerable populations, including young individuals, the elderly, and immunocompromised patients [82]. Therefore, developing sensitive, fast, and user-friendly biosensors for *E. coli* O157:H7 is critical for the food industry, environmental monitoring, and clinical diagnosis.

To improve sensor performance, Shi et al. designed and synthesized a composite material of functionalized nitrogen-doped carbonized polymer dots (N-CPDs@FLBP) in situ grown on a thin layer of black phosphorus, and used it as a modifier to establish a portable intelligent electrochemical DNA biosensor for the detection of *E. coli* O157:H7. The biosensor utilized nanogold as the immobilization matrix and methylene blue as the electrochemical indicator. Its analytical performance was evaluated using standard complementary ssDNA sequences, demonstrating a linear concentration range of 0.1 aM to 1.0 μM and a LOD of 0.033 aM [83]. To shorten analysis time, Tan et al. developed an integrated high-sensitivity and high-specificity detection strategy, transcribing amplification Cas14a1-activated signal biosensor (TACAS), for *E. coli* O157:H7 RNA detection. In this method, dual-function DNA (promoter probe and signal probe) was directly linked to the target RNA sequence via ligase SplintR. The ligation product was transcribed into Cas14a1 RNA activator by T7 RNA polymerase, generating fluorescent signals from the Cas14a1/sgRNA complex. This approach achieved a LOD of 1.52 CFU/mL within 2 hours and was successfully applied to *E. coli*–infected fish and milk samples [77]. Lin's group integrated *E. coli* O157:H7 isolation, lysis, purification, DNA amplification, and detection on a single microfluidic chip, driven by finger actuation (Figure 10). By modifying magnetic beads and RPA-Cas12a, the sensor converted low-concentration nucleic acids into fluorescent signals, enabling user-friendly, rapid (< 2.5 h), highly sensitive (< 100 CFU/mL), portable, and cost-effective (≈ $ 1.5 per sample) detection, which held great potential for detecting other bacteria in resource-constrained areas [84].

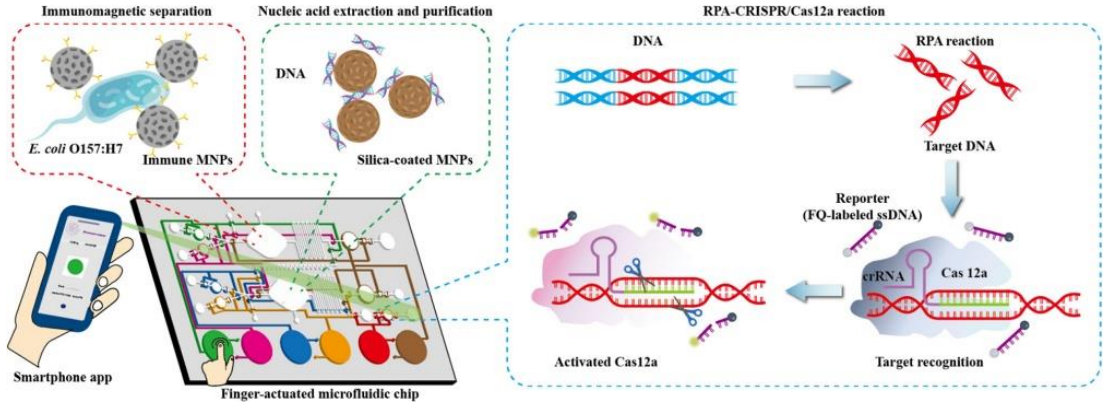

Figure 10. Fully integrated microfluidic biosensor with finger actuation, accomplishing the procedures from immunomagnetic separation, nucleic acid extraction, and purification, to the final signal detection based on the RPA-CRISPR/Cas12a reaction [84].

3.1.3. Mycobacterium tuberculosis

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (MTB) infection remains the world's leading infectious disease killer, with nearly 10 million new infections and approximately 1.5 million deaths annually. Due to the extremely low bacterial load in sputum samples, highly sensitive detection methods are essential. However, current diagnostic techniques, such as bacterial culture, sputum smear microscopy, and antigen–antibody response-based methods, fail to meet the demand for rapid and highly sensitive MTB detection [85]. The World Health Organization (WHO) approved the GeneXpert MTB/RIF test, developed by Cepheid (USA), which can detect MTB genes and rifampin resistance within 2 hours, but its high cost and equipment requirements limit accessibility [86].

