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ADVANCED REVIEW

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Sample-to-answer salivary miRNA testing: New frontiers in point-of-care diagnostic technologies

Zhikun Zhang[1,2] | **Tianyi Liu**[1] | **Ming Dong**[1] | **Md. Ahasan Ahamed**[1] | **Weihua Guan**[1,2]

[1]Department of Electrical Engineering, Pennsylvania State University, University Park, Pennsylvania, USA

[2]Department of Biomedical Engineering, Pennsylvania State University, University Park, Pennsylvania, USA

Correspondence
Weihua Guan, Department of Electrical Engineering, Pennsylvania State University, University Park, PA 16802, USA.
Email: wzg111@psu.edu

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Abstract

MicroRNA (miRNA), crucial non-coding RNAs, have emerged as key biomarkers in molecular diagnostics, prognosis, and personalized medicine due to their significant role in gene expression regulation. Salivary miRNA, in particular, stands out for its non-invasive collection method and ease of accessibility, offering promising avenues for the development of point-of-care diagnostics for a spectrum of diseases, including cancer, neurodegenerative disorders, and infectious diseases. Such development promises rapid and precise diagnosis, enabling timely treatment. Despite significant advancements in salivary miRNA-based testing, challenges persist in the quantification, multiplexing, sensitivity, and specificity, particularly for miRNA at low concentrations in complex biological mixtures. This work delves into these challenges, focusing on the development and application of salivary miRNA tests for point-of-care use. We explore the biogenesis of salivary miRNA and analyze their quantitative expression and their disease relevance in cancer, infection, and neurodegenerative disorders. We also examined recent progress in miRNA extraction, amplification, and multiplexed detection methods. This study offers a comprehensive view of the development of salivary miRNA-based point-of-care testing (POCT). Its successful advancement could revolutionize the early detection, monitoring, and management of various conditions, enhancing healthcare outcomes.

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KEYWORDS

cancer, infectious disease, microRNA, multiplexed detection, neurodegenerative disease, point of care testing (POCT), saliva

1 | INTRODUCTION

MicroRNA (miRNA) are a series of non-coding small RNAs, typically comprising 18–26 nucleotides, that play critical roles in gene regulation (Wronska, 2023). Initially discovered in 1993 in *Caenorhabditis elegans* (Reinhart et al., 2000), the miRNA database miRbase has made significant strides in cataloging these molecules. To date, it has listed 1917 precursor miRNA (pre-miRNA) and 2654 mature miRNA (Gebert & MacRae, 2019; Nemeth et al., 2023). miRNA are produced through a detailed multi-step processing pathway. It begins with the transcription of primary miRNA from DNA sequences in the nucleus. These primary miRNA subsequently evolve into precursor miRNA and eventually mature into miRNA in the cytoplasm (Garcia-Martin et al., 2022; Rani & Sengar, 2022). miRNA serves as crucial regulators in a multitude of biological processes, influencing gene expression by both repressing translation and degrading targeted messenger RNAs (mRNAs) (Jet et al., 2021). Almost every miRNA interacts with a multitude of mRNAs, acting as either "oncogenes" or "tumor suppressor" genes in cancer cells (Boriachek et al., 2018; X. Chen et al., 2019; Goh et al., 2016). miRNA are closely linked to a range of complex human diseases, including neurodegenerative disorders, concussions, and infectious diseases (Carè et al., 2007; Chmielarz et al., 2017; Gebert & MacRae, 2019; Hung et al., 2016; Masud, Na, et al., 2019). Consequently, miRNA are increasingly being recognized as promising biomarkers for diagnosing diseases and assessing the effectiveness of treatments across various medical conditions.

Point-of-care testing (POCT) offers rapid, convenient medical diagnostics at or near the location of patient's location, facilitating quicker clinical decisions and improved accessibility. Saliva is advantageous for POCT due to its non-invasive, safe, and easy collection methods (Song et al., 2023). Utilizing salivary miRNA as biomarkers for early disease diagnostics enables physicians to assess disease risk at the earliest possible stage. This approach contributes to increased survival rates, aids in the prevention of disease progression, and enhances public health outcomes by facilitating timely interventions. Addressing key challenges is essential for employing salivary miRNA as biomarker in POCT for disease diagnosis. First, the notably low concentration of miRNA in saliva, constituting just 0.01% of the total RNA in complex biological fluids, varies from femtomolar to picomolar levels (Jet et al., 2021). Second, the subtle differences in miRNA dysregulation between healthy individuals and patients pose significant challenges for accurate quantitative analysis. Third, disease-specific miRNA profiles often involve changes in expression levels of multiple miRNA types, further complicating the detection process (Cai et al., 2021; Ivanov et al., 2021; Jet et al., 2021; Ma & Zhang, 2023; P. Zhang et al., 2014). Hence, it is essential to develop techniques for the sensitive and quantitative analysis of multiple miRNA in saliva.

Conventional techniques used for miRNA detection include northern blotting, reverse transcription polymerase chain reaction (RT-PCR), and microarray (Masud, Umer, et al., 2019; Tavallaie et al., 2015; Zhou et al., 2019). Northern blotting, while offering high reliability, is impeded by lower sensitivity and labor-intensive procedures. RT-PCR, despite its exceptional sensitivity and specificity, the requirement for large, expensive thermal cyclers significantly hampers the application in resource-constrained environments and in POCT settings (Gorgannezhad et al., 2018). Likewise, microarray, while advantageous for simultaneous analysis of multiple miRNA, frequently faces challenges in sensitivity and specificity. These limitations highlight significant barriers to the effective application of these techniques in POCT for disease diagnostics. Given the limitations of traditional methods and the unique characteristics of salivary miRNA, new detection technologies have been developed for multiplexed and sensitive miRNA detection in POCT. Currently, a large family of isothermal miRNA detection techniques has successively been developed, such as rolling circle amplification (RCA) (Dong et al., 2022), exponential amplification reaction (EXPAR) (Jia et al., 2010), hybridization chain reaction (HCR) (Zhao et al., 2023), catalytic hairpin assembly (CHA) (Luo et al., 2022), strand-displacement amplification (SDA) (Xu et al., 2018), duplex-specific nuclease signal amplification (DSNSA) (Wu et al., 2020), loop-mediated isothermal amplification (LAMP) (Tang, Nouri, et al., 2022) and recombinase polymerase amplification (RPA) (Ahamed et al., 2024; Lobato & O'Sullivan, 2018). Isothermal detection techniques have transformed the landscape of diagnostics by offering faster detection speeds and more compact equipment without the need for thermal cycling, thus creating a solid foundation for nucleic acid-based POCT.

Exploring the connection between salivary miRNA and diseases establishes a crucial foundation for miRNA as biomarkers in POCT. This review provides a comprehensive analysis of the biogenesis of salivary miRNA, highlighting the latest techniques for extracting and detecting miRNA, focusing on advancements in multiplexing, sensitivity, and quantitative analysis techniques. Furthermore, we discuss the incorporation of these technological developments into POCT system. Our objective is to close the gap between research on salivary miRNA and their application in POCT diagnostics, aiming to foster the creation of innovative, clinically valuable diagnostic tools.

2 | SALIVARY miRNA BIOGENESIS AND FUNCTION

In humans, about 2654 types of miRNA have been found, and they control over 60% of our genes and are involved in many cell activities (Shu et al., 2017). miRNA can be first synthesized in the cytoplasm. Then, cells secrete miRNA into biological fluids, such as serum and saliva, in two forms: in complex with proteins (circulatory) or entrapped in microvesicles (Pittman et al., 2023). In both conditions, the miRNA are protected from degradation (Park et al., 2009; Patel et al., 2023). In this review, we provide a detailed introduction to the synthesis process of miRNA.

The biogenesis of miRNA contains mainly two steps in both the nuclear and cytoplasmic regions (Figure 1a) (Ning et al., 2023; Rani & Sengar, 2022; Tétreault & De Guire, 2013). The biogenesis of miRNA starts with the primary miRNA transcript (Pri-miRNA) that contains several thousands of nucleotides and stem-loop structures from the miRNA gene. These pri-miRNA are, for the most significant part, transcribed by RNA polymerase II. Following the synthesis of pri-miRNA, it undergoes cleavage, leading to formation of a small hairpin structure known as pre-miRNA. This reaction takes place in the nucleus and is accomplished by the nuclear RNAse III-type protein Drosha and the DiGeorge syndrome critical region gene 8 (DGCR8). Along with Drosha, DGCR8 forms a large complex, which cleaves the pri-miRNA into a ~70 nucleotide pre-miRNA with a stem–loop structure.

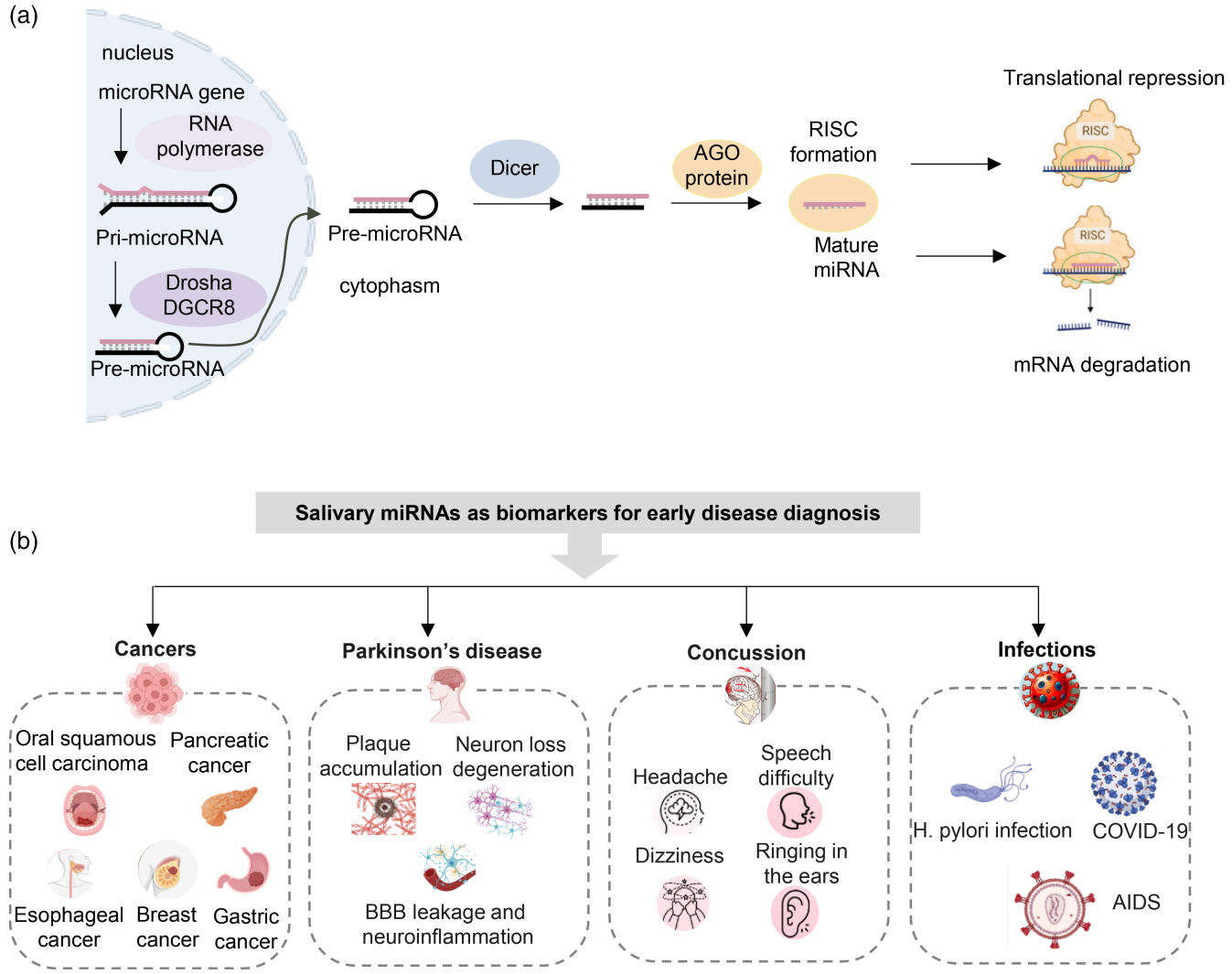

FIGURE 1 The biogenesis and regulatory mechanisms of miRNA and their relevance to various diseases (a) Schematic of miRNA biogenesis, processing, and mechanism of action in the network of gene expression by translational repression and mRNA degradation (b) salivary miRNA as biomarkers to its relevance to diseases for early disease diagnosis, including cancers, Parkinson's disease, concussion, and infectious disease.

The subsequent step in the process involves Exportin-5, which acts as a nucleo/cytoplasmic cargo transporter, exporting pre-miRNA from the nucleus to the cytoplasm. Afterwards, the hairpin precursors of pre-miRNA are processed into small double-stranded RNA (dsRNA) duplexes. These duplexes consist of the mature miRNA strand and its complementary strand. The miRNA duplex is then incorporated into an Argonaute (Ago) protein, forming a RNA-induced silencing complex (RISC). In this complex, the passenger strand of the miRNA is degraded. Subsequently, the mature miRNA is incorporated into the miRNA-induced silencing complex (miRISC), which is crucial in regulating gene expression. This process may result in the degradation of mRNA in cases where there is an exact match between the miRNA and its mRNA target, or alternatively, it can inhibit the translation of the mRNA.

