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Recent advances and challenges of biosensing in point-of-care molecular diagnosis

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ABSTRACT

Molecular diagnosis, which plays a major role in infectious disease screening with successful understanding of the human genome, has attracted more attention because of the outbreak of COVID-19 recently. Since point-of-care testing (POCT) can expand the application of molecular diagnosis with the benefit of rapid reply, low cost, and working in decentralized environments, many researchers and commercial institutions have dedicated tremendous effort and enthusiasm to POCT-based biosensing for molecular diagnosis. In this review, we firstly summarize the state-of-the-art techniques and the construction of biosensing systems for POC molecular diagnosis. Then, the application scenarios of POCT-based biosensing for molecular diagnosis were also reviewed. Finally, several challenges and perspectives of POC biosensing for molecular diagnosis are discussed. This review is expected to help researchers deepen comprehension and make progresses in POCT-based biosensing field for molecular diagnosis applications.

1. Introduction

Driven by the development of new techniques and the improvement of health consciousness, in vitro diagnosis, which judges diseases or body function by extracorporeal analysis of specimens like serum and tissue, has played a growing important role in our daily life \cite{1,2}. The value of in vitro diagnosis can be embodied in disease screening and prevention apart from treatment, and especially highlighted in the prevention and control of the recent COVID-19 pandemic \cite{3-8}. In vitro diagnosis predominates in time consumption and patient acceptance. The utility of in vitro diagnosis lies on implementation of a biosensor, which is defined by the International Union of Pure and Applied Chemistry (IUPAC) as a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals. The major types of in vitro diagnosis include biochemical examination, immunoassay, and molecular diagnosis. Biochemical examination always has a high demand of professional skill of operators and can scarcely avoid expensive implementation equipment. Immunoassay, which focuses on affinity reactions between target biomarker (antigen or antibody) and other molecules in a sample for limited binding sites provided by the immobilized capture reagent \cite{7}, has gained a mania in the thriving field of disease screening benefiting from its low cost and short turnaround time \cite{9}. Nevertheless, some immunoassays are dependent on antibodies in samples, whose appearance may lag several weeks after initial exposure, leading to false results in the early stage of infection \cite{5,10,11}. Besides, immunoassay often suffers from relative low specificity for possible cross-reactions due to similarity between the molecules \cite{3,12}. Molecular diagnosis, which refers to the strategy of detecting changes in the structure or expression of individual genetic substances like DNA and RNA using molecular biological methods, possesses excellent sensitivity and specificity comparably. Thus, biosensing for molecular diagnosis can indicate characteristics of genetic makeup that have a high correlation with certain conditions and allow assessment of body status for healthcare delivery \cite{13}.

Molecular diagnosis has reaped much appreciation and been regarded as the gold standard of some disease diagnoses \cite{10,14-16}. However, some lab-based tests rely on bulky and expensive instruments, hindering their widespread use, especially in resource-poor settings.

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where centralized laboratory facilities, funds, and trained personnel are in short supply. Point-of-care testing (POCT), defined restrictively by the College of American Pathologists (CAP) as ‘testing provided within the institution, but performed outside the physical facilities of the clinical laboratories, and without the requirement of permanent dedicated space but instead includes kits and instruments, which are either hand carried or transported to the vicinity of patient for immediate testing at that site’ [2,17], holds advantages of rapid results, unrestricted test sites and low professional skill requirements for operators [18–21]. Pushed forward by blazing market demands and the obvious bottlenecks of lab-based tests, POCT has blossomed in many fields such as glucose monitoring and pregnancy tests [22–24]. Remarkable advances in miniaturization, microcomputerization, and noninvasive testing procedures have enabled production of compact and portable POCT biosensing devices. Conceivably, armed with POCT biosensing techniques, molecular diagnosis can serve extensive scenarios in a rapid, robust, and cost-effective manner in patients’ bedside, physician offices, pharmacies, supermarkets, and airports, etc [2,6].

In this review, we first summarize the mainstream POCT techniques for molecular diagnosis, and describe the principles and characteristics of these techniques. Then, we discuss the construction of POCT biosensing systems for molecular diagnosis from the aspects of diagnostic platform, readout form and improvement factors. Furthermore, the application scenarios of POCT biosensing for molecular diagnosis are introduced. Finally, we make a brief conclusion and prospect the development of POCT-based molecular diagnosis.

2. Available techniques for POCT molecular diagnosis

Since the mid-20th century, genetics has become a distinct specialty and people began to utilize the study of gene expression and variation to serve disease diagnosis and clinical assistance [25]. However, the development and innovation were initially tardy and limited because most industry resources and interests are always concentrated on something with considerable commercial value. With the improvement of health consciousness along with living standards and the frequent blow of dreadful diseases, people gradually turn the spotlight on the value of molecular diagnosis. In recent decades, academia and industry have collaborated to facilitate a quantum leap forward in molecular diagnosis at an unparalleled pace. The tremendous and promising market has galvanized the advent of a cornucopia of effective techniques and vice versa. These techniques are targeted to the same topic of molecular diagnosis but emphasize different aspects such as detection speed, cost, throughput, sensitivity, specificity, and complexity. The major molecular diagnosis techniques for POCT are introduced as follows.

2.1. Nucleic acid amplification testing (NAAT)

NAAT is a mature technique to interrogate pathogens by generating multiple copies of nucleic acid sequences [26]. In NAAT assays, samples are always pretreated by lysis, purification and dilution prior to amplification and detection. Various types of samples including serum, nasopharyngeal or oropharyngeal swabs, bronchoalveolar lavage fluid, saliva, sputum, and stool are applied to NAAT assays [7]. Amplification reaction plays a pivotal role in the whole detection process and searches are devoted to developing a variety of effective amplification methods.

2.1.1. Polymerase chain reaction (PCR)-based testing

PCR has been widely used in clinical analysis due to its excellent sensitivity, specificity, and quantification capability [27,28]. In a PCR assay, the template gene is denatured by heating, and the complementary sequence of primer and single strand DNA (ssDNA) are renatured by cooling to form the complex. Then, the DNA is synthesized from 5’ to 3’ direction by heat-resistant DNA polymerase using the primer as the fixed starting point. After 20–30 cycles, the nucleic acid is amplified multiple times for detection.

PCR is generally regarded as the gold standard for nucleic acid detection. However, the highly specialized and energy intensive nature of the thermal cycling process extremely renders these assays bulky, tedious, and time-consuming, and limits their POCT application. In this regard, many efforts have been made to address this issue [29–36]. For instance, Cheong et al. exploited magneto-plasmic nanoparticles for plasmonic heating to achieve fast thermocycling [29]. One single cycle only took 8.91 s and the whole detection could be completed within 17 min. The approach of amplification based on Rayleigh–Bernard natural convection was reported, providing a protocol which could dramatically ease the burden of energy, cost and time compared with the conventional PCR assays [34,35]. Furthermore, the insulated isothermal PCR system has been verified to be suitable for POCT detection and possesses brilliant sensitivity and specificity [36]. Some commercial diagnosis systems also utilize optimized PCR assays to realize disease detection. Typically, Cepheid GeneXpert was able to detect many pathogens and drug resistance based on integrated real-time fluorescent PCR [37–39]. BioFire FilmArray system using nested multiplex PCR was known for the ability of detecting 24 pathogens in one serum test within 1 h, exhibiting extraordinary performance for early detection.