Currently, NAT has emerged as a critical tool for TB prevention and diagnosis, with biosensors targeting conserved insertion sequences like IS6110, IS1081, and rpoB, as well as 16S rDNA [87]. For instance, Xiao et al. integrated RPA, Cas12a, and fluorescence/lateral flow technologies to construct a dual-signal-output optical sensor for multiplex detection of MTB. This sensor achieves 1-h analysis time and 4 copies/μL sensitivity, outperforming Xpert MTB/RIF (63.6% sensitivity, 100% specificity) in clinical samples from 107 TB patients and 40 non-TB patients (74.8% sensitivity, 100% specificity) [88].

The amplification effect of traditional linear RCA is unsatisfactory, limiting its clinical application. Tian et al. further improved detection by introducing endonuclease into the RCA reaction (NickRCA) and developing an optical magnetic sensor based on magnetic nanoparticles (MNPs) for MTB rifampin-resistant mutation rpoB 531 detection, achieving a LOD of 15 fM and a 100-min total analysis time [89]. Three fluorescent biosensors have been constructed by Chen's group, utilizing LAMP-strand displacement reaction-molecular beacon [90], RPA-MNAzyme-quantum dot [62], and RPA-CHA-quantum dot systems [63], demonstrating their clinical applicability in sputum samples from TB patients and healthy individuals (Figure 11).

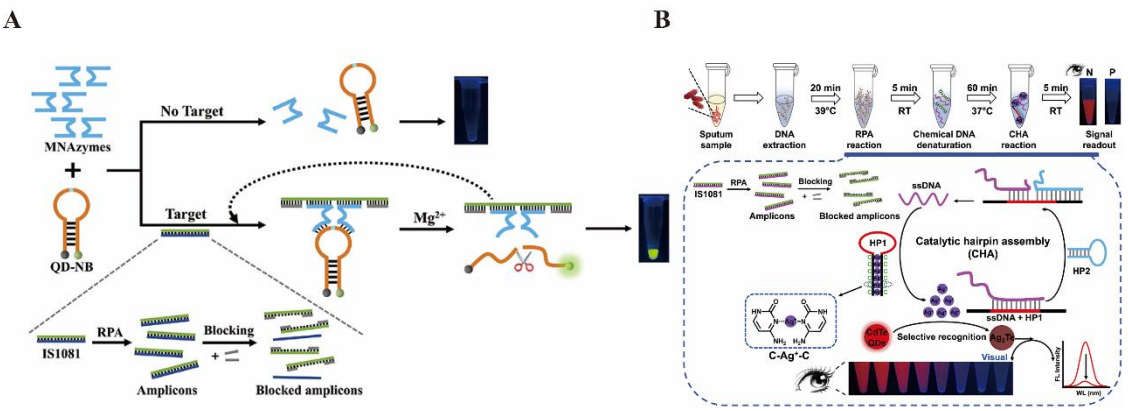

Figure 11. Chen's team analyzed the tuberculosis bacteria genes using the RPA-MNAzyme-quantum dot signal probe and the RPA-CHA-quantum dot signal probe [62,63].

3.1.4. Salmonella

Salmonella is a highly pathogenic Gram-negative bacterium and one of the most common foodborne pathogens causing food poisoning, acute gastroenteritis, and other health issues in both humans and animals. According to WHO data, a significant proportion of diseases and deaths are linked to *Salmonella* infections. Although *Salmonella* cannot directly be transmitted between individuals, fecal-oral transmission primarily occurs through the consumption of contaminated food [91]. Therefore, there is an urgent need to develop highly sensitive and selective *Salmonella* detection systems for rapid disease identification.

