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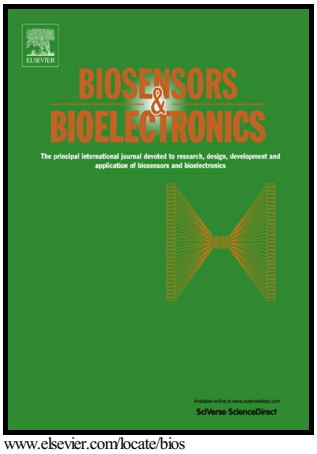

www.elsevier.com/locate/bios

PII: S0956-5663(18)30247-1
DOI: https://doi.org/10.1016/j.bios.2018.03.065
Reference: BIOS10391

To appear in: *Biosensors and Bioelectronic*

Received date: 26 January 2018
Revised date: 16 March 2018
Accepted date: 28 March 2018

Cite this article as: Hongjie Qi, Shuzhen Yue, Sai Bi, Caifeng Ding and Weiling Song, Isothermal exponential amplification techniques: from basic principles to applications in electrochemical biosensors, *Biosensors and Bioelectronic,* https://doi.org/10.1016/j.bios.2018.03.065

Isothermal exponential amplification techniques: from basic principles to applications in electrochemical biosensors

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ABSTRACT

As a conventional amplification technique, polymerase chain reaction (PCR) has been widely applied to detect a variety of analytes with exponential amplification efficiency. However, the requirement of thermocycling procedures largely limits the application of PCR-based methods. Alternatively, several isothermal amplification techniques have been developed since the early 1990s. In particular, according to the reaction kinetics, isothermal exponential amplification techniques possess higher amplification efficiency and detection sensitivity. The isothermal exponential amplification techniques can be mainly divided into two categories: enzyme-based isothermal exponential amplification and enzyme-free isothermal exponential amplification. Considering the advantages of high sensitivity and selectivity, high signal-to-noise ratio, low cost

and rapid response time, exponential amplification electrochemical biosensors have attracted considerable attention. In this review, we introduce the basic principles of isothermal exponential amplification techniques and summarize their applications in electrochemical biosensors during the past five years. We also highlighted the present challenges and further perspectives of isothermal exponential amplification-based electrochemical biosensors.

Keywords: Isothermal exponential amplification; Electrochemical biosensor; Signal amplification; Biochemical analysis; Biomolecules

1. Introduction

Based on whether the temperature changes during reactions, the signal amplification techniques can be divided into two categories. One kind is thermocycling amplification techniques, such as polymerase chain reaction (PCR), which has been considered as a conventional method for amplification detection of nucleic acids at extremely low concentrations (Dettloff et al., 2008; Fang et al., 2009; Mahjoob et al., 2008; Pješčić et al., 2010). The other is isothermal amplification techniques, which has been developed since the early 1990s (Zhao et al., 2015). Although PCR has been widely applied in various fields, it still has many disadvantages, such as easy contamination, high cost, and time consuming (Dahl et al., 2004; Niu et al., 2010; Wang et al., 2013). What's more, the requirement of a thermal cycler largely limits the applications of PCR in the resource-limited settings and point-of-care measurements (Deng and Gao, 2015). Alternatively, isothermal amplification can effectively avoid the shortcomings of PCR and amplify the targets in a rapid, simple and efficient way at a constant temperature (Zhao et al., 2015; Deng and Gao, 2015; Li and Macdonald, 2015; Notomi et al., 2015).

Based on the reaction kinetics, isothermal amplification techniques can be divided into three types, including linear amplification, cascade amplification and exponential amplification (Zhao et al., 2015). Among these, isothermal exponential amplification techniques provide higher amplification efficiency and detection sensitivity. Up to now, several isothermal exponential amplification techniques have been developed, such as exponential rolling circle amplification (E-RCA), loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), recombinase polymerase amplification (RPA), exponential amplification reaction (EXPAR) and enzyme-free exponential amplification methods (Asiello and Baeumner, 2011; Deng and Gao, 2015; Ding et al., 2017; Xuan et al., 2015; Xuan and Hsing, 2014). With the development of molecular biology, such as aptamers and DNAzymes, isothermal exponential amplification techniques have been used to detect a wide range of analytes, including nucleic acids, proteins, biological small molecules, ions, and even cells (Cheng et al., 2009; Goldmeyer et al., 2007; Mori and Notomi, 2009).