2.1.2. Nucleic acid sequence-based amplification (NASBA)

NASBA [40] was developed on the basis of transcription-based amplification system (TAS) [41] and self-sustained sequence replication (3SR) system [42]. As illustrated in Fig. 1A, NASBA assays, which include a non-cyclic phase and a cyclic phase, make use of AMV reverse transcriptase, RNase H, T7 RNA polymerase, and two specific primers to complete the whole amplification process. In the non-cyclic phase, primer 1 binds to template RNA and then is extended by AMV reverse transcriptase to form a cDNA copy. The produced DNA/RNA hybrid is hydrolyzed by RNase H and retains a single strand of DNA which then binds to primer 2 and forms a double strand DNA (dsDNA). Taking advantage of T7 RNA polymerase, dsDNA can generate many RNA copies which serve as templates in the cyclic phase. Primer 2 binds to these template RNAs followed by extension with the work of AMV reverse transcriptase as before. Then the formed DNA/RNA hybrids are hydrolyzed and finally form dsDNAs on the basis of primer 1 and RNase H. The cycle repeats to realize the amplification of target nucleic acid sequences. NASBA is similar to transcription mediated amplification (TMA) technique, while the latter uses MMLV reverse transcriptase with RNase H activity and reverse transcriptase activity instead of AMV reverse transcriptase and RNase H.

It’s noteworthy that NASBA can directly amplify specific nucleic acids with less cycle requirement compared to PCR, and the amplification temperature is kept at 41 °C to avoid sophisticated thermocycling operations. NASBA holds the ability for achieving 10³-fold amplification in 2 h [43], and the primer containing T7 promoter sequence results in excellent specificity. There is no need for denaturation steps as the amplified product of NASBA is single strand RNA. However, this makes the strategy not ideal for DNA analysis because precise temperature control and increased power consumption should be concerned when dsDNA is to be targeted [44,45]. Moreover, the efficient length of target amplification sequences is limited to 100–250 nucleotides [46], and the enzymes can not be added in advance due to the poor heat-resisting property.

NASBA has a wide use in many fields [47–50]. For instance, Molla-salehi et al. developed a rapid and cost-effective assay based on 16S rRNA gold nanoprobe-NASBA for food-borne pathogen detection [47]. Primers along with a thiolated oligonucleotide gold nanoprobe for one of the hypervariable regions of 16S rRNA gene were designed, which could enhance sensitivity and specificity. The whole detection time was optimized to about 80 min and the promising performance was verified using Salmonella genus. Multiplex NASBA detection was demonstrated in 1999 by Peter et al. [51]. Two individual mRNAs were detected
successfully in a single-tube amplification platform with great precision and accuracy. There seems to be some extent sacrifice in sensitivity of multiplex assays. Nevertheless, these effects are modest and decided by assay design optimization.

2.1.3. Strand displacement amplification (SDA)

The discovery of nicking enzymes, which can specifically identify nucleic acid sequences and shear the head or tail of the single strand sequence, facilitates a new nucleic acid amplification way instead of using heat denaturation. SDA is a novel nucleic acid amplification technique proposed by Walker et al. based on the nicking enzyme [52]. In an SDA assay as shown in Fig. 1B, the dsDNA sample is cleaved by the cleavage enzyme. After heat denaturation, the two ssDNA fragments bind to primer 1 and primer 2, respectively. Then DNA replications occur to form two dsDNA sequence fragments which contain a cleavage enzyme recognition site. HincII recognizes the specific site and nicks the DNA strand. The leaving 3’ end after nicking initiates an extension reaction by DNA polymerase and displaces the downstream DNA strand. The new produced sequences are also recognized and nicked by HincII. Thus, the next extension and displacement reactions carry on. The ssDNA displaced from one target-primer complex can serve as the template for the corresponding primer of the other complex, which resulting in the exponential amplification of the target DNA fragment. Prior to the amplification reaction, the restriction enzyme is used to generate the target DNA template, which makes the overall process sophisticated and reduces the selectivity of target DNA sequences. To address these issues, Walker et al. reported a new scheme which exploited the strand displacement reaction to generate the target DNA sequences as desired [53].

SDA can achieve 10^5-fold amplification in 2 h. The entire process is simple and easy-to-control, making it popular in fluorescence-labeled testing. However, there are also some unmet disadvantages of SDA assay. Firstly, although the temperature in amplification process is constant, thermal denaturation at 95 °C for target generation is often required when analyzing genomic DNA [43]. Second, SDA is not suitable for synthesizing long nucleic acid sequences due to the use of nonstandard nucleotides and may introduce contamination while adding enzymes after DNA denaturation.

Fang et al. used aptamer-mediated SDA to detect Salmonella enteritidis, which offered an alternative to conventional methods for foodborne pathogen detection [54]. Similarly, they converted this protocol into detection of Escherichia coli O157:H7 successfully [55]. Westin et al. chose SDA as the ideal rapid amplification approach for multiplex nucleic acid detection [56]. To improve the efficiency of amplification, they introduced electronic anchors of primers in distinct areas on the basic microchip, which could reduce the primer-primer interactions and created distinct amplification zones. However, the sensitivity of the anchored SDA may be limited because the single-stranded copies in SDA are physically unconstrained.

2.1.4. Rolling circle amplification (RCA)

RCA is an isothermal nucleic acid amplification technique developed
in 1995 [57] and refers to the replication process of circular DNA [58]. In an RCA assay as shown in Fig. 1C, a cyclized padlock probe binds to the target nucleic acid sequence and the 3’ end and 5’ end are ligated. The primer 1 binds to the padlock probe and is extended with the help of DNA polymerase. The extension results in long tandem DNA copy sequences and then primer 2 anneals to these sequences. The downstream sequences are displaced and work as new template DNAs of primer 1 for further amplification, which results in an exponential amplification of target DNA sequences. As for linear RCA, the primer 2 is circumvented and the final production is an ssDNA sequence.

RCA can generate billions of copies of the target nucleic acid sequences within 90 min at a constant temperature. It is worth noting that the amplification products can be used for genomic sequencing after phosphorylation treatment. However, the additional enzymes and procedural complexity in circularization process may increase the cost and hinder the conversion into POC sensors.

In general, RCA is a powerful technique and suitable for many applications of nucleic acid detection. There are plenty of reports which utilized RCA to develop POC biosensing devices for the detection of viruses [59], bacteria [60] and fungus [61,62]. For instance, Davarti et al. reported an RCA assay for the detection of Fusarium Head Blight-related species using custom padlock probes based on polymorphisms in the elongation factor 1-α gene [61]. This assay successfully amplified the target DNA in both environmental samples and contaminated heat samples and showed no cross-reactivity with other species. Murakami et al. proposed a new nucleic acid amplification protocol which termed ‘PG-RCA’ for DNA detection [63]. In the PG-RCA assay, ‘primer’ was generated as the reaction, avoiding the conventional use of exogenous primers in PCR. To improve the sensitivity of RCA which may limit by the 1:1 hybridization ratio of target strand and padlock probe, Long et al. came up with a target sequence recycled RCA (TR-RCA) [64]. Briefly, the dumbbell probe opened and triggered RCA reaction after the hybridization of target DNA and probe. And the target recognized and hybridized with another probe after it was strand displaced, triggering the next RCA cycle. The TR-RCA could conduct by mixing the reactants into one tube and reached a high sensitivity at fM level.

2.1.5. Loop-mediated isothermal amplification (LAMP)
LAMP was proposed by Notomi et al. in 2000 and soon harvested tremendous sight and interest [65]. As shown in Fig. 1D, LAMP can be divided into three steps: 1) starting material producing step, 2) cycling amplification, and 3) elongation and recycling step. The whole process involves four primers: forward inner primer FIP (F1c-F2, c strands for ‘complementary’), forward outer primer F3, backward inner primer BIP (B1c-B2, c strands for ‘complementary’) and backward outer primer B3. At the beginning, FIP binds to target DNA and is followed by F3 binding reaction. As F3 extends, the extension products of FIP are displaced and form a circle structure DNA sequence due to self-pairing at the 5’ end. The circle structure DNA sequences serve as new templates of BIP and B3 for binding and extension, which induces new displacement reactions and results in single-strand dumbbell structure DNA sequences. Then the single-strand dumbbell structure DNA sequences are extended using self-strand as the template to form double-strand stem-circle structure DNA sequences. The next pairing and extension reactions occur by introduction of FIP. Continuous cycling amplification and recycling amplification reactions are carried on when BIP and FIP bind to circle DNA sequences, producing many copies of target DNA sequences.