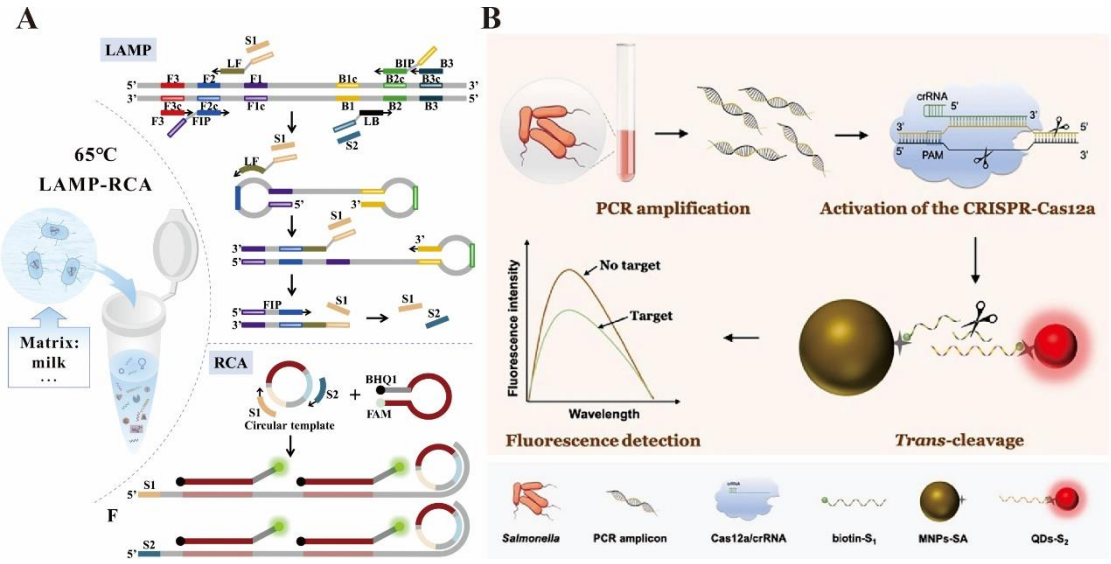

Figure 12. (A) Real-time LAMP-RCA fluorescent biosensor in *S. typhimurium* determination [66]. (B) QD-CRISPR-MNP biosensor for the detection of *Salmonella* [92].

Chen et al. established a universal fluorescent biosensor that integrates LAMP and RCA via a strand displacement strategy. This approach leverages the high specificity and simplicity of LAMP's

detection mechanism to target the *invA* and *malB* genes of *S. typhimurium* (Figure 12A). The method pre-hybridizes RCA primers with the ends of four LAMP primers, enabling flexible adaptation of detection sequences for various target-dependent assays. Additionally, RCA-based platforms are versatile for pathogen detection, offering great convenience for probe design [66]. Shen et al. proposed a quantum dot–based magnetic nanoparticle-assisted fluorescent biosensor (QDs-CRISPR-MNP) for *Salmonella* detection. In this system, the CRISPR/Cas12a system is activated by *Salmonella*-associated DNA PCR products, triggering trans-cleavage activity against linked ssDNA, which inhibits the coupling of MNPs and QDs. Fluorescence signal changes reflect *Salmonella* concentration, overcoming the limitations of traditional dye-based CRISPR platforms by improving fluorescence quantum yield and reducing background noise (Figure 12B) [92]. Huang et al. constructed a dual-mode biosensor combining CRISPR/Cas12a and DNA hydrogel rapid-reaction technology. Target DNA released from *Salmonella typhimurium* after thermal lysis is amplified by PCR to activate Cas12a trans-cleavage, leading to cleavage of fluorescently labeled DNA hydrogels and subsequent fluorescence recovery. Detection results can be visually observed using a fluorescent microplate reader, smartphone, or other portable devices. This method is operationally simple and cost-effective and provides a sensitive visual detection strategy for *Salmonella typhimurium* [93].

3.2. Viruses

Viral infections represent one of the most significant threats to human health, with the COVID-19 pandemic alone causing millions of fatalities globally. Beyond this, other viral diseases such as mosquito-transmitted infections and blood-borne pathogens also pose substantial risks, particularly in severe cases [94]. Currently, no specific antiviral drugs or therapies have been proven effective for treating these viral diseases in humans. Moreover, the rapid mutation rate of emerging viruses further complicates efforts to combat pathogenic strains [95]. Therefore, establishing rapid, accurate, on-site diagnostic methods and personalized treatment protocols is critical to curbing the spread of novel viral diseases.

3.2.1. Severe acute respiratory syndrome coronavirus 2

Since the beginning of 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to the global spread of COVID-19, resulting in millions of fatalities worldwide [1]. As a highly infectious and lethal respiratory virus, SARS-CoV-2 has imposed profound economic and social impacts on the global population and triggered multiple public health emergencies.