3 | CORRELATION BETWEEN SALIVARY miRNA AND EARLY-STAGE DISEASES

miRNA exhibit a distinctive capability to modulate a vast and intricate regulatory network of gene expression, encompassing a wide range of developmental and cellular processes (Hu & Coller, 2012; Huntzinger & Izaurralde, 2011; Salim et al., 2022). A certain class of miRNA has been found to have a strong connection and correlation with the disease based on their expression levels, including cancer (Carè et al., 2007), neurological diseases (Cressatti et al., 2020), infectious diseases (McDonald et al., 2021) (Figure 1b). These miRNA can facilitate early diagnosis of diseases, and are essential in the prevention and therapeutic management of a range of health conditions (Weber et al., 2010). This review presents an analysis of miRNA and their involvement in a variety of diseases, focusing on the array of miRNA types and their specific correlations with different pathologies. We will offer insights into their biological significance and implications for disease progression and treatment.

3.1 | Cancer

Oral squamous cell carcinoma (OSCC) is one of the most common cancer types in the world (D'souza & Addepalli, 2018). The majority of OSCC cases are diagnosed at an advanced stage, resulting in lower survival rates, with the 5-year survival rate for OSCC being less than 50%. Identification of novel molecular biomarkers is thus needed to aid in early diagnosis. In recent decades, salivary miRNAs have become recognized as significant contributors to carcinogenesis. miRNAs are involved in a complex system with their target genes (RhoA, FIH and ACOX1, etc.) and signaling cascades (the EGF-AKT signaling axis, the ERK-MMP9 cascade and the Hippo signaling pathway, etc.), that is essential for the development of OSCC (Lin, Han, et al., 2022; Lin, Liu, et al., 2022; Lin, Wu, et al., 2022). They find their way into the oral cavity from multiple sources, such as salivary glands, gingival crevicular fluid, and shed oral epithelial cells (Zahran et al., 2015). In our review, we summarize the changes in miRNA levels in OSCC. The pioneering research into salivary miRNA showed markedly decreased levels of miR-200 in individuals with OSCC relative to healthy subjects (Song et al., 2020). Furthermore, Ekanayake Weeramange et al. further researched and verified the experimental results, showing that miR-200 expression was significantly down-regulated in OSCC (Ekanayake Weeramange et al., 2023). The research has shown that miR-200 holds significant promise as a biomarker in the diagnosis of diseases. Other extensive studies focus on miR-21, which is high levels when people suffer from the OSCC (Al Rawi et al., 2021; Zahran et al., 2015). To date, there are also other important miRNA whose expression levels are strongly associated with OSCC in Table 1.

Additionally, different levels of miRNA expression have been identified across a variety of cancer types, including esophageal cancer (EC), gastric cancer (GC), pancreatic cancer (PC), and breast cancer (BC) (Rapado-González et al., 2018). Xie et al. reported significantly elevated levels of miR-10, miR-144, and miR-451 in the whole saliva of patients with EC (Xie et al., 2013). Similarly, Du and Zhang et al. found elevated expression levels of miR-10, miR-144, and miR-451, along with miR-98 and miR-363, have been identified in the saliva of patients with EC (Du & Zhang, 2017). These findings suggest that miR-10, miR-144, and miR-451 could play a role in modulating target genes associated with EC. Besides, GC is also associated with miRNA, including miR-140-5p and miR-301a (Li et al., 2018). Notably, these studies observed a significant decrease in the levels of these two miRNA candidates in GC patients compared to healthy individuals. A saliva-based panel including these miRNA could offer a non-invasive and early detection method for identifying individuals with GC (Kaczor-Urbanowicz et al., 2022). PC is among the most lethal malignancies, having a poor prognosis and a five-year survival rate of only 5% (Khalaf et al., 2021). PC is the world's

TABLE 1 Characteristic of the salivary microRNA level in OSCC patients.

No.	Study cohort	miRNA level changing	References
1	21: OSCC; 11: HC	miR-412-3P: ↑; miR-512-3P: ↑	(Gai et al., 2018)
2	15: HC; 15: OSCC	miR-21: ↑; miR-125a: ↓ miR-31: ↑; miR-200a: ↓	(Al Rawi et al., 2021; Shahidi et al., 2017)
3	5: HC; 12: OSCC	miR-1307-5p: ↑	(Patel et al., 2022)
4	23: OSCC; 21: HC	miR-140-5p: ↓; miR-143-5P: ↓ miR-145-5p: ↓	(Nisha et al., 2019; Patel et al., 2023; Zahran et al., 2015)
5	50: OSCC; 42: HC	miR-423-5p: ↑; miR-106B-5p: ↑ miR-193b-3p: ↑	(Romani et al., 2021)
6	5: OSCC; 3: HC	miR-31-5p: ↑	(Hung et al., 2016; Lai et al., 2018; Li et al., 2022)
7	45: OSCC; 10: HC	miR-24-3p: ↑	(He et al., 2020)
8	25: TSCC; 25: HC	miR-139-5p: ↓	(Duz et al., 2016)

Note: ↑ and ↓ indicate.

seventh leading cause of cancer mortality (Koopaie et al., 2022; Sung et al., 2021). Diagnosing pancreaticobiliary tract cancer presents challenges due to the absence of characteristic clinical symptoms and its anatomical positioning, particularly in early stages. Studies indicate that miR-1246 and miR-4644 found in salivary exosomes may serve as effective biomarkers for detecting pancreaticobiliary tract cancer in patients (Machida et al., 2016). Moreover, an increase in the levels of miR-103 and miR-107, along with a decrease in miR-155, was observed, which could help differentiate PC from normal controls (Roldo et al., 2006; Yang et al., 2021). Concurrently, miR-21 and miR-23 also exhibited distinct expression patterns in saliva samples from patients with malignant tumors compared to those without cancer, demonstrating high specificity and sensitivity (Humeau et al., 2015). Interestingly, a wide range of miRNA have been found to be associated with PC. Furthermore, BC is the most frequently occurring cancer worldwide and is the primary cause of death in women across the globe. Research into biomarkers for BC has primarily concentrated on miR-21. In patients with BC, higher miR-21 expression levels have been observed in their saliva, showing a significant reduction following treatments, including surgery, chemotherapy, and neoadjuvant therapy (Koopaie et al., 2021). This result demonstrates the significant potential of miRNA in the detection of BC.

3.2 | Concussion and Parkinson's disease

Concussion, often referred to as mild traumatic brain injury (TBI), primarily affects children and adolescents, representing about two-thirds of all such cases. These concussions, accounting for over 80% of mild TBIs, involve miRNA. These small molecules are crucial in gene expression regulation of TBI. While most children recover from concussion symptoms within 2 weeks, approximately one-third may suffer from prolonged concussion symptoms (PCS). Hicks et al. identified that, specifically, miR-27 shows remarkable accuracy in differentiating between individuals who have experienced concussions and those who have not, highlighting its potential as a reliable biomarker in concussion diagnosis (Hicks, Onks, et al., 2023). Moreover, analysis through logistic regression revealed that the levels of multiplexed miRNAs accurately indicated patients with prolonged symptoms, including miR-320, miR-133, miR-769, let-7a, miR-203, and miR-1307. Additionally, three specific miRNAs were found to correlate with certain symptoms 4 weeks post-injury: miR-320 was associated with memory problems, miR-629 with headaches, and let-7b-5p with fatigue (Johnson et al., 2018). Furthermore, miR-28 and miR-339 were identified as potentially playing a role in the underlying mechanisms of concussions (Hicks et al., 2020; Miller et al., 2022).

Parkinson's disease (PD), a progressive neurodegenerative condition, stands as the foremost movement disorder and the second most frequent neurodegenerative disease. It is estimated to affect about 2% of the global population aged over 65 years (Cressatti et al., 2020; De Lau & Breteler, 2006). At present, the identification of PD predominantly depends on the clinical symptoms, and since there are no established, effective biomarkers for diagnosis. Nonetheless, recent studies have indicated that miRNAs in saliva, which influence genes and pathways associated with PD, are experiencing changes in individuals who have the disease (Fyfe, 2020; Kamal et al., 2015). miRNA could play a role in

the development of Parkinson's disease through critical mechanisms like apoptosis, neuroinflammation, mitochondrial malfunction, and proteasomal breakdown. In addition, studies have shown that miRNAs can be regulators of genes associated with PD, including the *SNCA* gene, *PRKN* and *PARK7* gene, and so forth (Filatova et al., 2012; Leggio et al., 2017). Certain miRNAs, like miR-153 and miR-223, have been found to influence genes associated with PD and affect the accumulation of α-synuclein, either directly or indirectly (Cressatti et al., 2020). Expression levels of miR-153 and miR-223, when log-transformed, showed a notable reduction in the saliva of patients with PD compared to individuals without neurological disorders. The presence of miR-153 and miR-223 in saliva can differentiate PD with accuracies of 79% and 77%, respectively, making these miRNAs moderately effective diagnostic markers for idiopathic Parkinson's disease. Additionally, a combination of salivary miR-29 and miR-28 has emerged as a promising diagnostic biomarker for idiopathic PD (Y. Jiang et al., 2021; X. Zhao, Wang, Xiong, et al., 2020).

3.3 | Infectious diseases

miRNA are increasingly recognized as valuable biomarkers for detecting infectious disease from a range of pathogens, including Helicobacter pylori (H. pylori), SARS-CoV-2, HIV, and so on (Drury et al., 2017). Alterations in miRNA profiles can appear during the early phases of a disease, often preceding the direct detection of the pathogen itself. Zhou et al. found that the downregulation of miR-204 in pulp tissue, serum, and saliva indicated that people suffer from H. pylori infection (Zhou & Xu, 2019). The role of miRNA in regulating inflammatory pathways linked to H. pylori infection and their expression changes in pre-neoplastic lesions underscore their potential as biomarkers for early gastric cancer detection and as therapeutic targets (Săsăran & Bănescu, 2023). Additionally, children with severe SARS-CoV-2 infection exhibit altered salivary miRNA levels compared to those with milder cases, mainly showing a decrease in miRNA like miR-4495, miR-296, miR-548, and miR-1273. These miRNA are involved in pathways relevant to SARS-CoV-2, including viral processing and immune response, indicating a need for further research to establish their role as biomarkers in severe cases (Hicks, Zhu, et al., 2023). Saulle et al. found significant downregulation of miRNA let-7a, let-7b, and let-7c, and upregulation of miR-23a, miR-23b, and miR-29c, along with three immunomodulatory miRNA (miR-34a-5p, miR-181d-5p, and miR-146) in SARS-CoV-2 infections (Saulle et al., 2023).

Early diagnosis of diseases is challenging due to the lack of specific biomarkers and the absence of clear symptoms for cancer, concussion, PD, and infectious diseases. Consequently, many patients fail to receive treatment during the most effective window, leading to unfavorable outcomes. Utilizing miRNA as a biomarker can significantly enhance early detection and diagnosis. This early detection allows for timely intervention, potentially increasing survival rates and enhancing the quality of life for patients. Hence, utilizing miRNA as a biomarker to achieve early disease diagnosis is of critical significance and value for health and life. However, there is no widespread agreement on which miRNA are most suitable to serve as biomarkers. Many factors affect the expression levels of miRNA in patients, such as age, lifestyle and gender, and so forth. Furthermore, variations in laboratory practices for collecting, processing, and detecting these miRNA also impact their accurate detection. These differences present significant challenges in using salivary-based miRNA as biomarkers in POCT.