Electrochemical biosensor has been used to produce digital electrochemical signals by transducing the biological sensing element-target recognition events (Jia et al., 2016; Turner et al., 1987). Compared with optical biosensors, such as fluorescent, colorimetric and chemiluminescent assays (Cui et al., 2018), electrochemical biosensors have attracted considerable attention due to the advantages of high sensitivity and selectivity, high signal-to-noise ratio, simple equipment, low cost and rapid response time (Han et al., 2015; Jia et al., 2016; Wang and Dai, 2015). Moreover, the combination of nanomaterials with electrochemical biosensors can not only enlarge the electrochemically active areas but also accelerate the electron transfer efficiency between electrodes with detection materials (Jia et al., 2016). These remarkable characteristics result in better performances of electrochemical biosensors with ultrahigh sensitivity and stability (Jia et al.,

2016). In this review, we introduce the basic principles of different isothermal exponential amplification methods including enzyme-based and enzyme-free exponential amplification techniques, and further summarize their applications in electrochemical biosensors during the past five years. The present challenges and further perspectives of isothermal exponential amplification-based electrochemical biosensors are also highlighted.

2. Enzyme-based Exponential Amplification

2.1. Exponential Rolling Circle Amplification (E-RCA)

Rolling circle amplification (RCA) is an isothermal amplification technique, which has been widely used in various domains because of its simplicity and high efficiency (Ali et al., 2014). The mechanism of RCA is shown in Fig. 1a. The circular DNA template containing a small gap is to be filled by template mediated enzymatic ligation. Then, the circular template binds to a primer. The RCA reaction is immediately initiated by DNA polymerase or RNA polymerase with strand displacement activity in the presence of deoxynucleotide triphosphates (dNTPs) or nucleotide triphosphates (NTPs) solution mixture, resulting in the generation of a long nucleic acid product containing repetitive sequences which can further be tagged with probes for amplified detection of various analytes, such as DNAs (Dahl et al., 2004; Johne et al., 2009; Xu et al., 2012), RNAs (Christian et al., 2001; Li et al., 2009), small molecules (Ali and Li, 2009; Cho et al., 2005; Ma et al., 2011), proteins (Konry et al., 2011; Zhou et al., 2007) and even cells (Christian et al., 2001; Ding et al., 2012; Zhao et al., 2012).

To achieve ultrahigh sensitivity, E-RCA methods have been developed, including hyperbranched rolling circle amplification (HRCA) and padlock exponential rolling circle amplification (P-ERCA). HRCA was proposed by Lizardi et al. for the first time to detect point

mutations in a small quantity of human genomic DNA (Lizardi et al., 1998). The mechanism of HRCA is depicted in Fig. 1b. In contrast to traditional RCA, HRCA requires two primers. The first primer (P1) binds to the padlock probe to initiate the RCA reaction, forming a long single-stranded DNA (ssDNA) by DNA polymerase in a few minutes. The second primer (P2) immediately combines with the complementary sequence of the long ssDNA. As each primer extends to the downstream primer, strand displacement reaction occurs and leads to the accumulation of tandem repetitive sequences that are the same as the original circularized probe. The next cycle of extension process is initiated by P1. As a result, the two kinds of primers trigger a cascade of self-assembly and ever-increasing process called hyperbranched rolling circle amplification (HRCA), leading to the formation of a large amounts of double-stranded DNAs (dsDNAs). During HRCA, each circle can create more than 10^9 copies within 1.5 h at 60 ^{o}C, achieving exponential amplification efficiency (Nilsson, 2006). Compared with PCR, HRCA can be performed at a constant temperature without the requirement of thermal cyclers. What's more, HRCA possesses the advantages of ultrahigh detection sensitivity, simplicity, cost-effective performance and less time consuming (Li et al., 2016b). So far, HRCA has been widely applied to the detection of single nucleotide polymorphism (SNP) (Qi et al., 2001), viral RNAs (Wang et al., 2005), and proteins (Cao and Zhang, 2012; Matsuno et al., 1998; Zhu et al., 2013).