NAT has been widely used for diagnosing COVID-19. To address the limitations of false negatives and high costs in SARS-CoV-2 detection, Zhuo et al. developed a novel electrochemical sensor targeting the SARS-CoV-2 RdRp gene using TMSD and CRISPR technologies (Figure 13A). This sensor leverages the target-activated TMSD to generate abundant single-stranded RNA, which subsequently activates Cas13a-mediated digestion of Hoogsteen DNA, thereby preventing hybridization with DNA tetrahedrons and current generation. The sensor exhibits a low LOD of 89.86 aM, high stability, and excellent repeatability [96]. Similarly, Zhang et al. designed an ECL ratiometric biosensor based on a three-strand Y-type DNA (Y-DNA) probe to detect SARS-CoV-2 through hybrid chain reaction (HCR) induced by the proximity effect. By integrating two signal amplification strategies, this biosensor achieves high sensitivity with an LOD of 59 aM, enabling accurate and

efficient detection of target genes [97]. Li et al. further demonstrated the potential of CRISPR-Cas13a combined with ultra-sensitive graphene field-effect transistors (gFETs) for amplification-free detection of SARS-CoV-2, achieving a remarkable LOD of 0.6 copies/μL without pre-amplification [98].

However, relying solely on a single SARS-CoV-2 gene sequence may lead to unreliable diagnostic results due to insufficient sensitivity. To enhance detection reliability, Zhu et al. developed a multiplex reverse transcription loop-mediated isothermal amplification (mRT-LAMP) coupled with a nanoparticle-based lateral flow biosensor (mRT-LAMP-LFB). This method simultaneously amplifies the ORF1ab and N genes of SARS-CoV-2 using two sets of LAMP primers in a single-tube reaction, completing the analysis within 1 h [99]. Additionally, Zhang et al. innovatively established an ECL sensor that integrates dual-target regulation with single-signal output by serially connecting two identical signal amplification strategies (Figure 13B). This sensor targets the ORF1ab and N regions of the SARS-CoV-2 genome, utilizing nitrogen-doped carbon quantum dots as ECL beacons and CHA for signal amplification. It can achieve quantitative detection of SARS-CoV-2 in the range of 50 fM to 200 pM within 40 min. The detection results of 7 clinical pharyngeal swab samples are consistent with the RT-qPCR detection results [100].

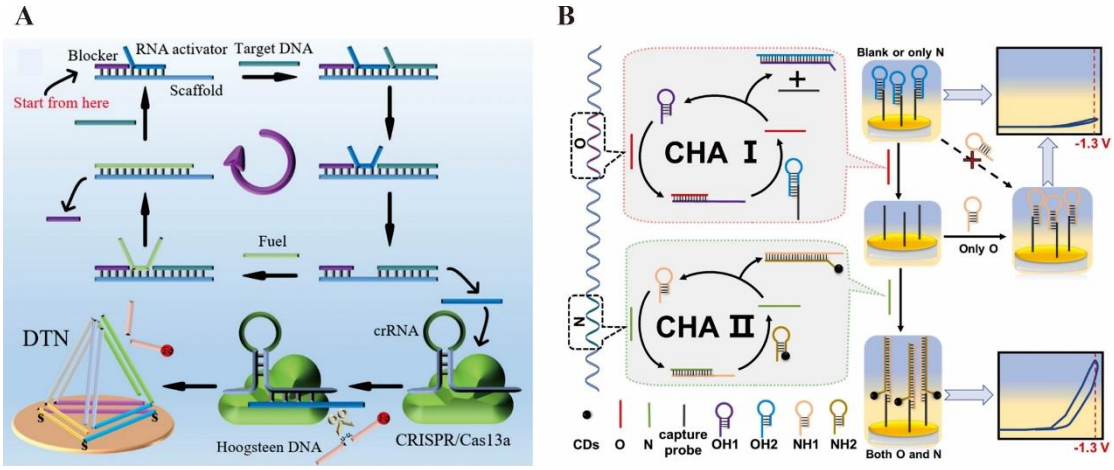

Figure 13. (A) Electrochemical biosensor based on target-triggered entropy-driven reaction displacing the RNA activator, triggering CRISPR/Cas13a activity [96]. (B) Dual-target responsive ECL sensor using a single ECL probe based on CHA signal amplification systems in series [100].

3.2.2. Hepatitis B virus

Viral hepatitis is a global health challenge, with approximately 2 billion people infected with the hepatitis B virus (HBV) worldwide. The initiator of hepatitis B is the hepatitis B virus (HBV), a partially double-stranded DNA virus belonging to the Hepadnaviridae family, with a genome length of approximately 3.2 kb [101]. The genome consists of four partially overlapping open reading frames (ORFs), namely S, C, X, and P. These regions play a crucial role in encoding viral proteins.