4 | EXTRACTION AND DETECTION OF SALIVARY miRNA AT POCT

POCT is defined as testing conducted near or at the site of the patient, and because it is easy to do, fast, and accessible. Saliva is a highly desirable body fluid for biomarker development for clinical applications because it provides a noninvasive, simple, and low-cost method for disease screening and detection. Saliva miRNA can be used as biomarkers for disease diagnostics (Li et al., 2004; Park et al., 2006, 2007). These findings suggest that the detection of miRNA in saliva can be used as a noninvasive and rapid diagnostic tool for early disease diagnosis, such as cancer, concussion, Parkinson's diseases and infection diseases (Park et al., 2009). Using salivary miRNA as a biomarker for early disease diagnosis involves a three-step process in traditional methods. This process includes preparing the sample through miRNA extraction, amplification of the miRNA, and detection for signal read-out. These methods provide high sensitivity and specificity, but they require expensive equipment, trained technicians, and complicated procedures. To effectively detect salivary miRNA through POCT, the detection platform needs to integrate all necessary steps on one device, making it more efficient and accessible for on-site testing. POCT can significantly reduce the detection time with simple operations to detect miRNA, thereby enhancing its feasibility and effectiveness in early disease diagnostics

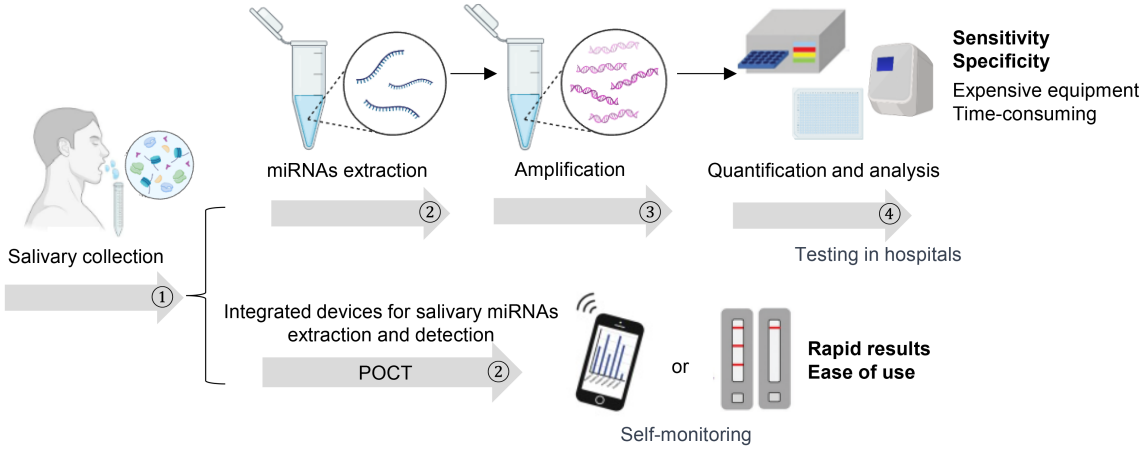

FIGURE 2 Schematic illustration of detection of salivary miRNAs through traditional techniques and POCT. Traditional methods involve four steps with expensive equipment for high sensitivity and specificity with a long detection time. POCT is conducted on-site by integrated devices to isolate and detect miRNAs to rapidly get results with simple operation.

(Figure 2). Herein, we primarily concentrate on reviewing the ongoing advancements in the fields of extraction, amplification, and detection for miRNA, and meanwhile, microfluidics and paper-based biosensors for miRNA detection have been well reviewed for the current progress of POCT. We intend to investigate the potential and practicality of creating POCT assays for miRNA in clinical diagnosis.

4.1 | Extraction of miRNA from human saliva

Saliva, a sophisticated and stretchy fluid, holds an abundance of biomolecules such as nucleic acids, enzymes, hormones, antibodies, and elements with antimicrobial capabilities. The proteins, ions, and lipids in complex biological matrices can cause the inhibition of miRNA amplification and the interference of miRNA detection (Gallo et al., 2012; J. Kim et al., 2017; Michael et al., 2010). To obtain high-quality miRNA, a series of procedures for sample preparation, such as lysis, extraction, concentration, and purification of ribonucleic acid (RNA), has been extensively applied before the amplification or detection system. Now, there are two main ways to purify and increase the amount of miRNA: liquid-phase and solid-phase extraction methods (Jeong et al., 2021).

4.1.1 | Liquid-phase extraction methods

Liquid-phase extraction, established as the standard method for RNA isolation, utilizes a combination of organic solvents and salts to isolate total RNA, which includes miRNA. The phenol-chloroform-based one-step technique, introduced by Chomczynski and Sacchi, has gained widespread popularity for its straightforwardness, effectiveness, and affordability. The technique is versatile and can be used with a broad range of samples from humans, plants, yeasts, and bacteria, encompassing fluids, tissues, and cells grown in culture. Additionally, a variety of readily available, cost-effective commercial reagents enhance their ease of use while reducing the consumption of reagents and the production of hazardous waste. Examples of this include reagents such as TRIzol™ from Thermo Fisher Scientific Inc., and QIAzol® from Qiagen Inc. These have been broadly utilized, either on their own or as part of the preliminary treatment in numerous solid-phase extraction kits designed for miRNA extraction. Furthermore, various alterations have been suggested to enhance recoveries, such as changes in the composition of the reagent (for instance, RNAzol® by Molecular Research Center Inc., which eliminates the need for chloroform-induced phase separation) or extra extraction and purification steps using chloroform or alcohol, along with more tailored adjustments for specific types of samples. Recent progress in the liquid-phase extraction of miRNAs has concentrated on exploring innovative extraction solvent for efficient extraction and purification of NAs from intricate matrices.

4.1.2 | Solid-phase extraction methods

Solid-phase extraction techniques rely on employing a suitable solid sorbent to capture miRNA from limited sample volumes. Typically, a modest quantity of sorbent materials, such as particles, fibers, or membranes, are packed or placed in devices like spin columns, filters, or centrifugal units to expedite the process across various extraction stages. Solid-phase extraction techniques present a superior option to liquid-phase methods for isolating miRNA due to their rapid and effective purification capabilities. They circumvent numerous challenges associated with conventional methods and often achieve comparable or superior yields in specific applications.

Silicon materials can be used to adsorb the negatively charged nucleic acids at high salt and low pH, and then desorb them by changing the pH value and concentration of salt. Using the silicon materials and a slidable chip, genomic DNA could be extracted from human whole blood inside the chamber packed with 70 mm-diameter silica beads. Boom and colleagues developed silica particles for nucleic acid adsorption, and presently, the majority of commercial nucleic acid extraction kits employ silica as the primary solid-phase sorbent. The binding of nucleic acids to the silica surface can be controlled by varying the pH and concentration of a chaotropic agent. Sorbents based on silica naturally tend to retain larger RNA molecules and are less effective for smaller RNA molecules (less than 200 nucleotides), such as miRNA (Mraz et al., 2009; Salim et al., 2022). Additionally, a novel sorbent based on SiC has been developed in commercial kits, which seem not to exhibit size bias. Walt et al. developed two version extraction methods (Ter-Ovanesyan et al., 2021). This technique utilizes carboxylated paramagnetic beads alongside a guanidinium thiocyanate lysis buffer for nucleic acid binding. It can be executed in two volumes: a small-scale version using 30 μL of saliva in a 96-well PCR plate, and a larger-scale version with 300 μL of saliva in a deep-well plate. Anderson et al. developed three hydrophobic MILs as miRNA extraction solvents to extract miRNA (Emaus & Anderson, 2021). MILs can be efficient miRNA extraction solvents.

4.1.3 | POCT devices for miRNA extraction from saliva

Recent advances in POCT devices have enabled the streamlined and efficient extraction of miRNA from saliva, a development that significantly enhances the convenience and non-invasiveness of diagnostic procedures, opening new avenues for real-time, patient-centered monitoring and early disease detection. Innovative developments have been made with the creation of microfluidics, and paper-based devices (Ahrberg et al., 2016; Kim et al., 2014; Thompson et al., 2016; Zhang et al., 2019). In this review, we focus on examining the advancements in POCT miRNA isolation using portable devices, exploring how these technological innovations are reshaping the landscape of miRNA-based diagnostics and offering new possibilities for rapid, efficient, and accessible health monitoring.

Microfluidic-based devices have emerged as a promising alternative, offering improved yield and purity in miRNA extraction. These devices provide a rapid, portable, and automated setup, enhancing efficiency and requiring minimal input fluid. Chen et al. integrated silica membrane-based DNA extraction from saliva, PCR amplification, and lateral flow-based amplicon detection in a microfluidic cassette (Chen et al., 2010). Furthermore, Oblath et al. combined aluminum oxide membrane-based nucleic acids extraction and real-time PCR amplification on a microchip (Oblath et al., 2013). Yang et al. introduced a budget-friendly and mobile setup by combining a magnetic bead-based solid-phase nucleic acid extraction with a digital RPA (Recombinase Polymerase Amplification) assay. This integrated system consisted of two parts: a cartridge and a controlling device (Yang et al., 2018). Within the cartridge, various solutions such as lysis buffer, magnetic beads, binding buffer, and washing buffers were stored in separate centrifuge tubes. Throughout the nucleic acid extraction process, these liquid reagents were systematically moved from one tube to another, emulating a standard benchtop nucleic acids extraction method. This combined apparatus showed an approximate 90% success rate in recovering M. tb from saliva samples that had been artificially contaminated. Chan and colleagues transformed a 3D printer into a device for nucleic acids detection from saliva. This adapted printer was capable of conducting magnetic particle-based nucleic acid extraction from as many as 12 samples at once, completing the process in just 15 min. Additionally, they employed the printer's heated bed to conduct an RT-RPA assay, facilitating swift detection of the Zika virus in saliva samples. In order to further simplify operation and improve extraction efficiency, Politza and colleagues used magnetic bead to create a portable, battery-operated, adaptable, and field-ready device for preparing nucleic acid samples (Politza et al., 2023). A versatile electromagnetic actuator was engineered to maneuver a magnetic robot (ProMagBot) across a two-dimensional X/Y space. This innovation allows for the easy transition of various magnetic bead-based sample preparation techniques from laboratory environments to POCT settings. The ProMagBot,

demonstrated in a model application, was utilized to isolate HIV viral RNAs from samples using two popular magnetic bead kits: ChargeSwitch and MagMAX beads. ProMagBot efficiently extracted viral RNAs from 50 μL plasma samples with viral RNA concentrations as low as 10^2 copies in just 20 min.

Paper-based devices have also been commonly utilized for extracting nucleic acids from saliva. Nucleic acids can bind to some laboratory-modified paper. After patterned with low molecular weight chitosan, nitrocellulose (NC) membrane-based devices capture miRNA via anion exchange chromatography, allowing for simultaneous purification and concentration of miRNA. Jiang and colleagues employed cellulose papers for isolating Zika viral RNA from saliva. Their miRNA extraction apparatus was divided into two sections: an upper buffer unit and a lower integrated mixing and detection unit (X. Jiang et al., 2018). The buffer unit featured four compartments for holding liquid reagents. To store these reagents, the researchers implemented valves operated by bearing balls. As the lower section slid beneath these compartments, a pin lifted the ball valves, enabling the release of the stored buffers to seep down through the cellulose paper.

4.2 | Multiplexed detection of salivary miRNA

The flexible multiplex testing of miRNA is essential to the research and diagnosis of diseases. Analyzing multiple miRNAs offers vital biological insights for both physiological and pathological cancer research, encompassing aspects like screening, monitoring, and prognostication. However, the inherent characteristics of miRNA, such as their short sequences, low abundance, and high level of homology (Jet et al., 2021), make their multiplex detection challenging in a convenient, sensitive, and precise manner for increasing the precision of disease diagnosis (He et al., 2017). In that section, we will review isothermal methods for sensitive and multiplexed microRNA detection, including in situ hybridization, amplification-free miRNA detection, and amplification strategies based on HCR/TMSD/EXPAR/RPA/ligation reaction/enzymes-assisted cyclic amplification (Figure 3).

4.2.1 | Multiple miRNA hybridization

Multiplex miRNA hybridization techniques are highly promising, enabling specific and sensitive detection of multiple miRNA without requiring amplification steps. In these strategies, miRNA capture and labeled complementary probes were used to form a multiplex sandwich structure with the target miRNA (Koo et al., 2016; Zhou et al., 2017). The primary advantage of this method over other miRNA detection methods is its capacity to track the cellular and subcellular distributions. Additionally, it facilitates the determination of their spatiotemporal expression profile. This is crucial for understanding the biological role and their pathologic roles in various diseases.