LAMP is able to produce 10^9 copies of the target nucleic acid sequences in less than 1 h, and it performs well in specificity due to little influence of the co-presence of non-target DNA. In virtue of a series of independent DNA sequences in the amplification reaction, the backgrounds that impede many amplification tests are reduced successfully. The main shortcoming of LAMP is the complicated primer design. To overcome this problem, web-based software was developed to design proper LAMP primers and loop primers [46].

LAMP is considered as an ideal candidate for POC-based detection of COVID-19 [6]. A myriad of researchers have dedicated to exploiting LAMP assay for rapid screening and spread control of this dreadful pandemic [66-70]. A series of improvements have been made and the applications have been expanded in a great scale, including detection of bacteria [71,72], virus [73], parasites [74] and genotyping [75]. Multiplex detections based on LAMP have also been developed [70, 76-80]. For example, Segawa et al. proposed and evaluated a molecular diagnosis system ‘Simprova’ based on LAMP for simultaneous determination of multiple pathogens [79,80]. Simprova was comprised of a master unit for control and a test unit for pretreatment. A multi-well testing chip containing 25 reaction wells enabled simultaneous dealing with multiple nucleic acids by LAMP within 30 min. This fully automated system showed great potential and power for streamlining the protocol of detection in POC test settings.

2.1.6. Helicase dependent amplification (HDA)
HDA is analogized by in vivo DNA replication and reported by Vincent et al. in 2004 [81]. As illustrated in Fig. 1E, HDA exploits Escherichia coli UvrD helicase to convert target DNA into single strand which then is stabilized by ssDNA binding proteins. By means of DNA polymerase, the ssDNA extension is initiated by the primer to form a new dsDNA which works as the template of the next cycle amplification reaction. The target DNA number gets expanded exponentially. An et al. cloned and purified thermostable UvrD helicase (Tt-UvrD) from Thermoaeroebacter tengcongensis to replace Escherichia coli UvrD in the HDA assay, which enabled amplification at a high temperature (60-65 °C) and thus resulted in a great improvement in sensitivity and specificity [82].

Ascribed to DNA helicase, HDA can complete the amplification and avoid heating operation for DNA melting, making the whole assay entirely keep at constant temperature, and thus is preferable for POC application. HDA has the capability for analyzing long DNA targets with kilobase regions, making it more competitive in clinical research. The efficiency, however, is limited when the samples are less than 100 copies. This issue can be minimized by optimized operations such as increasing enzyme concentration and enhancing helicase activity [83].

HDA is suitable for the detection of DNA and single nucleotide polymorphism (SNP). For example, Li et al. came up with an HDA-based assay for genotyping the genetic polymorphisms in the VKORC1 and CYP2C9 genes, which can help maintain the dose of anticoagulant warfarin and reduce the risk of drug adverse effects [84]. Meanwhile, some commercial molecular diagnosis devices using HDA were developed. BioHelix corporation has launched a HDA kit "BEST" for sensing a series of targets like Neisseria gonorrhoeae [85] and HIV-1.

2.1.7. Recombinase polymerase amplification (RPA)/recombinase aided amplification (RAA)
Referring to T4 phage DNA replication process, Piepenburg et al. developed RPA in 2006 [86]. In an RPA assay as shown in Fig. 1F, the recombinase is connected to the primer to form a recombinase-primer complex which can scan target dsDNA and anneal at cognate sites. Then the recombinase-primer initiates the extension reaction by DNA polymerase and strand displacement occurs. The ssDNAs binding proteins interact with the displaced single strand sequences for stabilization. The exponential expansion of target DNA is realized by continuously extension and displacement reactions. RAA is similar to RPA and the difference is the replacement of the recombinase derived from T4 bacteriophages with that derived from bacterial and fungal.

RPA/RAA can achieve millions of copies of target with a low limit of detection (LOD) down to a single target copy within 40 min, and RPA/ RAA exhibits excellent performance in sensitivity and specificity. However, the reaction conditions are always stringent and the analysis of crude samples may be challenging [44].

The ability of POC-based nucleic acid analysis using RPA/RAA has been demonstrated on microchip systems [87,88]. Real-time PRA is
utilized to study the genes of viruses and bacteria [89,90]. Crannell et al. set up an RPA-based assay to diagnose cryptosporidiosis, which was verified to be superior to PCR [91]. Ma and co-workers have used RPA/RAA to realize the detection of many infectious viruses including adenovirus, pertussis, respiratory virus and SARS-CoV-2 [92–95]. There are other researchers that have used RPA/RAA for rapid diagnosis of SARA-CoV-2 in the context of the new coronavirus pandemic [96–99].

2.2. Gene sequencing

Gene sequencing means detecting the whole base sequences of nucleic acids, which provides a direct and accurate approach to analyze genomic messages. Since the double helix structure of DNA was found in 1953 [100], many researchers have been dedicated to exploring the gene sequence. In 1977, Sanger et al. developed the first-generation sequencing technology which galvanized dideoxynucleoside triphosphate (ddNTP) to terminate the synthesis of DNA [101]. Later, genome sequencing for the first time in history was achieved in phage X174 [102]. The first-generation sequencing possesses exciting accuracy. However, it fails to meet the needs concerning quality and throughput. Next-generation sequencing (NGS) was a hot topic in the last few decades. The main NGS techniques include Solexa sequencing technique [103], high throughput pyrosequencing technique [104], and SOLID sequencing technique [105], which depend on the principles of sequencing by synthesis or sequencing by ligation. NGS techniques achieve high throughput sequencing and reduce cost compared with the first-generation sequencing, but there are unmet issues in read-length and raw accuracy. This being so, the third-generation sequencing (TGS) which features single molecular sequencing came out with the demand. The representative techniques are Single Molecule Real-Time (SMRT) sequencing and nanopore sequencing. SMRT sequencing can induce specific fluorescence in the base pairing stage by using distinct fluorescence to label 4 bases. Thus, we can recognize the base by the wavelength and peak of the fluorescence. SMRT achieves long read-length about 10^4–10^6 bp and high throughput, and it can achieve real-time data acquiring and direct RNA detection. Besides, the nanopore assay can be optically fulfilled by hybridizing the nucleic acids with fluorescent reporters as well (Fig. 2B) [110]. To sum up, TGS can greatly improve the read-length and sequencing speed although the test cost is still challenging, holding the potential of more effective and simpler molecular diagnosis.

Matthew et al. utilized the MinION (Oxford Nanopore Technologies) to sequence and assemble the reference genome of the human GM12878 Utah/Ceph cell line, which produces 91.2 Gb of sequence data and ~ 30× theoretical coverage [111]. Ultra-long reads (NS0 > 100 kb, read lengths up to 882 kb) were achieved. MinION was also used by Richard et al. for profiling the microbiota and antimicrobial-resistant pathogens of preterm infants [112]. They successfully identified Klebsiella pneumoniae and Enterobacter cloacae and their corresponding antimicrobial resistance gene profiles. The results were verified by whole-genome sequencing and antibiotic susceptibility testing, demonstrating MinION could offer a meaningful approach for genome analysis in clinical research. Gene sequencing can also work as a powerful auxiliary tool of other methods like NAAT. For instance, Wu et al. reported a two-stage SARS-CoV-2 testing strategy defined as INSIGHT, which combined NASBA with NGS to realize population-scale testing [48]. The highly multiplex sequencing step can help confirm the near-patient testing results and centralize data collection.

Although the test cost of gene sequencing continues to plummet, implementation as a convinced and widely-used POCT tool remains in its infancy due to the requirements of specialized skillful person and time-consuming process, and there is much room to further improve throughput and reduce the error rate. There is still a long way to strive, but luckily, we are cheered up and encouraged enough by the amazing and incredible speed and improvement that have been achieved.