HBV detection primarily relies on serological antibody assays and NAT to directly detect viral nucleic acids. Huang et al. developed an electrochemical biosensor for HBV DNA detection by integrating Exo III-RCA, a metal-organic framework composite (MB@ZIF-90), and an

MXene/multiwall carbon nanotube–based triple amplification system (Figure 14A). The circular probe design facilitates HBV-DNA hybridization, triggering target cycle amplification assisted by Exo III, exposes the looping sequence, forms a loop under the action of poly-T primers and *E. coli* ligase, and initiates RCA. This process generates a pyrophosphate byproduct, which induces methylene blue release from the MB@ZIF-90 composite. The released dye is adsorbed onto an MXene-modified glass carbon electrode, producing a measurable electrochemical signal. This sensor exhibits a low LOD of 0.5 pM, a wide dynamic range, and high specificity in spiked human serum samples [102]. Tao et al. developed a fluorescent biosensor for HBV-DNA analysis based on CRISPR/Cas12a signal amplification using metal nanoclusters as luminescent nanoprobes. The sensor operates by activating Cas12a trans-cleavage activity in the presence of target DNA, which degrades DNA probes and inhibits the formation of luminescent metal nanoclusters, resulting in weak fluorescence (Figure 14B). This system outputs CuNCs as the fluorescent signal, enabling detection within 25 min with a LOD of 0.54 pM, achieving label-free, fast, and simple HBV bioanalysis [103]. Zhang and his colleagues combined isothermal multiplexed cross-displacement amplification (MCDA) with gold nanoparticle–based lateral flow technology to create a sensitive and cost-effective HBV DNA sensor. This device achieves a low LOD of 10 copies/response, rapid detection within 50 min, and high specificity, making it a promising POCT tool for resource-limited settings [104].

3.2.3. Human immunodeficiency virus

Acquired immunodeficiency syndrome (AIDS), caused by human immunodeficiency virus (HIV) infection, is a severe infectious disease. Early antiviral treatment for infected individuals can significantly prolong life and reduce transmission risk. However, in low-income and developing countries, HIV-related testing remains costly and inaccessible, preventing early diagnosis and treatment evaluation [105]. Despite the availability of commercial rapid self-testing kits for HIV self-testing, the false-positive and false-negative rates remain relatively high. Additionally, after antiviral therapy, low plasma HIV viral load often falls below the LOD of conventional methods (e.g., < 50 copies/mL), rendering them ineffective for detecting low-level viral replication.

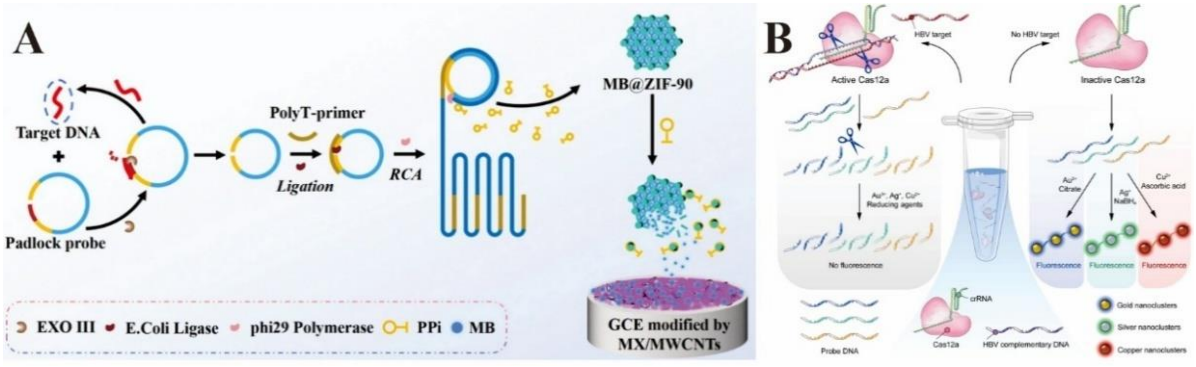

Figure 14. (A) Three-stage signal amplification strategy for label-free electrochemical detection of HBV-DNA based on MX/MWCNTs/GCE [102]. (B) CRISPR-Cas12a-triggered synthesis of metal nanoclusters for fluorescence HBV DNA detection [103].