For instance, Zhou et al. developed a new approach for miRNA detection using Surface Enhanced Raman Scattering (SERS) by the hybridization of sandwich structure between miRNA and probes (J. Wu et al., 2021). They engineered DNA-coded Raman dye within a core-shell structure composed of uniform stellate fractal gold nanoparticles (F-AuNPs). Three F-AuNPs as probes were employed as probes for the simultaneous detection of miRNA-122, miRNA-21, and miRNA-223 (Figure 4a). The limit of detection (LoD) achieved for three miRNA was notably low, with 349 aM for miRNA-122, 374 aM for miRNA-223, and 311 aM for miRNA-21. Based on the sandwich structure strategy, many microRNA detection methods have been developed. Liu et al. innovated a novel approach for multiplexed microRNA analysis that is amplification-free by a microreactor and various gold nanoparticle reporters (GNP) on SERS (Lu et al., 2021). This microreactor incorporates antibodies, serving as a universal module to bind nucleic acids. At the same time, a pool of SERS reporter gold nanoparticles exists, with each batch of GNPs tagged with a Raman identifier molecule and a DNA probe specific to the target miRNA. The presence of the target miRNA facilitates the selective attachment of these SERS reporter GNPs to the microreactor, significantly amplifying the miRNA-triggered SERS signals when observed under a confocal Raman microscope. To enhance the integration of numerous binding sites for the nanoscale capture of diverse target miRNAs, DNA origami nanoarray is uniquely designed to enable distance-dependent recognition of miRNA using super-resolution microscopy techniques (Kocabey et al., 2023). The sensor is capable of detecting up to four different miRNA, either individually or in combination. The detection method exhibits remarkable sensitivity, achieving a detection limit as low as the femtomolar range, specifically between 11 and 388 fM. Fluorescent signal devices are more convenient and faster compared to the SERS and super-resolution microscopy, making it more suitable for application in POCT. Zhou et al. developed an innovative fluorescence-based platform for

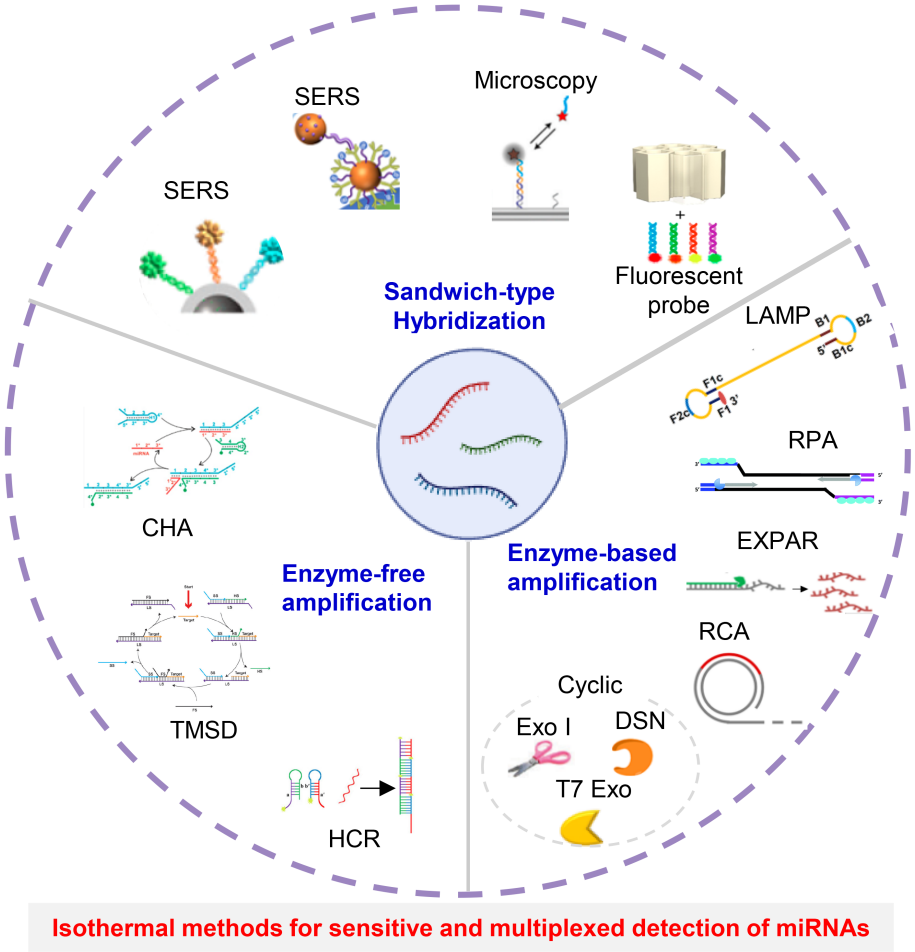

FIGURE 3 Isothermal methods for sensitive and multiplexed detection of miRNAs by in situ hybridization, enzyme-free amplification, and enzyme-based amplification.

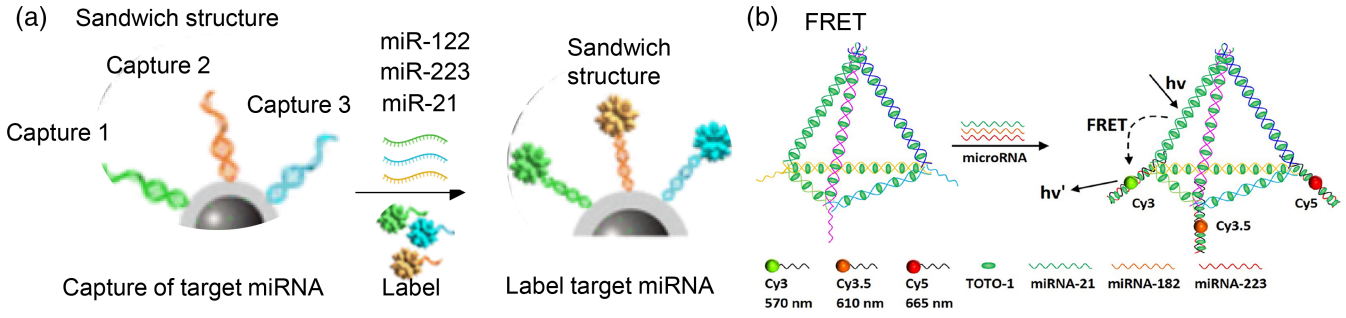

FIGURE 4 In situ hybridization-based miRNA detection (Adapted with permission from J. Wu et al., 2021). (a) Sandwich structure-based multiplexed miRNA detection. (b) DNA tetrahedron nanotag for the multiplexed detection of miRNA based on FRET from TOTO-1 to dyes (Adapted with permission from H. Zhao, Wang, Xiong, et al., 2020).

the specific detection of sequence-specific miRNA. This platform employs fluorophore-labeled DNA in conjunction with metal–organic frameworks (MOFs) (S. Peng et al., 2020). It is capable of quenching the fluorescence emitted by four different fluorescent dyes labeled on single-strand oligodeoxynucleotides (ssODNs). When the target miRNA are present, they induce the recovery of fluorescence. The intensity of the recovered fluorescence correlates linearly with the concentration of miRNA, within a range of 1.25–100 nM. This fluorescent sensor platform is designed to detect multiple types of miRNA simultaneously within the same system, offering high specificity and minimal cross-reactivity. Meanwhile, the fluorescent resonance energy transfer (FRET)-based multiplexed technique has become a powerful tool

for the simultaneous detection of multiple miRNA by employing multiple donor and acceptor pairs (Ahamed et al., 2022; Qiu & Hildebrandt, 2015; Thapa et al., 2022). Chen et al. devised a novel assay for the simultaneous detection of multiple miRNA, employing DNA tetrahedron nanotags coupled with FRET between the nucleic acid stain TOTO-1 and three different organic dyes (Figure 4b) (Zhao, Wang, Xiong, et al., 2020). DNA tetrahedral nanostructure (DTN) has three adaptor oligos on its vertices. TOTO-1, as a fluorescent donor, can be embedded into the native nucleic acid backbone of DTN. Three organic dye-functionalized strands are fluorescent receptors. When target miRNA are present, they undergo hybridization with the FRET oligonucleotides and adaptor oligonucleotides positioned at the vertices of DTN. This interaction leads to the formation of stable DNA tetrahedron nanotags. Consequently, TOTO-1 is brought into close proximity to the three fluorescent dyes. This proximity facilitates efficient FRET enabled the effective detection of miR-21, miR-122, and miR-223. Liu et al. also used TOTO-1 and three labeled fluorescence dyes to develop multiplexed MicroRNA (miRNA-155, miRNA-182, and miRNA-197) detection platforms using FRET (Y. Liu, Wei, et al., 2017).

Recent advancements in technology have led to the development of numerous in situ hybridization-based methods for the multiplexed detection of miRNA (Cai et al., 2021; Huang et al., 2017). These methods often employ a variety of intricately designed sandwich structures to facilitate signal transformation. In sandwich structures, different miRNA captures were designed to capture multiplexed miRNAs. Labeled complementary probes were used to achieve signal transformation for quantitative analysis of the target miRNA at low concentrations. This strategy effectively solves the problems of low concentration and quantitative detection of multiplex miRNAs in salivary miRNA-based disease detection. A key focus in the ongoing evolution of these technologies is the design of simpler structures for miRNA captures and labeled probes that can effectively accomplish signal transformation. This simplification is crucial for enhancing the practicality and efficiency of miRNA detection methods in various applications.

4.2.2 | Enzyme-free isothermal amplification of multiplexed miRNA detection

Multiplexed miRNA amplification methods are a critical aspect in the detection of trace target molecules, enhancing the sensitivity and selectivity of molecular diagnostics. Isothermal amplification, in particular, has become a prominent method for miRNA quantification, demonstrating its efficacy in creating highly specific and sensitive miRNA assays without the need for precise and complex thermocycling. A diverse range of isothermal amplification techniques for multiplexed miRNA has successively been developed, such as HCR (Zhao et al., 2023), SDA (Feng et al., 2023), RCA (Yao et al., 2021), EXPAR (Reid, Le, & Zhang, 2018), DSNSA (Shi et al., 2019; Tian et al., 2017; Wu et al., 2020), and LAMP (Tang, Cui, et al., 2022; Tang, Nouri, et al., 2022). These isothermal amplification methods are mainly based on enzyme-based replication, digestion, or enzyme-free strand displacement processes. In this review, we will comprehensively examine the technologies developed for multiplexed miRNA detection, providing a detailed analysis of their advantages and limitations.

Enzyme-free systems are simpler and more stable compared to enzyme-dependent systems. They do not rely on enzymes, which can be sensitive to environmental conditions and require stringent storage requirements, such as HCR and toehold-mediated strand displacement amplification (SDA) and catalytic hairpin assembly (CHA). The HCR has emerged as a powerful signal amplification technique, showing an enhanced application due to its nonenzymatic and isothermal features. HCR offers a sophisticated, effective, and isothermal method for signal amplification, commonly employed in miRNA detection. This process includes the linear extension of double-stranded nicked DNA, single-stranded initiator DNA, and a pair of hairpin-shaped fuel DNAs. For instance, Park et al. developed a method that integrates a self-priming hairpin-triggered cascade reaction with the adsorption properties of graphene oxide (GO). This innovative approach is utilized for the multiplexed detection of miRNA (Song et al., 2022). In this platform designed by Park et al., miRNA interacts with the self-priming hairpin probe (SHP), causing it to unfold and rearrange into a primer. This process enables the extension reaction to displace the target miRNA, allowing it to be reused for opening another SHP. This results in the formation of a double-stranded SHP with an elongated stem region. The presence of a nicking enzyme recognition site within this double-stranded SHP leads to continuous nicking and extension reactions, generating a substantial amount of the trigger sequence. In the second phase of the reaction, this trigger sequence also transforms a single-stranded target template probe (TTP) into its double-stranded form (dsTTP) and simultaneously creates multiple target mimic strands in the same fashion. These mimic strands can activate the first reaction phase, simulating the target miRNA. The double-stranded forms (ds SHP and ds TTP) resist adsorption by graphene oxide (GO), resulting in intense fluorescence upon the application of GO. In contrast, the single-stranded forms of the probes

exhibit negligible fluorescence in the absence of the target miRNA. This system achieves a remarkably low limit of detection (LoD), reaching attomolar levels (42.63 aM for miRNA let-7a, 13.08 aM for miRNA-141, and 10.14 aM for miRNA-98) within 30 min. Hildebrandt et al. used a HCR to construct the platform of multiplexed detection of miRNA via time-gated FRET (Figure 5a) (Guo et al., 2019). Liu et al. created a framework nucleic acid (FNA)-mediated microarray. This innovative approach is designed for the quantitative analysis of multiple miRNAs (Qu et al., 2018). Liu and team expertly crafted framework nucleic acids (FNAs) of varying dimensions, thereby effectively altering both the surface density and the side-to-side interactions of the DNA probes on their microarray. This methodology provided a robust means for programmable customization of both the efficiency and the dynamics of hybridization at the biosensor interface. Notably, they found that as the size of the FNA increased, so did the hybridization efficiency, with FNA-17 exhibiting optimal performance. Coupled with a hybridization chain reaction amplification approach, this precisely engineered FNA microarray emerges as a highly sensitive and selective tool. It is adept at performing simultaneous multiplexed detection of various miRNA biomarkers, such as FNA-miR-652, FNA-miR-627, and FNA-miR-629, making it particularly useful in identifying key biomarkers associated with gastric cancer.