2.3. CRISPR/Cas system

In 1987, Ishino et al. found that there was a strand of repeat gene sequences which had regular interspaces located on the upstream of the iap gene of Escherichia coli [113]. Then Jansen et al. demonstrated that this repeat gene sequence family existed in many domains of bacteria or Archaea and named this family as Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) [114]. CRISPR is a kind of immunization tool to resist the invasion of phages and viruses. It can capture the...
specific gene sequence of exosomes and restore between two repeat units. The gene sequence will be identified and cut by CRISPR-associated proteins (Cas) when the exosomes invade again [115]. This immune system of bacteria shed light on the hope of precise DNA cleavage. In 2012, Emmanuelle Charpentier and Jennifer Doudna found the family of endonucleases that cleaved dsDNA breaks under the guidance of dsRNA, and revealed the great potential of CRISPR/CAS system for gene editing under the guidance of RNA. This discovery then aroused overwhelming research focuses on CRISPR and help the two discoverers win the 2020 Nobel Prize in chemistry [116]. In 2013, Zhang et al. reported that CRISPR/cas9 could be used to edit the genome of mammalian, which was the first use of CRISPR for gene editing in human cells [117].

Cas9 predominates in the play of CRISPR for the ability of cleaving target dsDNA with the help of guide RNA. It has a wide implementation in gene regulation, gene imaging, and gene screening [118]. The combination with nucleic acid amplification can surmount the low sensitivity caused by few target DNA amounts. Pardee et al. reported a NASBA-based CRISPR/Cas9 system which termed "NASBACC" to discriminate lineages of Zika virus [119]. They designed a guide RNA with a specific protospacer-adjacent motif (PAM) which covered a unique gene site in American-lineage sequence. Thus, the target American virus sequences were cleaved and distinguished while other sequences kept integrity and were activated to generate detection signals. Nuclease-deactivated Cas9 (dCas9) is a kind of mutated Cas9 and retains the ability of binding to dsDNA, which makes it a desirable tool to detect target nucleic acid sequences. Qiu et al. combined CRISPR/dCas9 system with RCA to achieve microRNA detection [120]. Two dCas9 effectors were decorated by half of the split horseradish peroxidase (HRP) protein. When they bound to target DNA sequences, the split HRP reconstituted, generating a colorimetric signal for detection.

The collateral cleavage activity of Cas13a was found in 2016 [121], which broadened the horizon of CRISPR/Cas system application. In CRISPR/Cas12 or Cas13 system, both of Cas12 and Cas13 use guide RNA to find matching or complementary nucleic acid sequences. Cas12 recognizes and cuts DNA while Cas13 recognizes and cuts RNA. This process is called CIS cleavage. After finding the target, Cas12/Cas13 turns on the trans cleavage which occurs on different locations of the protein and involves cutting DNA or RNA that doesn’t bind to guide RNA. Signal expression produced by trans cleavage reaction will be achieved by adding florescence or colorimetric reporters. Based on the collateral cleavage activity of Cas12/13, dozens of outstanding detection systems have been developed by researchers around the global. One of the representatives is SHERLOCK (Specific High Sensitivity Enzymatic Reporter Unlocking) which developed by Zhang et al. [122] and has a wide application in disease detection such as COVID-19 [123]. The schematic of SHERLOCK system is illustrated in Fig. 3A. It utilizes the crRNA-programmed collateral-cleavage activity to detect RNA in vivo by triggering programmed cell death or in vitro by nonspecific degradation of labeled RNA. An ortholog of Cas13a from Leptotrichia wadei (LwCas13a) with excellent RNA-guided RNase activity is used. RPA is chosen to improve further sensitivity while it can couple with T7 transcription to allow subsequent sensing amplified nucleic acid by LwCas13a. SHERLOCK exhibits marvelous performance for nucleic acid detection with attomolar sensitivity and single-base mismatch specificity, which was demonstrated by identifying several virus and bacterial pathogens. Cas13b was utilized for proposing SHERLOCKv2 system later [124]. Several other improvements for SHERLOCK were also established by researchers [125–127]. Furthermore, Cheng et al. harnessed Cas12a and Cas12b to develop the CRISPR-based detection system, i.e., HOLMES (Fig. 3B) [128,129] and HOLMESv2 [130]. Similarly, DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) was developed by Chen et al. [Fig. 3C] [131]. These systems show great sensitivity in nucleic acid detection or virus genotyping.

CRISPR/Cas system has attracted rising interest and capital recently. There is a spectacular market with scale of billions of dollars and tremendous companies competing to participate in the feast of CRISPR/Cas development. However, some challenges must be overcome to achieve next innovation in clinical utility [132,133]. Firstly, CRISPR effector proteins guided by crRNA can recognize target sequences that are complementary to crRNA, but the available sequences for effector proteins are limited. The choice of sensing region and effector proteins is crucial for CRISPR/Cas detection. Secondly, few detection systems based on CRISPR/Cas can realize quantitative detection. Some quantitative systems rely on sophisticated methods like real-time PCR, which

![Fig. 3. Schemes of CRISPR platforms. (A) SHERLOCK. Reprinted with permission from Ref. [122]. Copyright 2017 American Association for the Advancement of Science. (B) HOLMES. Reprinted from Ref. [129], Copyright 2018 Shi-Yuan Li et al. (C) DETECTR. Reprinted with permission from Ref. [131]. Copyright 2018 American Association for the Advancement of Science.](image-url)
involves bulky facilities and time-consuming processes. Moreover, CRISPR/Cas systems are always combined with isothermal amplification techniques to realize POC implementation. However, the mismatch of the best temperature between amplification and CRISPR/Cas recognition is problematic. Separating the two parts in whole system may be a feasible approach to solve this issue. Denaturation of the proteins is a main limitation for the implementation of CRISPR/Cas system. This issue is emphasized when the biosensing device needs to be transmitted to remote districts with poor conservation conditions. Finally, the cost of the involved effector proteins is expensive. How to avoid the use of the unaffordable material or achieve comparable performance with minor material is of great matter. By the way, there is a consensus that nature will never cease to inspire human with its biological toolbox. We hold the mission to unveil the biological essential for developing more valuable and powerful tools like CRISPR/Cas system.

2.4. Other techniques

In addition to the aforementioned techniques, there are many alternatives that hold the potential of POC-based molecular diagnosis applications. The exponential amplification reaction (EXPAR) and signal-mediated amplification of RNA technology (SMART), which fall under the umbrella of isothermal NAAT, can offer the possibility for PCT-based molecular diagnosis and have been used for different desires [134,135]. Besides, Yin et al. utilized a cascade enzymatic polymerization reaction to detect and quantify microRNA by employing DNA polymerase with the isothermal strand-displacement property and restriction endonuclease with nicking activity [136]. With the elimination of thermal cycling requirements and gel analysis, this method can be applied for low-cost, portable PCT testing. Connolly et al. came up with a protocol to rapidly detect DNA with two functional molecular switches [137]. One switch was activated by the target DNA and the DNA polymerase, and the primer was used to lock the molecular beacon. Then the other switch was initiated to control the amplification reaction and signal transduction. Both switch reaction was kept at 40 °C, making this protocol suitable for rapid and simple implementation. Gao et al. developed a G-quadruplex DNAzyme-based chemiluminescence biosensing platform which combined Exonuclease III-assisted signal amplification and carbon nanotubes to reduce the background for effective DNA detection [138,139]. Li et al. developed a primase-based Whole Genome Amplification (pWGA) which had a simple isothermal reaction step and ultrafast speed for quantification of target DNA and held the potential of POC application [140].

3. Construction of biosensing systems for POC molecular diagnosis

For the purpose of POC application, the biosensing system for molecular diagnosis should always satisfy with the following indicators: 1) portable equipment; 2) minimal sample preparation; 3) minimal biosafety requirements; 4) no requirement for a temperature-controlled environment; 5) rapid response from sample collection to readout. All these indicators stimulate lots of thoughts in the construction of biosensing systems for molecular diagnosis, which are embodied in both the whole framework and the single component.