HIV nucleic acid detection encompasses the identification of both HIV RNA and proviral DNA,

which serve as critical markers for early diagnosis due to their comparable specificity and sensitivity. Liu et al. developed an ultrasensitive biosensor for HIV DNA detection by employing highly stable fluoronitride co-doped carbon dots (FNCDs) as an ECL emission material, coupled with Exo III–mediated target cycle amplification (Figure 15A). This strategy converts trace amounts of HIV DNA fragments into amplified ssDNA via the Exo III reaction, triggering a horizontal hybridization chain reaction (H-HCR) to form a Y-shaped three-dimensional DNA nanostructure. This structure immobilizes a large amount of doxorubicin-diferrocarboxylic acid (ECL quencher), which significantly improves the sensitivity of this biosensor, with a LOD of 6.34 aM [106]. To further improve the biosensing sensitivity for HIV DNA, Shi et al. constructed a homogeneous reaction system based on red blood cell membrane/magnetic nanoparticles as the reaction interface, integrating CHA for signal amplification (Figure 15B). The target DNA induces CHA to form a methylene blue–modified Y-shaped structure on magnetic beads, which are then captured by magnetic glassy carbon electrodes for electrochemical readout. This approach leverages both magnetic enrichment and target cycle amplification, yielding an LOD of 35 aM and enabling differentiation between healthy and HIV patients [107]. Inspired by the multi-compartment structure of living cells, Li et al. proposed an artificial cascade reaction system incorporating nanoporous membrane-based separation to integrate CRISPR-mediated reactions into a microfluidic platform (CRISPR-MCR biosensor). This biosensor enables HIV DNA and RNA detection using a glucose meter, with LODs of 43 copies for DNA and 200 copies for RNA [108].

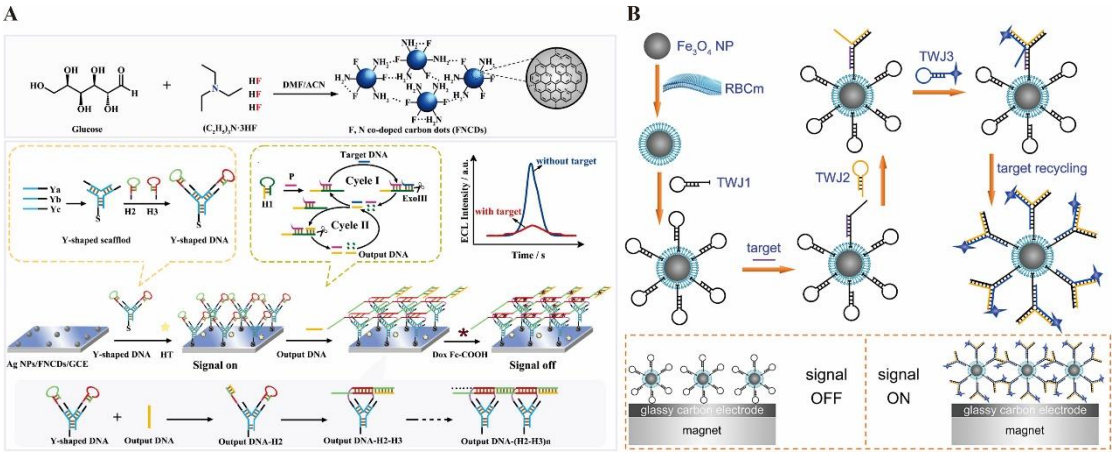

Figure 15. (A) ECL biosensor constructed by H-HCR coupled with Exo III to detect HIV DNA [106]. (B) RBCm-cloaked magnetic nanoparticles for amplified detection of HIV DNA [107].

4. Nucleic acid biosensors used in nucleic acid detection of cancer markers

Biosensors have emerged as versatile tools for detecting various biological analytes, including pathogens and cancer biomarkers. Their application in pathogen detection has been well-established for rapid and accurate diagnosis of infectious diseases, and their underlying principles, such as high specificity, real-time monitoring, and integration with advanced transduction mechanisms, have been seamlessly adapted to detect cancer biomarkers. For instance, similar to how biosensors identify pathogenic agents through biomarker recognition, they now enable the detection of cancer-specific

proteins, nucleic acids, and other molecular markers in bodily fluids like blood or saliva.