SDA is an enzyme-free molecular tool to exchange one strand of DNA or RNA (output) with another strand (input). It is based on the hybridization of two complementary strands of DNA or RNA via Watson-Crick base pairing (Kabza et al., 2022). Li et al. engineered a DNA molecular motor, which comprises two stem-loop DNA structures. These structures have identical stems but feature complementary loop domains, a design that facilitates the multiplexed detection of miRNA (Figure 5b) (Wang et al., 2016). miRNA can bind with a specific stem-loop DNA, causing the stem to open and create a miRNA/DNA hybrid along with a single-stranded DNA (ssDNA). This ssDNA then pairs with another stem-loop DNA, ultimately forming a double-stranded DNA (dsDNA) and freeing the miRNA. One stem-loop DNA in this setup is marked with both a fluorophore and a quencher, leading to effectively suppressed fluorescence. The generation of dsDNA triggers a distinct fluorescence signal, aiding in miRNA detection. The liberated miRNA perpetuates the subsequent hybridization between the two stem-loop DNAs, establishing a cycle-operating DNA molecular motor. This cycle leads to significant fluorescence amplification. The LoD for this method can be as low as 1 picomolar/L for the miRNA target, with an extensive dynamic range spanning from 1 picomolar/L to 2 nanomolar/L. Wei et al. integrated the strand displacement amplification (SDA) reaction to create a platform capable of detecting multiple miRNAs (Xu, Feng, et al., 2021). The designated miRNA selectively interacts with the template, triggering the strand displacement amplification (SDA) process, which generates a large quantity of amplification products (triggers). These triggers are then involved in the ACHA (Asymmetric Chain Amplification) reaction on the electrode's surface, leading to a reduced electrochemical response. Utilizing dual amplification methods, a linear correlation is observed between the electrochemical signals and the concentrations of miRNA-122 and miRNA-21, ranging from 0.1 to 10 fM, with detection limits of 0.012 and 0.075 fM, respectively. This electrochemical sensor is versatile and can be adapted to detect various miRNAs by simply altering the template strands. Moreover, the electrode can be refreshed for reuse by incubating it with specific DNA strands. Li et al. also use AgNCs as signal molecules and TMSD as amplifier to constructed

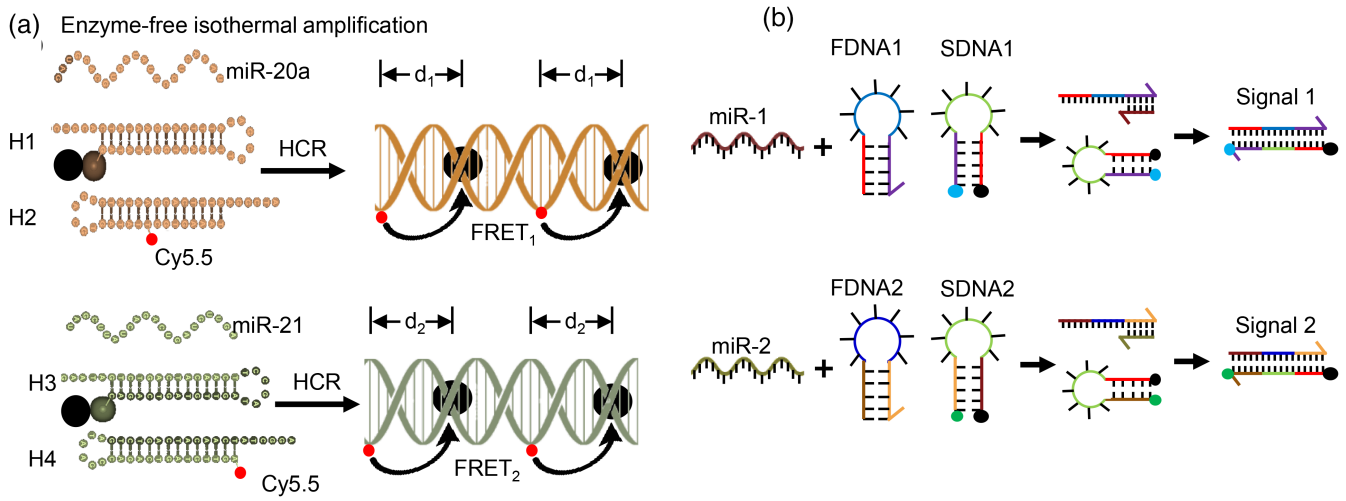

FIGURE 5 Enzyme-free isothermal amplification for miRNA detection. (a) HCR-TG-FRET amplification for duplexed miRNA (miR-20a and miR-21) detection. (b) miRNA-initiated DNA molecular motor-based multiplexed miRNA detection.

target-triggered recycling amplification biosensors (Li, Li, et al., 2021). The LoD can reach as low as 9.8/6.1 pM for miR-21/miR-141, respectively. Additionally, Zhang et al. engineered a straightforward, highly sensitive, and selective method for detecting multiple miRNAs. This approach leverages the fluorescence quenching properties of graphene oxide (GO) in combination with an isothermal strand-displacement polymerase reaction (ISDPR) (Zhang et al., 2014). The ISDPR amplification greatly enhances the sensitivity of Zhang et al.'s miRNA detection method. The strong affinity between single-stranded DNA (ssDNA) and GO results in the fluorescent DNA probe exhibiting very low background fluorescence under normal conditions. However, when a specific target miRNA is recognized, an ISDPR is initiated. This reaction generates a large number of specific DNA-miRNA duplex helices, which, due to their weaker interaction with GO, emit a strong fluorescence signal. This miRNA biosensor achieves an impressive sensitivity, capable of detecting concentrations as low as 2.1 fM, and it offers a linear detection range spanning four orders of magnitude. Additionally, the expansive planar surface of GO enables the simultaneous quenching of multiple DNA probes, each tagged with different fluorescent dyes. This feature allows the creation of a multiplex biosensing platform that is both highly sensitive and selective. Such a platform holds significant promise for applications in profiling miRNA expression patterns and in various biomedical research fields. Additionally, a significant quantity of hairpin-structured nucleic acids was designed to enhance the TMSD techniques, the design for multiplexed detecting miR-155 and OPN mRNA was based on multi-donor iFRET (Li, Li, et al., 2021). This technique demonstrates detection limits in the subfemtomolar range for two RNA sequences, specifically 0.5 fM for microRNA-155 and 0.3 fM for osteopontin mRNA.

CHA possesses the capability to convert and intensify signals for use in analytical applications (Xu, Feng, et al., 2021). Lu and colleagues innovated a CHA-based system, leveraging the multiplexing ability of the Luminex xMAP platform, for the sensitive and concurrent detection of multiple miRNAs. This method amplifies the fluorescent response through the repetitive use of target miRNA, creating a significant quantity of H1-H2 duplexes. This approach enabled the simultaneous detection of liver tumor-associated miRNAs—miRNA-21, miRNA-122, and miRNA-222 with a LOD of 2 pM. Overall, this proposed technique, which first integrates CHA with the Luminex xMAP system, establishes a sensitive platform for multi-miRNA detection, offering potential applications in both biomedical research and clinical diagnostics. The simplicity of enzyme-free systems makes them easier to integrate with various detection and analytical platforms, including microfluidics, biosensors, and POCT devices.

Enzyme-free amplification for miRNA detection can effectively and rapidly enhance the signal from the target miRNA molecule without enzyme in low temperature. With this strategy, target miRNAs can be quantitatively detected with high sensitivity at low temperatures by means of rapid amplification, enabling detection of the clinic samples. However, a multiplex miRNA system based on enzyme-free amplification needs multiple groups of ssDNA probes, which can impact the specific amplification of miRNA. This may result in non-specific background noise, reducing sensitivity and specificity. Hence, the pursuit of creating an enzyme-free amplification method that offers high specificity and sensitivity is not just a challenging endeavor but also one with substantial potential.

4.2.3 | Enzyme-assisted isothermal amplification of multiplexed miRNA detection

Recent progress in the field of molecular biology has resulted in the creation of a new isothermal amplification method, termed enzyme-assisted isothermal amplification. This method offers remarkable amplification efficiency, capable of exponentially amplifying target nucleic acids within a significantly reduced timeframe. Notable methodologies encompassing this technique include EXPAR, LAMP, RPA, RCA, and so forth. Enzyme-based isothermal amplification for miRNA has high sensitivity without the thermal cycles at constant temperature. It also involves complexity, potential for contamination, dependency on enzyme quality and RNA integrity, and can be time-consuming and expensive. For isothermal amplification, there will be a significant background signal, which might result from the undesired products from the possible cross-dimerization with isothermal amplification procedures (Kim et al., 2023; Meagher et al., 2018). This will decrease the sensitivity. Meanwhile, similar to enzyme-free amplification, there was also the issue of multiplexed miRNA detection. Enzyme-based amplification requires that the detection system contain several groups of ssDNA probes for specific recognizing miRNA. It also increases the background signal to reduce the sensitivity. So, future research methods should focus on designing the platform to achieve effective and highly sensitive miRNA detection (Table 2).

EXPAR demonstrates exceptional amplification efficiency, achieving 10^6 to 10^8-fold amplification in less than 30 min. This efficiency renders it particularly suitable for miRNA detection, as illustrated in (Figure 6a) (Reid, Le, & Zhang, 2018; Van Ness et al., 2003). The EXPAR system employs a uniquely designed template featuring two repeat

TABLE 2 Summary of the main isothermal amplification approaches for multiplexed miRNA detection.

Method	Enzyme	Target	LoD	Linear range	Time	Temperature	Ref.
Argonaute-EXPAR	Nt.BstNBIDNA, polymerase	miRNA-21 miRNA-92a miRNA-31 miRNA-141	1 aM	1 aM to 10 nM	20 min, 15 min	55°C, 80°C	(Lin et al., 2022)
Expar/CHA		miR-21 miR-122	0.012 fM 0.075 fM	0.1 to 10 fM	45 min	55°C	(Xu, Feng, et al., 2021)
RT-LAMP	DNA polymerase	miR-16 miR-191 miR-423 miR-1307 miR-1246	10^3–10^6copies per 50 μL	–	2 h	65°C	(Hashimoto et al., 2019)
RCA/ CRISPR/ Cas9	Taq DNA ligase, DNA polymerase	miR-21 miR-221 miR-222	90 fM	100 pM	2 h, 10 min	37°C, 65°C	(Wang et al., 2020)
Ligation/RPA	Ligase, recombinase polymerase	SARS-CoV-2 ORF1ab gene, N gene	1copies	–	30 min	37°C	(Wang et al., 2021)
T7 Exo. cyclic amplification	T7 Exo.	miR-221 miR-222	0.78 pM	1–100 pM	12 h	30°C	(Liu, Zhang et al., 2017)
Cycle amplification	DSN	miR-21 miR-210 miR-486	98 am 120 aM 300 aM	0.5 fM to 10 pM	2 h	55°C	(Djebbi et al., 2022)
DSN-MASS	DSN	miR-141 miR-21 let-7a	42 pM 41 pM 95 pM	–	1 h	60°C	(Shi et al., 2019)

regions. One of these regions is designed to be complementary to the miRNA sequence. During the process, a DNA polymerase endowed with strand displacement activity extends this region. This enzymatic action results in the formation of double-stranded DNA (dsDNA) that incorporates a NEase (nicking endonuclease) recognition site. Subsequent to this extension, the NEase acts on the extended trigger DNA strand at the recognition site. Following this, the DNA polymerase initiates extension from the 3′-end of the nicked site, concurrently displacing the newly synthesized DNA strand. The displaced strand is then capable of hybridizing with another template molecule, instigating a subsequent round of amplification, leading to exponential amplification (2n) of the target molecule. For example, Liu et al. developed a highly sensitive method for the detection of multiplexed miRNA (Jia et al., 2010). The EXPAR for miRNA amplification is performed at 58°C. The LoD of miRNA can reach as low as 0.1 zmol by a real-time method with a wide dynamic range of more than 10 orders of magnitude. EXPAR provides outstanding amplification efficiency but has limited sensitivity because of its nonspecific background signal (Reid, Paliwoda, et al., 2018); in order to address this issue, Kong et al. used TtAgo to assist exponential isothermal amplification for multiplex miRNA detection (Lin, Han, et al., 2022; Lin, Liu, et al., 2022; Lin, Wu, et al., 2022). This system possesses single-nucleotide discrimination and high sensitivity, down to attomolar concentrations with a rapid reaction time (30–35 min). Due to the single-nucleotide accuracy of TtAgo, the system exhibited strong multiplexing abilities, enabling the concurrent detection of four miRNA targets and distinguishing between members of the let-7 family. Although EXPAR method is renowned for its high sensitivity in detecting miRNA, a key area for further investigation remains the reduction of background noise in these assays. This background interference often poses a challenge in achieving accurate quantification and detection, particularly in samples with low target concentrations. Continued research is focused on optimizing the assay conditions,