3.1. Diagnostic platform of biosensing

With the development of micro electro mechanical systems (MEMS) and flexible electronic technology, the biosensing for molecular diagnosis prefer to be devised as miniaturized and highly integrated platforms for meeting POC application. These platforms usually include microfluidics, lateral flow assay (LFA), and capillary platform.

3.1.1. Microfluidics

Microfluidics, i.e. lab-on-a-chip, is a kind of technology which manipulates and processes fluids in the dimension of tens of micrometers to achieve chemical or biological needs [141]. Microfluidic platforms generally consist of kinds of elements such as micro to submillimeter fluid channels, reaction or detection chambers, filters, and sensor units, which are always fabricated based on micro-machining technology such as soft lithography on silicon, metal, polymer and glass substrates, to name a few. Notably, as shown in Fig. 4A, thousands of chambers and other elements can be integrated into one microfluidic platform to facilitate the capability of high-throughput screening and powerful analysis [142]. A series of functions, including sample and reagent introduction, purification, separation, fluid movement and reaction, are centralized on a miniaturized chip.

Through integrating multiple sensing and processing units into one micro-scale device, microfluidic platforms can complete POC molecular diagnosis with a number of advantages including portable volume, minimal quantity of samples and reagents, high sensitivity and resolution, low cost, short time consumption, small footprint, parallel processing capability, and disposability. For example, Burns et al. integrated microfabricated fluid channels, heaters, temperature sensors, and fluorescence detectors into a portable device [143]. All the components were microfabricated on a single glass and silicon substrate by photolithographic techniques. This portable device could achieve nanoliter DNA sample analysis at low unit cost. Microfluidic platforms can also facilitate multiplex detection. For instance, Fang et al. reported an eight-channel microfluidic system for quantitative detection of pathogens using LAMP technology [144]. This system had a minor sample volume requirement and could fulfill diagnosis in a parallel and high-throughput manner. Benefiting from the aforementioned advantages, microfluidic platforms have been applied for molecular diagnosis in many scenarios such as virus detection and bacteria differentiation [145–148]. For instance, Luo et al. developed a LAMP-based microfluidic chip for real-time detection of three acute upper respiratory tract infections related bacteria: Mycobacterium tuberculosis (MTB), Haemophilus influenza (HIN), and Klebsiella pneumonia (KPN) [148]. This microfluidic chip was cost/time effective and easy-to-operate. This microfluidic chip could complete the whole analysis within 45 min with the LOD (S/N = 3) of 28, 17, and 16 copies µL−1 for MTB, HIN, and KPN, respectively.

3.1.2. Lateral flow assay

LFA is a kind of detection platform based on the movement of liquid samples through a strip-attached molecule which can specifically recognize and interact with the analyte to provide a visible signal [149]. Generally, the LFA platform as presented in Fig. 4B is in a dipstick or cassette format and involves sample pad, conjugate pad, test pad and absorbent pad [150]. The sample pad receives sample and acts as a filter to aid flow. Some sample pads have the capability of adjusting sample properties like pH or viscosity. The specific molecules on the conjugated pad recognize and bind to the targets in the sample. The test pad usually contains a test line and a control line, on which capture reagents are immobilized. The liquid sample continues to flow along the pad by capillary action and is intercepted by the test line and the control line. The binding reaction in the test line conveys the message of positive results and the visual control line ensures a correct running process. The labels of the molecules are chosen depending on complementarity with the test components, and provide color expression for readout by naked eyes or a lateral flow reader. The final absorbent pad helps flow the samples and collects any excess liquid.

LFA tests can be configured to work with a range of liquid samples either whole or prepared, and commonly completed within 5–10 min from sample introduction. It is of great advantage including high specificity, low cost, rapid response and minimal expertise requirement, facilitating extensive usage in POC-based molecular diagnosis assays. In comparing with microfluidics platform, LFA tests can offer direct data report which can be captured by naked eyes. For instance, Lie et al. developed an LFA-based device that utilized strand-displacement...
polymerase reaction and gold nanoparticles to detect nucleic acids [151]. The colorful bands in the sensing zone demonstrated the existence of target nucleic acids. The optical density of the colorful bands could be utilized for quantitative analysis. This device provided a simple, low-cost, and sensitive approach to detect nucleic acids with an LOD of 0.01 fm. One of the advantages of LFA tests is disposable characteristic [152,153]. For example, Tang et al. developed an integrated paper-based device which combined HAD and LFA for nucleic acid analysis [152]. This fully disposable device utilized a paper strip for naked-eye detection and could achieve the detection of *Salmonella typhimurium* with a low LOD of $10^2$ CFU ml$^{-1}$ in wastewater and egg, and $10^3$ CFU ml$^{-1}$ in milk and juice, which is available and cost-effective for POCT implementation even in resource-limited settings. LFA has wide applications for the detection of pathogens like bacteria or viruses [54,154]. For instance, Mukama et al. reported an LFA-based biosensor for the detection of HPV using LAMP and CRISPR/Cas12a technology [154]. The *Lachnospiraceae bacterium* Cas12a reaction was conducted to trans-cleave an ssDNA reporter upon target DNA recognition. In case target DNA existed and got amplified by LAMP, the trans-cleaved ssDNA reporter couldn’t bind to the specific molecule in the test line of LFA biosensors, thus inducing the absence of color in the test line. This LFA-based biosensor provided a new protocol for pathogen detection and the feasibility was demonstrated by determining and distinguishing HPV16 and HPV18 from pathogens of other types.

### 3.1.3. Capillary platform

Technically, the capillary-based assay falls within the ambit of microfluidics but can serve as a special and promising way for bioanalysis. Capillary platforms work based on the capillary action which circumvents the use of external pumping equipment. The liquid flow in the capillary overcomes the effect of gravity and viscosity owing to the surface extension and wetting property of the capillary [155]. Porous materials such as paper and nitrocellulose, which are inherently hydrophilic or wettable after surface treat, are always utilized to form flow paths. Some capillary valves or flow resistance are possibly needed for ensuring appropriate control of flow and reaction.

Capillary platforms can be applied to the satisfaction of POCT applications by providing with multiplex capability, small time and money cost, simple operation, and portable deployment. They can easily be stacked or connected in tandem for devices with separate functions for filtration, reagent addition, and multiple tests in distinct areas [156]. For instance, a capillary system combined with LAMP was also devised by Xu et al. as illustrated in Fig. 4C [157]. The analysis steps, including sample purification, enrichment, amplification, and data report, are separated by a series of plugs. The entire DNA analysis process can be completed by manually controlling magnetic beads to migrate through the capillary. Plugs targeting different genomic DNA were added in a single container and separated by mineral oil to enable simultaneously detect two types of sexual transmitted diseases, which exhibits

![Fig. 4.](image-url)
comparable performance with real-time PCR. Capillary platforms have been widely used for POC detection of various pathogens including virus [158] and bacteria [159]. For instance, Liu et al. developed a fully integrated capillary platform for parallel detection of *Mycobacterium tuberculosis* with small cost of fabrication and analysis based on LAMP and droplet technology [159]. The liquid samples and reagents in the capillary formed droplets which served as the processing place of DNA extraction, nucleic acid amplification, and fluorescence detection. This platform enabled high-throughput detection of tuberculosis with small footprint size, reduced reaction volume and low fabrication cost. It can complete the entire detection process automatically within 50 min and achieved a limit of 10 bacteria with a sensitivity of 96.8% and specificity of 100% in clinical samples.

3.2. Readout form

There is generally an indispensable need of a readout procedure to report the detection result following the recognition or amplification process. An outstanding and simple readout system is significant for the simplification and generalization of POC biosensing systems for molecular diagnosis. The mainstream readout ways include fluorescence, colorimetric assay, and electrical signal.