Cancer remains the second leading cause of death globally, responsible for approximately 7.6 million fatalities annually and accounting for 13% of all global deaths in 2010. Projections indicate that cancer-related mortality could exceed 13 million by 2030, underscoring the urgent need for improved early detection strategies [109]. Notably, early-stage cancer diagnosis significantly enhances survival outcomes, with five-year survival rates for most malignant tumors increasing substantially when detected at earlier stages. This highlights the critical importance of developing reliable biomarker-based detection methods.

As cancer progresses, distinct biological alterations occur in body fluids, including DNA, RNA, proteins, and other small molecules at the cellular level. These molecular changes enable the quantification of specific biomarkers as valuable diagnostic parameters [110]. Among these, nucleic acids have emerged as promising biomarkers due to their differential expression patterns between cancer patients and healthy individuals. Unlike many other biomarkers, nucleic acids exhibit remarkable stability in bodily fluids, offering unique advantages for cancer diagnosis. However, the low abundance of certain nucleic acids in clinical samples presents challenges for detection, as they are susceptible to interference from other biological components [111]. Developing highly sensitive, rapid, and reliable nucleic acid biosensors remains a critical priority for advancing cancer diagnostics.

MicroRNA (miRNA) is a small non-coding RNA molecule that regulates approximately 60% of protein-coding genes closely associated with human health [112]. Studies on circulating miRNAs in blood environments have shown their strong correlation with cancer development, suggesting that detecting changes in blood-related miRNA levels could help identify tumor traces and determine tumor progression stages [113]. Based on a miRNA-triggered binary "locked" nucleic acid ("Lock" DNA) structure that induces the chain displacement amplification reaction of DNA polymerase, Peng et al. developed a fluorescent biosensor using CRISPR/Cas12a as a signal amplification strategy for miR-21 detection in breast cancer cells. This sensor exhibits high specificity and a low LOD of 28.8 aM, enabling effective differentiation between cancer cells. The programmability of this system allows for detecting various nucleic acid markers by modifying the "Lock" DNA sequence without altering the crRNA spacer sequence [114].

To address clinical diagnostic needs, Zhao et al. designed an electrochemical biosensor combining peptide nucleic acid (PNA) as a capture probe with a ternary structure (PNA-DNA2 three-way junction, 3WJ) and CHA-HCR cascade amplification. In this biosensor, PNA-DNA_2 3WJ establishes a dense molecular layer on the sensor surface, effectively reducing background signal interference and precisely adjusting the surface density of the HCR probe binding site. This improves HCR amplification efficiency, thereby reducing the LOD of this sensor (2.9 aM) [115]. Further, Zhou et al. have developed a novel multi-miRNA detection platform, named RCA-SDA-CRISPR (RS-CRISPR), which integrates RCA, strand displacement amplification (SDA), and CRISPR/Cas12a technologies. This platform utilizes RCA products as the template for SDA, maximizing miRNA binding sites through spatial arrangement to overcome the limitations of traditional enzymatic digestion methods. This platform enables rapid and simultaneous detection of multiple miRNAs. The RS-CRISPR platform achieves a LOD of 57.8 fM for target miRNAs, offering new insights into RCA with SDA technology in CRISPR/Cas assays [71]. Liang developed an ultrasensitive sandwich-type electrochemical sensor for miR-155 detection using DNA tetrahedrons, improved TMSD, and a synergistic catalytic nanoprobe, achieving a LOD of 0.13 fM [116]. However, material synthesis and sensor assembly introduce uncertainties in results. To solve this, we integrated modified NickRCA

with CHA in a single-step process, fundamentally simplifying the analysis process. In addition, the introduction of protective agents can inhibit the elongated reaction caused by binding padlocks to RCA products, slow down nonspecific reaction kinetics, and reduce the LOD of the sensor to 0.025 amol. Serum spiking experiments validated this sensor's consistency with RT-PCR results, demonstrating its potential for biomedical research and clinical applications [117].