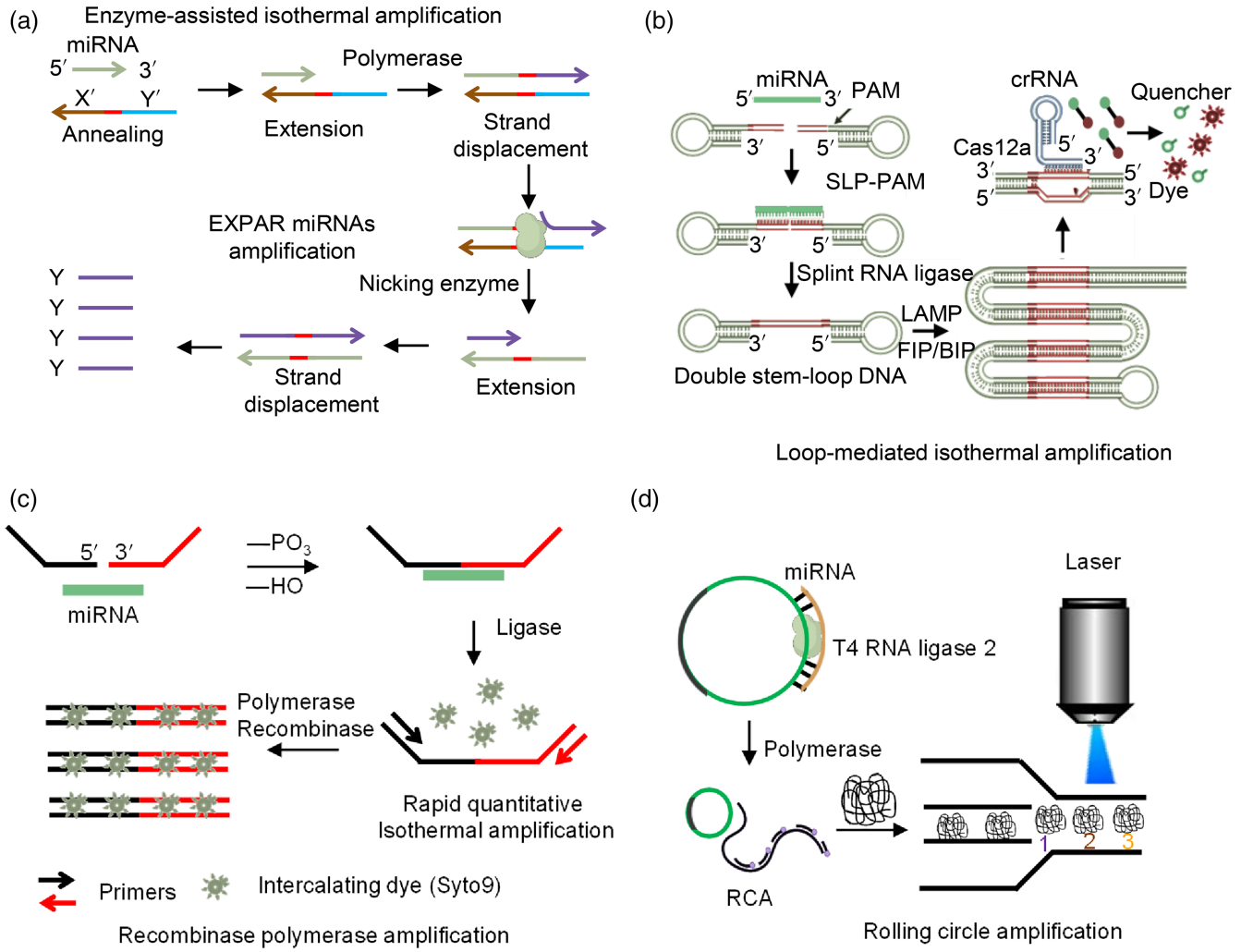

FIGURE 6 Enzyme-assisted isothermal amplification (a) the principle of exponential isothermal amplification, (b) illustration of CAL-LAMP for imRNAs detection. (c) The ligation/RPA-based multiplexed miRNA detection. (d) Digital multiplex miRNA detection procedure.

such as the enzyme concentrations and reaction times, as well as exploring novel biochemical strategies to enhance specificity. These improvements could potentially minimize non-specific amplifications and other sources of background noise. The development of such refined techniques will be crucial in broadening the applicability of EXPAR in various fields, including clinical diagnostics, environmental monitoring, and forensic science. Future studies may also explore the integration of EXPAR with advanced detection platforms, such as microfluidic systems and nanotechnology-based sensors, to further enhance its sensitivity and reduce background interference.

LAMP stands out as a highly efficient technique for nucleic acid amplification, notable for its operation under isothermal conditions. In a conventional LAMP reaction, four primers are designed from an extremely long single-strand DNA (ssDNA) template, which forms a concatemerized amplicon that ensures exponential amplification from a continuous strand displacement DNA synthesis. The final LAMP product comprises large-molecular-weight concatemers that contain self-priming hairpins and four single-stranded free loops. Meanwhile, some studies have reported the detection of miRNA by combining LAMP with other amplification methods. In a notable study, Hashimoto et al. ingeniously integrated reverse-transcription and elongation reactions to devise a miRNA detection system. This system LAMP on an electrical chip (Hashimoto et al., 2019). The LAMP primers for amplifying the lengthened miRNA were adsorbed and immobilized on the surface of the liquid-flow channel at five different positions. This method demonstrated the capability to simultaneously detect five different miRNA, with concentrations ranging from 10^3 to 10^6 (10^3 to 10^6) copies per 50 μL, within 2 h. In order to simply the operation process, Cai et al. developed a detection system that uses ligation-initiated, phosphorothioated primer-based loop-mediated isothermal amplification. This system is specifically designed for the detection of miR-146b-5p and miR-199b-5p (Talap et al., 2023). The system incorporates two linker

probes derived from dumbbell-shaped amplicons of LAMP, which are ligated to form an active amplicon only upon perfect hybridization with the target miRNA, ensuring high specificity. Additionally, the introduction of phosphorothioate (PS) modifications into the inner primers (BIP/FIP) enhances hairpin formation and extension in the LAMP process, markedly boosting the system's sensitivity. Further, to decrease the non-specific background, Li et al. developed a detection platform that combines CRISPR/Cas12a with ligation-initiated loop-mediated isothermal amplification (CAL-LAMP). This innovative approach is designed for the highly specific detection of microRNA (Zhang et al., 2021). The CRISPR/Cas12a component in this detection system plays a crucial role in sequence-specific identification of LAMP products. This method effectively eliminates the impact of non-specific amplification that might arise from primer dimers and spurious amplicons (Figure 6b). Additionally, the system involves crafting a pair of target-specific stem-loop DNA probes. These probes can be ligated together to form a double-stem-loop DNA template in the presence of the target, thereby initiating the LAMP reaction. This process achieves one-nucleotide resolution, thanks to the highly specific nature of the ligase reaction. Employing MicroRNA (miRNA) as model targets, the CRISPR/Cas12a-assisted ligation-initiated loop-mediated isothermal amplification (CAL-LAMP) method is capable of sensitively detecting miRNA concentrations as low as 0.1 fM with remarkable specificity. Due to its potent amplification capacity, LAMP employing complex primers can occasionally produce non-specific amplification products. This characteristic has the potential to result in false positive outcomes. Differentiating between very similar miRNA sequences can be challenging. Ongoing research is likely to focus on enhancing the specificity of LAMP in differentiating between closely related miRNA sequences. This includes refining primer design and optimizing reaction conditions.

RPA emerges as a highly sensitive and selective technique for isothermal nucleic acid amplification within the temperature range of 37–42°C. Its capability to amplify as few as 1–10 copies of miRNA in under 20 min. Wee and their team utilized a ligation reaction and RPA to devise an isothermal method that allows for the rapid, sensitive, accurate, and multiplexed detection of miRNAs (Wee & Trau, 2016). The target-specific sequences in probes are simply designed to be complementary to the half-sequence of miRNA at the 3′- and the 5′-terminal to synthesize long ssDNA (Feng et al., 2019; Zhang et al., 2013, 2014). This method is capability of detecting picogram levels of total RNA input (or ∼40 copies/pg) and discriminating between closely related miRNA (Figure 6c). Meanwhile, Gao et al. employed a ligation/RPA assay for rapid detection of SARS-CoV-2 genes (P. Wang et al., 2021). The assay demonstrated an impressive sensitivity, detecting as low as 101 viral RNA copies per reaction, a performance comparable to RT-quantitative polymerase chain reaction (RT-qPCR) and other methods for detecting SARS-CoV-2 nucleic acids. Moreover, the assay offered a quick and easy operation, with results available in less than 30 min. RPA is a rapid, sensitive, and versatile nucleic acid amplification technique, operating effectively at lower temperatures (37–42°C) for energy-efficient field application, requiring minimal sample preparation, and capable of amplifying both DNA and RNA targets with high specificity, all while eliminating the need for thermal cycling due to its isothermal process. RPA faces challenges such as a heightened risk of contamination due to its high sensitivity, nonspecific background and probe design is very expensive (Reid, Paliwoda, et al., 2018; Xu et al., 2023).

RCA is an isothermal enzymatic process where a short DNA or RNA primer is amplified to form a long single-stranded DNA or RNA using a circular DNA template and special DNA or RNA polymerases (Yao et al., 2021). The RCA product is a concatemer containing tens to hundreds of tandem repeats that are complementary to the circular template. The power, simplicity, and versatility of the DNA amplification technique have made it an attractive tool for biomedical research and nanobiotechnology. Zhang et al. introduced an innovative method for the homogeneous multiplexed digital detection of miRNA, utilizing ligation-rolling circle amplification with fluorescence flow cytometry (Hu et al., 2020). By this methods, multiplexed miRNA could be transformed into several kinds of nanoflower balls at the same time and detected by flow cytometry directly, enabling simple, sensitive, and multiplex detecting of miRNA in one reaction directly (Figure 6d). This digital microRNA analysis is anticipated to hold significant application potential in clinical diagnosis and related domains. miRNA-141, miRNA-21, and Let-7a. Chung et al. developed the visualization of two-dimensional fluorescent ring patterns to multiplexed detect nucleic acid targets (Lee et al., 2023). A small amount of test is deposited onto a cellulose nitrate membrane, and upon radial chromatographic flow and evaporation of the solvent, fluorescent patterns become visible when exposed to UV light. The target nucleic acid is amplified isothermally and promptly hybridizes with fluorescent oligonucleotide probes within a single reaction. They developed an integrated fluorescent ring assay in conjunction with isothermal amplification.

The cyclic enzymatic amplification method (CEA), a novel approach employing nucleases, represents a significant advancement in nucleic acid detection. This method is predicated on the principle that a single target initiates multiple cycles of target-dependent nuclease cleavage of reporter probes, thereby amplifying the output signal (Nie et al., 2024). Characterized by its simplicity, rapidity, sensitivity, and cost-effectiveness, CEAM offers a promising alternative to

traditional nucleic acid detection techniques, broadening the scope for applications in various fields such as diagnostics, molecular biology, and genetic research (Yang et al., 2023). Exonuclease I (Exo I) displays an excellent preference for degrading excess single-stranded oligonucleotide in a reaction system containing double-stranded extension products. Zhou et al. employed Exo I to develop multiplexed detection of miRNA based on an enucleation reaction by a conformational switch of hairpin probes (Figure 7a) (Zhang et al., 2016). The stem of the molecular beacon (MB) probe was opened upon hybridization of the target miRNA with the loop region of the corresponding MB probe. Subsequently, Exo I was employed to efficiently degrade the single-stranded overhang of the unfolded-state MB probe in the 3′ → 5′ direction. As a result, the fluorophore or quencher at the 3′ end was permanently cleaved from the MB probe, leading to the emission of the fluorescence signal. Besides, Ding et al. utilized exonuclease III to develop multiplexed fluorescence detection of miRNA-141 and miRNA-21 based on a cycle amplification strategy (Jie et al., 2017). The hairpin (HP) DNA was firstly linked to the magnetic bead@Au (MB@Au) complex, then the present target miRNA hybridized with HP, which then initiated the exonuclease III-aided target recycling process, leading to the generation of numerous intermediate DNA sequences (s1) on MB@Au, changing fluorescence signal of QDs. Zhang et al. developed highly efficient wavelength-resolved electrochemiluminescence of carbon nitride films for multiplex miRNA detection (Fang et al., 2023). The CN films with tunable ECL emission were developed from blue to green (410, 450, 470, and 525 nm). miRNA-21 and miRNA-141 were simultaneously measured and quantified via a one-pot ECL assay with superior low detection limits of 0.13 fM and 25.17 aM, respectively. Ai et al. developed a novel photoelectrochemical biosensor for the sensitive detection of dual miRNA using molybdenum carbide nanotubes as nanocarriers and energy transfer between CQDs and AuNPs (Wang et al., 2019). Two hairpin probes (H1 and H2) carrying the Au NPs were used to "switch off" and "switch on" the PEC signal of the CQDs, with a close approach of the tagged AuNPs to the CQDs quenching the PEC signal. The introduction of different miRNA (miRNA-159b and miRNA-166a) altered the interparticle distance between the AuNPs and CQDs, thereby affecting the intensity of the PEC response. The platform achieved the exceptional sensitive detection of both miRNA-159b and miRNA-166a, and the LoD were 0.15 fM and 0.21 fM, respectively. Miao et al. developed quantification of multiplex miRNA by mass spectrometry with duplex-specific nuclease-mediated amplification (Qian et al., 2023). The pretreatment procedure involves the utility of Fe_3O_4@AuNP mediated magnetic separation and DSN-catalyzed reaction cycles to enrich DNA mass tags. Strömberg et al. developed optomagnetic detection of miRNA based on DSN-assisted target recycling and multilayer core-satellite magnetic superstructures (Figure 7b) (Tian et al., 2017). The miRNA detection approach relies on optomagnetic readout, target recycling with the assistance of DSN, and core-satellite magnetic superstructures. When target miRNA is detected and target recycling is facilitated by DSN, the core-satellite magnetic superstructures release their satellite components into the suspension. These satellite components can then be precisely quantified using an optomagnetic setup. The total assay time is around 70 min, with an LOD of 4.8 fM with a linear detection range of approximately 6 orders of