### 3.2.1. Fluorescence

In a fluorescence-based detection assay, fluorescent dyes are always utilized to label the report probe or mix with the sample reagent. The fluorescent signal can be amplified or activated by the specific reaction for result readout with a fluorometer or even naked eyes. The intensity of fluorescence can be utilized for quantification of nucleic acids.

Fluorescence readout can offer a simple and easy-to-build way for data reports, which has been extensively used in many POC platforms like microfluidics. For example, Dou et al. developed a fluorescence-based microfluidic platform to detect *Neisseria meningitidis* which was the main bacteria accounting for meningitis [160]. In the LAMP zone of the integrated PDMS/paper hybrid chip, calcein was used as the fluorescent indicator, whose fluorescent signal can be quenched by combination with manganese ions before amplification. The pyrophosphate ions generated in the LAMP reaction process as the by-product would deprive of the manganese and thus result in fluorescence emission as illustrated in Fig. 5A. The free calceins could combine with magnesium ions to strengthen the fluorescent signal [161]. This one-step protocol can avoid the implementation of expensive specialized equipment for adapting to POCT applications. Likewise, the strategy, which has the aid of specific reactions activated by target nucleic acid to output fluorescence signals, is also utilized in devices serving for the current pandemic. Woo et al. developed a highly sensitive and portable system for the detection of SARS-CoV-2 based fluorescence reports [162].

![Fig. 5.](image-url)
Specifically, a promoter probe and a report probe which contained a template sequence for a dye-binding RNA aptamer would hybridize with the target DNA. The two probes were ligated by SplintR ligase when the target DNA was present and further amplified with a set of PCR primers. The full-length ligated probe was used as the template for the transcription of RNA aptamer by T7 RNA polymerase. Then the fluorescent dye bound to the RNA aptamer and then the planar structure of the fluorescent dye is stabilized, which could output the fluorescence signal. The transcription products containing the target RNA sequence could act as the splint for unligated probes, forming an amplification cycle to dramatically improve sensitivity. The system could complete the entire detection assay within 30–50 min and demonstrate its performance in SARS-CoV-2 samples with a sensitivity of 95% and a specificity of 100%.

3.2.2. Colorimetric assay

Colorimetric assay has extensive applications in POC biosensing for molecular diagnosis. Chromogenic materials are usually added into samples, and the generated color change can be captured either with the naked eyes or an image capture machine like an optical camera. We can also utilize the gray value or absorbance of the color expression to realize quantification detection.

The colorimetric assay provides a simple and intuitionistic way for the detection of various pathogens, which commonly avoids the use of additional result report equipment. Trinh et al. reported a colorimetric system based on silver nitrate and combined with some NAAT methods like PCR and LAMP to detect multiplex food-borne pathogens [163, 164]. As shown in Fig. 5B, the silver ions bound to the base of target nucleic acids like pyrimidine and purine to form a complex, which resulted in a color change under the UV light while the solution remained colorless in the absence of target nucleic acid. Compared with the single colorimetric assay, dual complementary colorimetric assay can provide cross-validation to enhance sensitivity and minimize the influence of environment factors. For instance, Zhou et al. used CRISPR/Cas12 and LAMP for nucleic acid detection and developed an orthogonal complementary colorimetric approach for readout [165]. On the one side, the ssDNA could crosslink two DNA functionalized gold nanoparticles which converted the red color into blue. On the other side, ssDNA could inhibit bare gold nanoparticles from salt-induced aggregation to maintain the solution color. Consequently, the LAMP amplicon presented a blue-to-red transition along with an opposite red-to-blue transition, whereas a blank of off-target amplicon maintained original color. Colorimetric assay is always harnessed in the LFA platform that depends on the color exhibition in the test line to report the result as mentioned above [14,151,154]. Many nucleic acid sensing devices have been fabricated based on combination of colorimetric assay with NAAT techniques like NASBA [119], HDA [166], and RCA [167].

3.2.3. Electrical signal

The specific nucleic acid detection reactions are often utilized to form a closed circuit or change electrical parameters like resistance or capacitance, which renders readout in the form of electrical signal feasible. Actually, the fluorescence and color signal can also be converted into electrical signals by adding a medium like a photovoltaic converter. Electrical signal readout allows real-time detection and long period monitoring, which is normally unable using fluorescence or colorimetric assay.

POC devices based on Electrical signal readout can be easily built, which benefits from the mature and cheerful MEMS technology. And there is always a great time stability and no need for large energy consumption. A silicon-based integrated transducer (TriSilix) was devised for pathogen detection [168]. In detail, a large number of amplicons were produced using RPA or PCR when the target nucleic acid was present. Then methylene blue would interact with the G-C base pairs and terminate the participation in the electron-transfer reactions with the working electrode, which resulted in a decreased electroanalytical signal generated by the redox-active reporter. This TriSilix chip is portable and easy to fabricated, and what’s more, holds the capability of running up to 35 tests with a battery with a 4000 mAh capacity (a typical battery capacity of a smartphone). Russell et al. developed an electrical sensor which combined RCA and electrical readout to detect DNA [Fig. 5C] [169]. A long ssDNA product was generated by RCA and hybridized with oligonucleotide-functionalized gold nanoparticles. A silver or gold salt solution was utilized to metализe the ssDNA threads for forming metal wires. The electrical signal was detected based on the change in resistance upon metallization. This sensor showed a high signal-to-noise ratio in the detection of Escherichia coli genomic DNA.

3.3. Improvement of POCt molecular diagnosis biosensing

In order to build a biosensing device applicable to POCt setting for molecular diagnosis, it always needs to pay attention to many aspects such as detection speed, economy cost, sensitivity, specificity, stability, complexity, and even biosafety. A series of sub-procedures including sample preparation, amplification, hybridization and readout can be optimized by utilization of functional materials, special designs, artificial intelligence and so on.

3.3.1. Functional materials

Material is critical for the construction of POC biosensing devices for molecular diagnosis as it can dramatically influence the cost, stability, sensitivity, and deployable ability of biosensing devices. Some rigid materials such as silicon, glass and quartz are widely used to establish the detection platform such as microfluidics or LFA. The rising development of flexible materials has opened a new horizon for miniaturization and integration of biosensing devices. For example, poly (dimethylsiloxane) (PDMS) possesses prevalent utilization for excellent flexibility, biocompatibility, moderate cost, minimal toxicity, and ease of microfabrication [160,170]. Other flexible materials like polyvinyl alcohol (PVA), polyethylene naphthalate (PEN), and paper are also employed as a substrate. Especially given the low cost, portability, and biodegradable property, paper material is preferred in many platforms such as LFA [16,152,160], Dou et al. developed a PDMS/paper hybrid microfluidics for bacterial pathogen detection. The results showed a much better stability compared with those of a paper-free system [160]. Similar to PVA and PEN, some other polymers are also introduced to improve the performance of biosensing. For instance, Annabestani et al. chose ethylene vinyl acetate (EVA) as an alternative of PDMS to build a microfluidic device [171]. This device exhibited great potential for POCt application due to its biocompatibility, flexibility, hydrophobicity, terahertz transparency [49,172], and absorption of EVA. Poly (methyl methacrylate) (PMMA) was applied as a promising substrate to fabricate biosensing devices for clinical analysis, taking advantage of machinability, low cost, and optical transparency. A number of carbon-based materials such as carbon nanotubes [173] and graphene oxides [174] may also play a magical role in biosensing for signal transmission and reaction facilitation.