Circulating tumor DNA (ctDNA) in the peripheral blood of cancer patients is a cell-free DNA fragment actively released from apoptotic or necrotic tumor cells, carrying highly specific genomic information of the original tumor. As a non-invasive biomarker, ctDNA has gained increasing attention in liquid biopsy research due to its potential for early cancer screening, real-time monitoring of tumor burden, and personalized treatment strategies. Long et al. pioneered a novel electrochemical biosensor for ctDNA detection by integrating iron single-atom nanoenzyme-carbon dots (SA Fe-CDs) as signal carriers with a DNA walker cascade amplification strategy triggered by Exo III. This triple-amplification system, combining reduced graphene/nanogold particles, Exo III-driven DNA walkers, and SA Fe-CDs, achieves a wide linear range (5 aM to 50 nM) and a low LOD of 1.26 aM, enabling sensitive detection in complex serum environments [118]. Wu et al. further advanced this field by developing a non-purified fluorescent biosensor through a dual isothermal amplification approach combining endonuclease-mediated strand displacement and CHA reactions, achieving a nearly 10^7-fold signal amplification with a LOD as low as 2 aM [119]. In another breakthrough, Wu et al. constructed an electrochemical biosensor (MME-CRISPR) based on manganese metal–organic framework (MOF)-enhanced CRISPR to detect epidermal growth factor receptor (EGFR) L858R mutations in ctDNA. By co-assembling enzyme stimulators (Mn^{2+}) with Cas12a/crRNA into MOF complexes, the proximity effect of MOFs ensures continuous Mn^{2+} supply for sustained Cas12a/crRNA interaction, enhancing trans-cleavage activity. In addition, the introduction of MOFs enhances the sensor's protection ability against various external interferences. This design achieves an ultra-low LOD of 0.28 fM without pre-amplification, demonstrating its clinical utility in detecting single-nucleotide variants and guiding targeted therapies [120].

5. Conclusions and prospects

The COVID-19 pandemic has had a profound and lasting impact on global public health, economies, daily life, and human physical and mental well-being. Over the past decade, nucleic acid amplification techniques and advanced nucleic acid sequencing technologies have been widely adopted for the diagnosis and treatment of diverse infectious and non-communicable diseases. Traditional methods such as PCR, NGS, and colony culture remain the gold standard for pathogenic microorganism detection. However, these approaches pose significant challenges to clinical laboratories, particularly at grassroots levels, due to their high economic costs, environmental demands, and personnel burdens. Centralized laboratories also struggle with maintaining rapid turnaround times for infectious disease diagnostics.

From this perspective, we explore the application of nucleic acid biosensors in detecting pathogenic microorganisms and disease markers. These interdisciplinary research efforts aim to address critical clinical challenges in disease management. DNA nanotechnology enhances detection sensitivity through effective signal conversion and amplification, while biosensors translate biological signals into measurable forms (e.g., electrical and optical) that can be easily detected by simple instruments. This enables real-time disease diagnosis through intuitive operator interfaces.

Engineering advancements have driven the development of portable biosensor devices, with biochip and microfluidic technologies enabling trace sample analysis.

Despite these advancements, further research is needed to enhance the stability and reproducibility of biosensors, especially in complex biological matrices, to mitigate false results caused by sequence similarities and interferences. Miniaturization of biosensor devices and integration of microfluidic technologies remain key challenges for commercializing POCT solutions.

Moreover, the integration of artificial intelligence (AI) with biosensors is poised to revolutionize healthcare and environmental monitoring by enabling real-time, personalized, and predictive analytics [121]. Emerging trends highlight the development of intelligent, miniaturized biosensors capable of seamless integration with wearable devices and the Internet of Things, facilitating continuous health monitoring and disease detection. AI-driven algorithms will enhance data processing capabilities, allowing for rapid pattern recognition, noise reduction, and early warning systems in medical diagnostics, while nanotechnology advancements will further boost sensitivity and multiplexing functionalities. The synergy between AI and biosensors will also drive the creation of large-scale sensor networks for public health surveillance, enabling timely interventions during outbreaks or chronic disease management. Future innovations will likely focus on autonomous, self-calibrating systems that minimize human intervention and expand applications beyond clinical settings to include personalized wellness tracking and environmental remediation. This convergence of AI, biosensors, and emerging technologies promises a paradigm shift toward proactive, data-centric healthcare solutions with unprecedented precision and accessibility.

To summarize, while nucleic acid biosensors present opportunities for pathogen and disease marker detection, their clinical translation faces obstacles such as regulatory hurdles and scalability issues. Nevertheless, given the limited diagnostic options available for early disease detection, prioritizing policy support and resource investment in relevant research is essential. Every effort should be made to ensure timely medical access and reduce the fear of disease among populations.

Use of generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Zuanguang Chen: Writing–review & editing, Supervision, Project administration, Conceptualization. Zhixian Liang: Writing–review & editing, Project administration. Yiting Huang: Writing–review & editing. Yunfei Sun: Writing–review & editing.

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