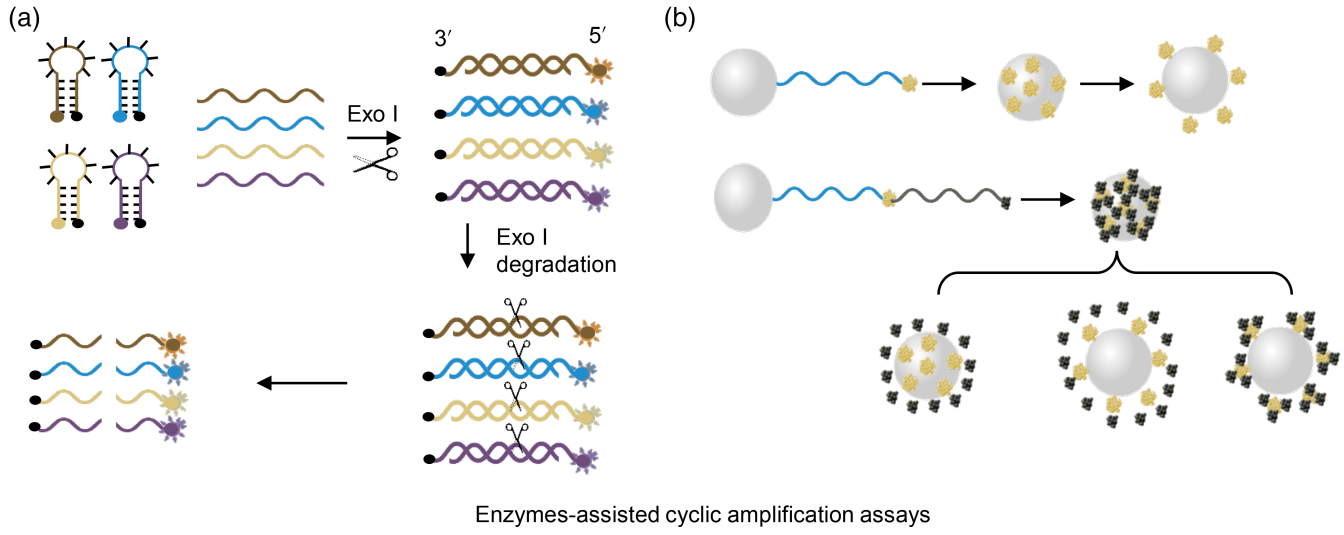

FIGURE 7 Enzyme-assisted cyclic amplification for miRNA detection. (a) Exo I-based multiplexed miRNA detection based on the fluorescence enhancement of molecular beacon (MB) probes. (b) DSN-assisted target recycling and multilayer core-satellite magnetic superstructures.

magnitude. Enzyme-assisted cyclic amplification stands out in miRNA detection for its exceptional sensitivity and specificity, efficiently detecting low miRNA levels and distinguishing similar sequences, alongside rapid processing that surpasses traditional methods. This technique operates at lower temperatures than PCR-based methods, simplifying the setup, and offers the capability for multiplexing, enabling simultaneous detection of multiple miRNA targets.

Enzyme-based isothermal amplification for miRNA has high sensitivity without the thermal cycles at constant temperature. This detection system is capable of rapid quantitative detection of miRNA at low concentrations for clinical samples. The simple operation at low temperatures and the fast reaction speed make these methods well suited for POCT. But, it also involves complexity, potential for contamination, dependency on enzyme quality and RNA integrity, and can be time-consuming and expensive. For isothermal amplification, there will be a significant background signal, which might result from the undesired products from the possible cross-dimerization with isothermal amplification procedures (Kim et al., 2023; Meagher et al., 2018). This will decrease the sensitivity. Meanwhile, similar to enzyme-free amplification, there was also the issue of multiplexed miRNA detection. Enzyme-based amplification requires that the detection system contain several groups of ssDNA probes for specific recognizing miRNA. It also increases the background signal to reduce the sensitivity. So, future research methods should focus on designing the platform to achieve effective and highly sensitive miRNA detection.

4.2.4 | POCT device for multiplexed miRNA detection

The advanced methodologies that facilitate highly sensitive and multiplexed miRNA detection offer promising avenues for the development of POCT for various diseases in limited resource sites. The integration of microfluidic technology into POCT systems for the detection of miRNA has fundamentally transformed biomarker analysis and detection. The review will explore these cutting-edge developments, examining how they have transformed the landscape of microRNA detection and amplification and their implications for future diagnostic practices and personalized medicine.

As for the microfluidics-based miRNA detection, diffraction techniques, there are two main methods based on the transducer types, namely electrochemical and optical detection (Chand et al., 2018). Bruch et al. first introduced the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas13a-powered microfluidic, an integrated electrochemical biosensor for the on-site detection of miRNA via amplification-free strategy (Bruch et al., 2019). miR-19b and miR-20a can be simultaneously detected with a readout time of 9 min. The LoD can reach 10 pM concentrations of miRNA with a measurement volume under 0.6 μL. This platform did not have error-prone amplification steps for miRNA amplification (target amplification-free) with simple steps. Watanabe et al. also used Cas13a to construct CRISPR-based amplification-free digital RNA detection (SATORI), by combining CRISPR-Cas13-based RNA detection and microchamber-array technologies (Shinoda et al., 2021). The concurrent utilization of several distinct guide RNAs heightened the sensitivity, resulting in the ability to detect SARS-CoV-2 Ngene RNA at approximately 5 fM concentrations. Bruch et al. created a CRISPR-based electrochemical microfluidic biosensor capable of multiplexed, amplification-free diagnostics for miRNA (Bruch et al., 2021). The team designed various multiplexed forms of their electrochemical microfluidic biosensor by segmenting its channel into different parts, resulting in four unique chip configurations. These configurations enabled the concurrent, amplification-free measurement of up to eight miRNAs on the CRISPR-Biosensor X. They also developed a one-step model assay with amperometric readout, integrated with a 2-min stop-flow protocol to assess the fluidic and mechanical aspects and constraints of the device's different models. The most effective sensor was then chosen for a Cas13a-enabled preliminary test to measure two miRNAs (miRNA-19b and miRNA-20a), known to be altered in the blood of children with medulloblastoma. This same device was used to quantify these miRNAs, including concurrent control tests. In addition, optical biosensors were incorporated into the microfluidics setup. Zhao and colleagues introduced a novel enzyme-free method for detecting multiple miRNAs, employing shape-coded hydrogel microparticles along with HCR in a microfluidic chip (Figure 8a) (Zhao et al., 2022). Cascades of hybridization between carboxyfluorescein (FAM)-labeled hairpin probes were triggered to form long dsDNA products in hydrogel microparticles, which led to the deposition of FAM in them. the method achieved LODs of 27.8 pM for miRNA 21 and 24.7 pM for miRNA let-7a. Further enhancement was achieved by integrating this technique with a microfluidic chip, which markedly reduced the detection time, increased the overall throughput, and simplified the operational procedures. A microfluidic paper-based sensor was designed using laser-induced fluorescence to detect miRNA-21 and miRNA-31 by Cai et al (X. Cai et al., 2020). In this research, FAM-labeled probes and quenching groups within the eclipse were introduced into the hydrophilic regions of a microfluidic paper-based analytical device. Also, DSN was loaded on other circles. miRNA were added to related circles, leading to the hybridization with probes.

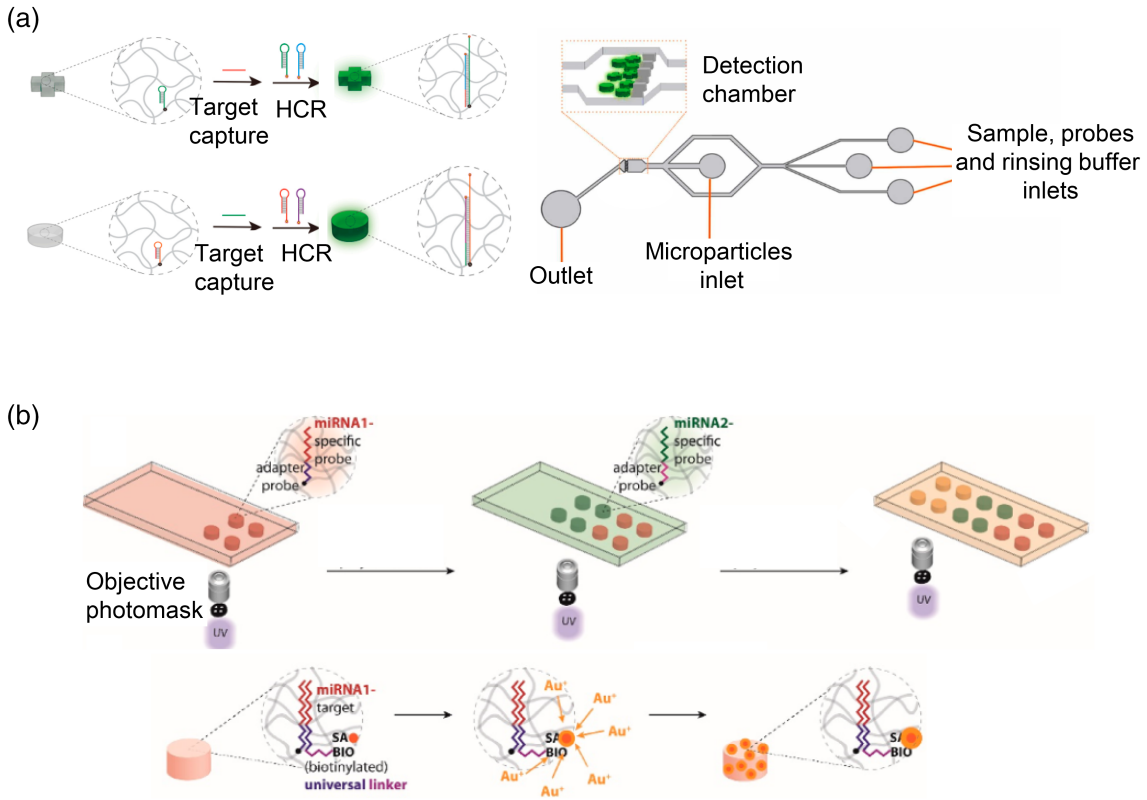

FIGURE 8 Microfluidic biosensor for miRNA detection. (a) Schematic illustration of multiplexed miRNA detection based on shape-coded hydrogel microparticles integrated with HCR (Adapted with permission from K. Zhao et al., 2022). (b) miRNA assay scheme: target hybridization, universal linker ligation, gold nanoparticle labeling, and gold ion deposition-based signal amplification on chip (Adapted with permission from H. Lee et al., 2020).

After folding and pressing the paper, DNA strands of probes were selectively digested by DSN, leading to the release of miRNA, FAM, and eclipse. The fluorescence intensity was enhanced as FAM became far from eclipse. The miRNA-21 and miRNA-31 were determined in the range of 0.50–200 and 1.0–200 fM with LODs of 0.20 and 0.50 fM, respectively. Doyle and Lee et al. created a hydrogel-based colorimetric assay that allows for the multiplexed detection of miRNA within a microfluidic device. This method is notable for not requiring intricate external apparatus for fluid handling and imaging (Figure 8b) (Lee et al., 2020). The assay exhibited a detection limit of 260 fM, and it was capable of multiplexing small groups of miRNA in both healthy and cancerous samples Integrated miRNA extraction and detection for POCT.

Merging the processes of miRNA extraction, amplification, and detection within a single platform can greatly advance personalized medicine. Such integration is particularly vital in the realm of POCT diagnostics, where the emphasis is on achieving speedy, precise, and convenient testing procedures. Recent progress in salivary miRNA detection has been largely centered on creating microfluidic devices and paper-based methods. Microfluidic devices have revolutionized traditional laboratory processes by miniaturizing and automating them, thereby enhancing speed and efficiency. Paper-based detection involves the use of paper as a substrate for bioassay development. The inherent simplicity, low cost, and disposability of paper-based devices make them highly attractive for widespread screening and monitoring purposes.

4.3 | Microfluidics for plasma miRNA sample extraction and detection

Due to their swift, compact, and automated processes, microfluidic devices are emerging as an effective alternative for miRNA detection with minimal input saliva. For example, Wang and Lee et al. utilized in situ hybridization to develop an integrated microfluidic system equipped with highly sensitive field-effect transistors to perform extracellular vesicles

(EV) extraction, EV lysis, miRNA (miR-21 and miR-126) isolation and detection within 5 h (Cheng et al., 2018). The LoD fell into the physiological range (fM) for two miRNA. The chip integrated the extraction and detection of extracellular vesicle (EV) miRNA and achieved multiple miRNA detection. However, the operation of this platform is quite intricate, requiring multiple steps, extended time for processing, and a relatively low LoD. To simplify the operation and improve the sensitivity, Guan et al. employed an RT-LAMP-based amplification system to develop a fully integrated SARS-CoV-2 device using a self-collected saliva sample (Tang, Cui, et al., 2022). There are four chambers for storing cell lysis reagents and RT-LAMP amplification reagents. A ready-to-use reagents cartridge, an easy-to-use smartphone interface, and an ultra-compact analyzer are used to extract RNA, amplification, and detection with 5 copies/μL of LoD in just 45 min. This testing device achieves integrated microfluidics for RNA extraction, detection, and signal processing with low sensitivity. However, it can only detect a single RNA and is unable to simultaneously detect multiple miRNA. To develop simultaneous detection of multiple miRNA, Lu et al. created a portable microfluidic system that utilizes EXPAR for the isothermal amplification and detection of exosomal multiplexed miRNA (Figure 9a) (Qian et al., 2022). The system is designed as a compact unit, comprising two interconnected flow cells. One is used for handling exosomes, and the other is dedicated to the detection of miRNA. Multiple reagents for the EXPAR were stored in the chip to simultaneously analyze multiple exosomal miRNA (miRNA-127, miRNA-146b, miRNA-210, and miRNA-223), respectively. The rapid exosomal miRNA assay's benefits include its ability to detect multiple targets simultaneously, use of an affordable and disposable sensor with femtomolar sensitivity, a swift 30-min detection time, and the convenience of bypassing RNA extraction for complex samples. The portable microfluidic system integrates several steps to extract, amplify, and detect multiple miRNA with a short detection time to achieve an all-in-one assay. However, designing multiple detecting probes is required for the EXPAR system, which makes the detection system complex.