3.3.2. Specific structure

In addition to functional materials, some researchers pursue special structure design to achieve improved biosensing performance, which embodies in the entire process or sub-procedures. Sandwich structure is an effective design to simplify the workflow and minimize the space of biosensing devices, and has been introduced in many scenarios [15,153,168,175–177]. Some attempts have been made to modify the conventional molecular diagnosis platform like LFA or microfluidics by introducing additional components [178–180]. For instance, Tang et al. added a sponge shunt between the nitrocellulose membrane and the conjugation pad of the LFA platform, which could prolong the reaction time by delaying the fluid flow rate, resulting in an enhanced sensitivity by virtue of the inverse correlation between analytical sensitivity and the reaction time [180]. It is a common way of microfluidics platforms to fabricate more microchannels in a single substrate for high-throughput
3.3. Smartphone

Benefiting from the thriving computer intelligence, biosensing systems are functionalized for the user-friendly operation and further data processing by combining with a smartphone. The smartphone can realize wireless connectivity with the biosensing system through blue tooth or a WIFI interface for device control, data collection, display, and analysis [182]. It can also serve to capture the image of the reaction products and communicate with cloud-connected computing systems for further image processing by professional software or algorithms [183-185]. In particular, the colorimetric assay-based biosensing systems are often combined with a smartphone to capture the result image and analyze the hue value of RGB channels, which can provide a more promising quantitative conclusion compared with naked eyes. For instance, Nguyen et al. integrated a smartphone into a pathogen detection system and designed a mobile app for real-time recording and processing using the hue value as the indicator of color transition [184]. Furthermore, as machine learning and deep learning technologies flourish, it’s a convincing and reliable way to combine biosensing systems with artificial intelligence by a smartphone to improve sensitivity and specificity of molecular diagnosis testing [186].

4. Application scenarios of POCT molecular diagnosis biosensing

With the vigorous development of various methodological approaches, molecular diagnosis has played a more important role in our society. More interests and passion of researchers are devoted and the industry market has been expanded in an overwhelming trend. The application of POC biosensing for molecular diagnosis has been achieved in a series of scenarios.

4.1. Virus diagnosis

The spread of viruses often causes unimaginable and uncontrollable disasters, as a consequence of an increase in population and integration of the global economy and politics. Screening capacity in large scale becomes critical to inhibit outbreaks and expansion, which is authenticated and highlighted in the current COVID-19 pandemic. Numerous researchers have struggled for this and gained some remarkable achievements [4,29,48,66-70,95-99,123]. For instance, Lu et al. used an LAMP assay to detect SARS-CoV-2 based on its N gene and obtained results by observing a colorimetric change from red to yellow [68]. The whole assay can be completed within 30 min with a low LOD of 118.6 copies per 25 µL, and its results agreed well with those from RT-qPCR assay. Similarly, Thai et al. developed a rapid single-tube accelerated RT-LAMP assay to detect SARS-CoV, a virus homologous with SARS-CoV-2, in less than 1 h [187]. Multiplex detection is a meaningful strategy for practical application. Fang et al. reported an octopus-like multiplex microfluidic platform for predicting viruses based on LAMP technology [188]. This platform was able to identify eight swine viruses as well as differentiating three human influenza A subtypes. In addition, there are many other viruses which have not escaped the interest of POC biosensing for molecular diagnosis, such as Influenza A H1N1 virus [189], Rift Valley fever virus [190], and Dengue virus [191].

4.2. Bacteria/fungus diagnosis

Bacteria or fungus diagnosis is also a hot spot of application for POC biosensing for molecular diagnosis. Mycobacterium tuberculosis is the causative pathogen of tuberculosis which is the leading cause of death from bacterial infectious diseases. Cepheid Inc. developed a portable and accurate assay “MTB/RIF” based on their world leading molecular diagnosis platform “GeneXpert” for rapid diagnosis of Mycobacterium tuberculosis and assessment of rifampin resistance. This fully automated assay utilizes heminested real-time PCR and can complete the whole diagnosis process within 2 h with minimal technical expertise [38]. Other protocols for Mycobacterium tuberculosis diagnosis have also been reported [185,192]. Escherichia coli can exist as beneficial probiotics in the symbiosis of the digestive tract, as well as toxic pathogens in food and environment, which makes it a major cause of many diseases, such as food poisoning, meningitis and urinary tract infections [193]. Safavieh et al. devised a microfluidic electrochemical LAMP-based platform to detect and quantify Escherichia coli [194]. This platform could avoid the requirements of probe immobilization, DNA extraction, and sample purification step, holding the potential for POCT application. Likewise, Chen et al. combined LAMP and lateral flow assay to develop a POCT-based biosensor for P. aeruginosa, which is a major pathogen responsible for some infections such as septicemia, pneumonia and keratitis [195]. Some fungi exist in soil or organisms of animals and plants, which can damage the grain harvest and vegetation growth. Rapid and effective detection of these fungi is meaningful for agriculture development and environment protection. Davari et al. utilized RCA and well-administered databases to successfully detect toxigenic Fusarium species in cereals, and the detection system showed no cross-reactivity with uncontaminated wheat or related species [61].

4.3. Cancer examination

Cancer is one of the biggest threats to human health and accounts for millions of deaths every year. Molecular diagnosis provides a protocol for early screening, prediction, and monitoring of tumors over time through directly detecting pertinent pathogen genes or variation events. MicroRNA is regarded as an ideal biomarker for cancer examination. Some POC systems have been reported based on microRNA diagnosis for cancer detection including prostate cancer, gastric cancer and cervical cancer [136,196,197]. For instance, Muto et al. devised a LAMP-based assay to detect cytokeratin 19 microRNA for rapid diagnosis of micro-metastasis of gastric cancer [196]. This assay could output the result within 75 min and showed the potential as an alternative to the conventional diagnosis method. Some cancers are closely related to virus infection, and thus detection of these viruses is beneficial to cancer early screening. For example, human papillomavirus (HPV) genome is always associated with cervical carcinomas. Wang et al. proposed a portable platform for the detection of three genotypes of HPV DNA based on a microfluidic chip and a personal glucose meter [175]. This platform is easy to fabricate and operate without the requirement of specialized skillful personnel, which makes it suitable for the development of POCT-based applications.

4.4. Other application scenarios of POCT molecular diagnosis biosensing

There are many other application scenarios where POC biosensing for molecular diagnosis plays a significant role. One of the representative applications is prenatal testing. It is necessary and important to detect the development status of the embryo or fetus like chromosome number abnormality, which will help choose the appropriate time for intrauterine treatment if possible. Besides, POC biosensing for molecular diagnosis can be used in agriculture and food safety requirements, for instance, the detection of genetically modified organisms. Huang et al. came up with a simple and effective LAMP-based assay for the detection of phytase genes in genetically modified maize [198]. This assay...
presented a high sensitivity which is 33.3 times higher than the traditional PCR assay. A portable and integrated paper-based microdevice was developed by combining a LAMP assay and an FTA card to detect multiplex foodborne pathogens including *Salmonella* spp., *Staphylococcus aureus*, and *Escherichia coli* O157:H7 with a fast response [199].

5. Challenges and perspectives

In recent years, the sublimation of healthcare consciousness and the urgent need in various scenes have given rise to an immense concern of POC biosensing for molecular diagnosis both in academia and industry. The enthusiasm poured into this field has been shedding light on the essence of gene expression and spurring a flood of techniques. NAAT is gradually moving on to maturity. Especially, isothermal NAAT techniques such as NASBA and LAMP can provide with a simply and rapid means by obviating cumbersome thermal cycle procedures. The characteristics of isothermal NAAT techniques are presented in Table 1. Gene sequencing is still in its infancy although obvious progresses have been achieved by tremendous endeavors of researchers. The application of gene sequencing in actual emergencies has been sporadic owing to the requirement of skilled operators and high costs. Nevertheless, after many false dawns for POC implementation, TGS techniques have blossomed with the sharp decline of the sequencing cost, and it holds a great potential for gene sequencing techniques to be applied into more real-world settings. CRISPR is an innovative and convinced tool in our arsenal to meet the challenges of healthcare and has garnered much attention. Although it hasn’t experienced a long-time precipitation, CRISPR has shown a compelling ability to meet the need of developing POC biosensing for molecular diagnosis. The molecular diagnosis platform has been moving in the direction to personal or micromachine and evolved into advanced types. Microfluidics for genomic analysis, as a miniaturized and highly integrated platform, has reached a mature stage for commercialization. Capillary platform provides a new way for multiplex screening and portable deployment and holds a great potential in clinical applications. LFA is widely used for immunosassays as well as molecular diagnosis testing. Paper-based platform is highly praised for low cost, portability and biodegradable property, arousing an intensive interest in academia and industry [200,201]. The detection result of biosensing can be reported in various forms like turbidity [202], fluorescent, colorimetric, and electrical signals. The performance can be improved by the utilization of functional materials, specific structure design, and the introduction of the smartphone. Taking advantage of well-performed nucleic acid detection strategies and miniaturization techniques, biosensing for molecular diagnosis have been applied in many scenarios in a field-used or bedside manner. Developing the POC biosensing devices for molecular diagnosis always plays a crucial role in disease screening and control, which has been highlighted by some catastrophic pandemics such as H1N1 and COVID-19. Detection of bacterial and fungi is a popular field for the application of biosensing for molecular diagnosis, which is of great value to agriculture, environment, food safety, etc. Furthermore, it can serve for cancer diagnosis/treatment through early screening, therapy prediction, and tumor burden monitoring over time, which owns a broad market and has gained many successful achievements such as the diagnosis of prostate cancer. Some other scenarios have also witnessed the value of biosensing for molecular diagnosis like prenatal diagnosis and drug resistance tests.

Although the remarkable progress in POC-based molecular diagnosis field has been made through nucleic acid diagnosis techniques and device manufacturing engineering, the current testing capacity and availability of biosensing devices cannot completely meet the unprecedented global demands for effective, reliable and widely accessible molecular diagnosis [203]. Before biosensing for molecular diagnosis can serve various scenarios even in resource-poor areas in various scenarios, there are still a number of challenges hindering the way forward.

There is always a trade-off between the cost and accuracy of biosensing devices. For the best performance, developing a molecular diagnosis protocol that can reduce the cost of time and money yet remain a dependable accuracy has become imperative. The introduction of a number of ubiquitous functional materials like paper and plastic [204] can effectively decrease the whole construction cost of biosensing systems. Similar to the strategy of isothermal NAAT, optimizing the diagnostic protocol to circumvent bulky instruments and time-consuming steps is a promising road to decrease the cost of money and time. To improve sensitivity and specificity of biosensing, it commonly works well to cascade two or more techniques, which is likely to enhance signal outputs or minimize interferences [43,205].

Some biosensing systems for molecular diagnosis utilize advanced techniques to achieve ultrafast speed, excellent accuracy, and reliable stability. However, the necessity of professional training overshadows the advantages of detection performance. The training procedure means extra time and labor cost which may be out of reach in some resource-poor settings. Fabrication of biosensing systems with simple workflow and user-friendly operation is one of the major goals for developers.

There are usually tedious sample preparation or data processing steps in a diagnostic system, which vastly increase the complexity and may introduce manual errors in the sub-procedures. A "sample-in, answer-out" device has been designed to overcome such challenge, which is very suitable for POCT applications [206]. The integrated construct and systematic workflow are nuclear to realize this concept.

The detection capacity is extremely crucial to ensure screening at a large scale for curbing the spread of infections. This being so, multiplex detection has garnered more attention in practice [207]. It is noteworthy that the cross-contamination is a crucial challenge during the multiplex detection assay and multiplex assays usually have a stringent demand of the dose of sample regent. Moreover, we should pay attention to guard

Table 1 Characteristics of isothermal NAAT techniques.

| Technique | Major reagents | Temperature (°C) | Reaction time (h) | Efficiency | Advantages | Disadvantages |
|-----------|----------------|------------------|-------------------|------------|------------|---------------|
| NASBA     | 2 primers, reverse transcriptase, RNase H, RNA polymerase | 41 | 1.5–2 | $10^6$–$10^9$ | Great specificity; No denaturation steps | No-suitability for DNA analysis; Limited length of target sequence; Enzymes are not thermostable; Precise temperature control |
| SDA       | 4 primers, DNA polymerase, RNase HincI | 37 | 2 | $10^7$ | Simple and easy-to-control workflow; Great sensitivity | Additional thermal denaturation for analyzing DNA; Not suitable for synthesizing long nucleic acid sequences; Possible contamination from enzymes |
| RCA       | 1 probe, ligase, polymerase | 60 | 1–3 | $10^9$ | Direct usage for gene sequencing; Great specificity | High cost; Procedural complexity |
| LAMP      | 4 primers, DNA polymerase | 60–65 | < 1 | $10^3$ | Great specificity | Complicated primer design |
| HDA       | 2 primers, helicase, DNA polymerase | 37–65 | 0.5–2 | $10^7$ | Ability for analyzing long DNA targets | Non-suitability for analyzing samples with less than 100 copies |
| RPA/RAA   | 2 primers, recombinase, DNA polymerase | 37–42 | 0.5–1.5 | $10^4$ | Great sensitivity and specificity | Stringent reaction conditions |
against sensitivity attenuation which is often encountered in multiplex detection assays.

It is of great importance to ensure the biosafety of a diagnostic platform. Although the goal of POC biosensing for molecular diagnosis is to get rid of the dependence of the biological laboratory, it doesn’t mean the ignore of testing biosafety. There is a necessity to effectively dispose excess reaction samples and regents. The entire process including sample preparation, specific nucleic acid reaction, and result report should allow undergoing without abnormal cross-reaction, variation, etc., and present strong stability to temperature and pressure for eradicating biological hazards.

Data security should also be taken into consideration in all the biosensing steps, including data collection, transmission and conservation. Molecular diagnosis testing often involves the most private messages of the human body in essence. Introduction of a smartphone allows the whole data processing mechanism mechanized and automatic, while cloud communication system is frequently utilized and large-scale data might participate in virtue of artificial intelligence techniques. There is a momentous need to provide with a reliable proof for the data security of biosensing for molecular diagnosis. Whether the result is visible or sharable should be concerned when proposing a scheme. Besides, building strict, standardized and authoritative regulations is pivotal to guarantee data security of biosensing and address privacy issues.

The utilization of biosensing for molecular diagnosis is mainly focused on some scenarios like virus diagnosis as mentioned above; however, this technique has been playing a more and more important role in many other applications. For example, Lu et al. found that there is fetal free DNA in maternal blood and thus developed the Non-invasive Prenatal Testing (NIPT) [208]. NIPT can take the venous blood of the pregnant woman and then sequence the free DNA fragments in maternal peripheral blood to get the genetic information of the fetus, which can effectively detect chromosomal diseases like Down’s syndrome. However, it was limited to be available in a few senior medical institutions and can seldom be applied for the POCT application. Moreover, although some studies have taken advantage of detecting tumor biomarkers like microRNA to realize cancer examination, very little oncology testing can jump out of the lab to field implementation due to social, economic, and technical barriers [209]. To broaden the boundary of POC biosensing for molecular diagnosis is one of tasks on the road ahead.

As the times progress and society advances, there are unprecedented demands for cost-effective and well-behaved POC biosensing systems for molecular diagnosis and we are witnessing incredible developments in many aspects. However, many challenges go along for improvement and innovation and tremendous efforts are on the way. The answer to all the aforementioned questions is imagination, ingenuity, and practice. We anticipated that the full potential and wide utilization will be realized through the great collaboration among engineers, biologists, and clinicians around the globe.

CRedit authorship contribution statement

Hongwei Chu: Conceptualization, Investigation, Writing – original draft, Writing – review & editing, Visualization. Conghui Liu: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. Jinsen Liu: Investigation, Writing – review & editing. Jiao Yang: Investigation, Writing – review & editing. Yichun Li: Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration. Xueji Zhang: Conceptualization, Investigation, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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