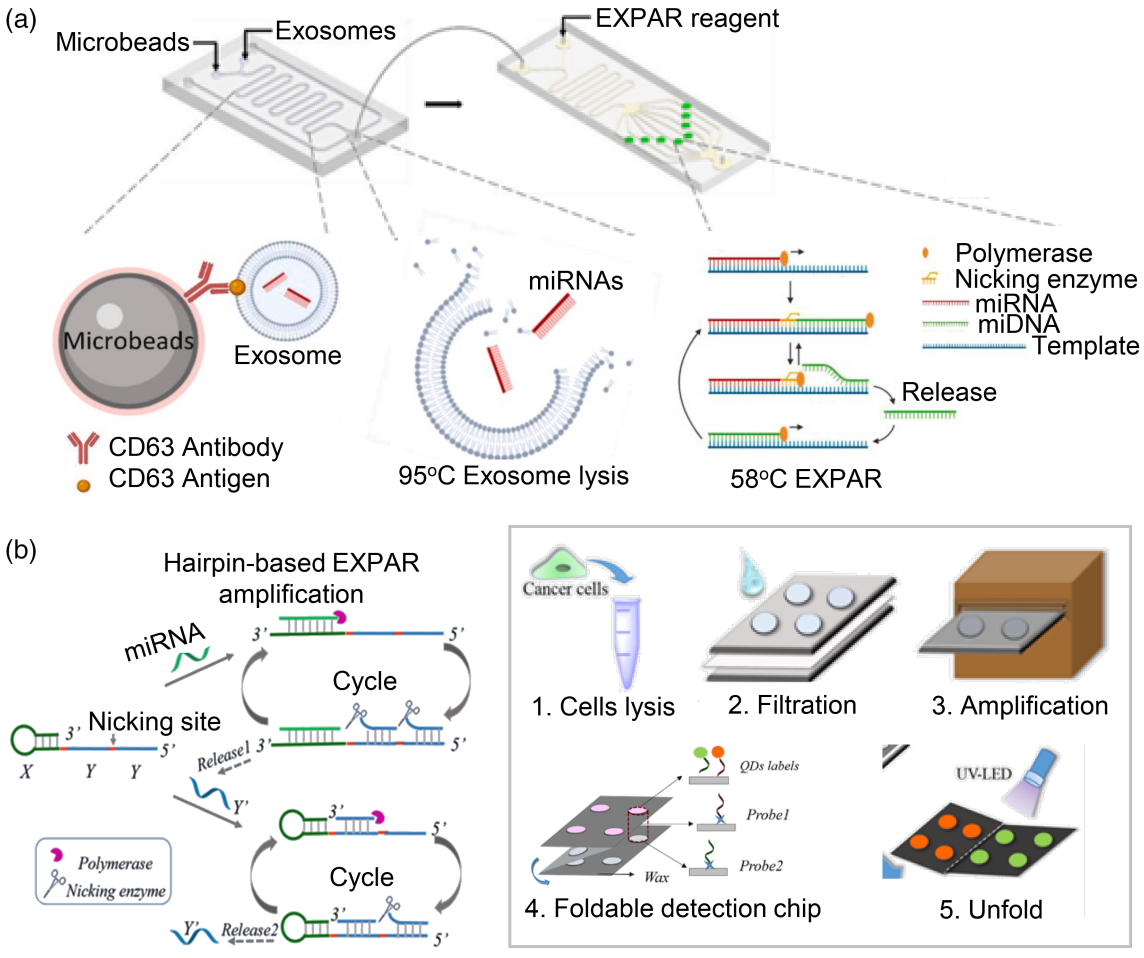

FIGURE 9 Paper-based miRNA extraction and detection on integrated devices. (a) EXPAR amplification-based multiplexed exosomal miRNA assay through the on-chip (Adapted with permission from H. Deng, Zhou, et al., 2017). (b) Hairpin-EXPAR amplification-based multiplexed miRNA assay on the paper fluidic chip (Adapted with permission from J. Qian et al., 2022).

4.4 | Paper-based cancer cell miRNA extraction and detection platform

The development of POCT diagnostics has increasingly focused on paper-based analytical platforms due to their rapid assay capabilities, minimal sample volume requirements, cost-effectiveness, and ease of use (Parolo & Merkoçi, 2013; Paul et al., 2020). The paper-based analytical techniques also need to integrate extraction and amplification steps into one system for miRNA detection. To achieve signal readout, a paper-based detection platform must use the strategies of in situ hybridization to capture the target molecules to readout. Meanwhile, owing to the low level of miRNA in samples, miRNA amplification should also be used to increase the signal. For example, Xing et al. designed a new hairpin probe–exponential amplification reaction (HP–EXPAR) to develop a paper fluidic chip device for small RNA extraction, amplification, and multiplexed miRNA (miR-21 and miR-155) analysis (Deng, Zhou, et al., 2017) (Figure 9b). In that strategy, EXPAR enables highly efficient amplification of short oligonucleotides and is capable of multiplex analysis. EXPAR yielded large amounts of products that hybridized with specific probes immobilized on the reaction zone, generating fluorescence signals with UV light excitation. This assay achieve a satisfactory sensitivity range from 3×10^5 to 3×10^8 copies, with a low detection limit of 3×10^6 copies. Tang et al. developed a portable POCT system to diagnose lung cancer through the detection of exosomal miRNA with low concentrations in urine and saliva (P. Zhou et al., 2020). A strip-based POCT system to diagnose lung cancer through the detection of exosomal miRNA. A novel strategy involving the use of Fe_3O_4@SiO_2-aptamer nanoparticles (FSAs) as a "catcher" to capture and accumulate lung cancer exosomes and DSN as an amplification tool to amplify miRNA signals was proposed. This novel strip-based POCT system successfully achieved real noninvasive diagnostics of lung cancer using clinical salivary and urine samples with high sensitivity and selectivity, that is with results comparable to those of the standard quantitative qRT-PCR method (Cao et al., 2023; Zhao et al., 2015).

The future direction of POCT devices is the integration of miRNA extraction, amplification, and signal readout in microfluidic and paper-based devices, enabling convenient, rapid and effective self-testing for patients and improving disease prognosis. Both microfluidic and paper-based devices necessitate the extraction and amplification of miRNA samples to enable ultra-sensitive detection. Compared to microfluidics, the paper-based detection methods require an additional design of in situ hybridization for signal readout, making this method more complex than microfluidics. Isothermal amplification presents a viable option for miRNA amplification in POCT. To detect multiple miRNA, a design incorporating multi-channel detection is required, with each channel specifically amplifying targeted miRNA molecules. However, this approach does not represent true simultaneous multiplex detection but rather parallel detection. The concurrent detection of multiple miRNA using a single system poses significant challenges and is an area ripe for further research and development.

5 | SUMMARY AND FUTURE PERSPECTIVE

Salivary miRNA are emerging as powerful early biomarkers, forging new directions in molecular diagnostics, prognosis, and personalized disease management (Ekanayake Weeramange et al., 2023; Hicks, Zhu, et al., 2023; Hsu et al., 2023). The incorporation of salivary miRNA extraction and amplification into POCT systems represents a notable advancement, yielding quicker diagnostic results and greater accessibility for patients. Extraction of miRNA mainly relies on liquid-phase or solid-phase extraction to isolate miRNA for the amplification or detection process. Multiplexed miRNA detection predominantly depends on techniques like in situ hybridization and isothermal amplification (Jet et al., 2021). In situ hybridization facilitates the detection of several miRNA simultaneously within a single sample, which is crucial for examining complex miRNA-involved regulatory networks. On the other hand, isothermal amplification methods are recognized for their exceptional sensitivity, enabling the detection of low levels of multiple miRNA, thus serving as an effective and practical substitute for traditional amplification techniques such as PCR.

Despite the many advancements in technologies for salivary miRNA detection, and we need to stress a few critical points about POC miRNA diagnostics, establish reliable biomarkers, simple samples extraction methods, multiplexed and sensitive assays, fully integrate devices with simple to answer.

5.1 | Establish specific biomarkers

Numerous factors influence miRNA expression levels, including age (Eshkoor et al., 2022; Jin Jung & Suh, 2012), gender (Lin, Han, et al., 2022; Lin, Liu, et al., 2022; Lin, Wu, et al., 2022), lifestyle (Panico et al., 2021), and an individual's

disease history (Li & Kowdley, 2012). Multiple factors collaboratively influence the expression level of miRNA. For example, miRNA-21 is all involved in many cancer types. The expression levels were reportedly upregulated in patients with gastric cancer (Zheng et al., 2012), but it was downregulated in patients suffering from breast cancer (Erbes et al., 2015). This means that the contribution of miRNA in various types of cancers differs. There is no general consensus regarding which miRNA are suitable as biomarkers. Taking these factors into account, certain miRNA have been identified with relatively low specificity and reproducibility, which poses challenges for their use in disease diagnosis. In the future, we need to establish specific miRNA biomarkers and standardize the detection methods and the indicators for diagnostic purposes.

5.2 | Samples extraction

Currently, the extraction of salivary miRNA in most detection methods is primarily conducted using kits, followed by the addition of these miRNA into the detection process (Politza et al., 2023; J. Qian et al., 2022). Nonetheless, this technique for salivary miRNA extraction complicates the operational processes of POCT. The pursuit of simpler and more convenient extraction technologies will greatly benefit the integration with detection processes. The integration of extraction and detection for salivary miRNA has a great promise in the future (Tang, Cui, et al., 2022). Such advancements ensure that miRNA testing becomes more accessible and less dependent on sophisticated laboratory infrastructure. This opens up a wide range of possibilities for widespread application.

5.3 | Multiplexed assay

Multiplexed assays include amplification-free (in situ hybridization) and isothermal amplification strategies (Deng, Zhou, et al., 2017). In situ hybridization facilitates the detection of multiple miRNA but does not include an amplification step, which consequently limits its sensitivity (Oishi & Juji, 2021). Amplification techniques of enzyme-free and enzyme are effective in increasing the number of target molecules derived from miRNA, thus increasing the sensitivity of the detection process with a fast amplification rate (Cao et al., 2023; Peng et al., 2024; Zhou et al., 2022). However, these methods necessitate the use of extra probes or primes and enzymes, which might result in background noise or unintended amplification. Most importantly, the simultaneous detection of multiple miRNA using these methods is complicated due to the intricate design required for primers and enzymes, which will generate the obvious background signal to affect the sensitivity. Meanwhile, this design complexity is a significant hurdle for multiplexed miRNA detection by POCT. Moreover, isothermal amplification faces other challenges in its application. A significant issue is the occurrence of nonspecific background amplification. This problem mainly arises from non-specific binding between probes or templates, which serves as a major contributor to this limitation (Reid, Paliwoda, et al., 2018; G. Xu et al., 2023). The field of multiplexed miRNA-based disease diagnostics is facing a significant challenge: create novel approaches that allow for the simultaneous, multiplexed, and sensitive detection of miRNA through simple systems and straightforward operations.

5.4 | Fully integrated device

The device also needs to be developed for simple-to-answer for layperson use (Khashayar et al., 2022; Zhang et al., 2017). Furthermore, there is a focused endeavor to develop advanced devices capable of facilitating the loading of extraction and amplification reagents for the simultaneous, multiplexed detection of miRNA, aiming to simplify the detection process in POCT systems. Additionally, to enhance the longevity and transportability of reagents and chemicals (Liu et al., 2023; Noviana et al., 2021), the adoption of lyophilization technology is being explored. This approach would allow for extended storage and easier transportation of these materials, further improving the practicality and accessibility of miRNA detection technologies.

AUTHOR CONTRIBUTIONS

Zhikun Zhang: Conceptualization (equal); writing – original draft (equal); writing – review and editing (equal). **Tianyi Liu:** Writing – original draft (equal); writing – review and editing (equal). **Ming Dong:** Conceptualization

(equal); writing – review and editing (equal). **Md. Ahasan Ahamed:** Writing – review and editing (equal). **Weihua Guan:** Conceptualization (equal); funding acquisition (lead); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

ORCID

Zhikun Zhang https://orcid.org/0000-0002-0945-7176
Md. Ahasan Ahamed https://orcid.org/0009-0006-9962-2994
Weihua Guan https://orcid.org/0000-0002-8435-9672

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