In 2013, Lin and coworkers successfully established a label-free electrochemical aptasensor based on HRCA for ultrasensitive and specific detection of platelet-derived growth factor B-chain (PDGF-BB) (Wang et al., 2013). The principle of this biosensor is presented in Fig. 1c. First, cDNA was immobilized on the surface of gold electrode through Au-S interaction, and the aptamer partially hybridized with cDNA to form the DNA duplex. In the presence of PDGF-BB, it bound to the

aptamer, resulting in the removal of aptamer from the electrode. Then, the free cDNA bound to a padlock probe with a small gap. The padlock probe was filled by *E. coli* DNA ligase, generating a circular padlock probe. Primer 1 then hybridized with the circular padlock probe, resulting in the 3' end extension catalyzed by DNA polymerase to form multiple ssDNAs, which were provided as the templates to hybridize with primer 2. Thus, primer 2 immediately extended forward in the presence of DNA polymerase. As a result, a mass of dsDNAs with different lengths were generated. Finally, methyl blue, as electrochemical indicators, were inserted into the dsDNAs to produce electrochemical signals, which were proportional to the concentrations of PDGF-BB. Therefore, a HRCA-based label-free electrochemical aptasensor was fabricated, achieving a detection limit of 1.6 fM PDGF-BB and high specificity to distinguish PDGF-BB from other proteins of lysozyme, human serum albumin (HSA), and thrombin.

Furthermore, Yi et al. developed an electrochemical biosensor by combing HRCA with molecular beacon-mediated circular strand displacement polymerization for specific detection of DNA (Li et al., 2016b). The molecular beacon was first immobilized on the surface of gold electrode. The hairpin structure was opened when target DNA was introduced to form the duplex DNA. The unfolded stem then hybridized with biotin-tagged primer, leading to the DNA strand polymerization with the assist of Klenow fragment (KF exo$^-$) DNA polymerase and dNTPs. The target DNA was thus released to initiate the next cycle of hybridization process with molecular beacons, leading to the accumulation of biotin-labeled DNAs. Through strong and specific biotin-streptavidin interaction, a large amount of streptavidin was immobilized on the electrode, and the HRCA reaction was initiated in the presence of circular templates and HRCA primers (primer 1 and primer 2). As a result, a lot of dsDNAs with different lengths were produced. Finally, the biotin-modified detection probes

specifically bound to the dsDNAs, which further linked to the streptavidin-alkaline phosphatase (ST-AP). The electrochemical signal catalyzed by alkaline phosphatase (AP) was acquired by the irreversible conversion of α-naphthyl phosphate (α-NP) to electroactive products, which was recorded by differential pulse voltammetry (DPV). This method achieved an ultrahigh sensitivity with a detection limit as low as 8.9 aM of target DNA.

In addition to HRCA mentioned above, another E-RCA method called padlock exponential rolling circle amplification (P-ERCA) was proposed as illustrated in Fig. 1d (Liu et al., 2013). The padlock probe containing a small gap is specifically hybridized with the target and further ligated by ligase. Then, the RCA reaction is initiated under DNA polymerase/dNTPs. The repeated sequences of the RCA products containing the nicking sites are recognized and immediately cleaved by nicking enzyme. After continuous process of polymerization and cleavage, a large number of P-ERCA products (triggers) are generated to initiate more RCA reactions, which thus results in the exponential amplification of target.

Recently, P-ERCA has been developed for electrochemical detection of microRNA-21 (miR-21) using $CoFe_2O_4$ magnetic nanoparticles (MNPs) for non-substrate nanoelectrocatalysis (Yu et al., 2017). The continuous process of polymerization and cleavage leaded to the exponential growth of P-ERCA products (triggers). Meanwhile, capture probe I (CP I) was immobilized on the surface of electrode, which can bind the triggers and further induce the immobilization of CP II/Au@$CoFe_2O_4$/Tb-Gra composites on the electrode. Ultimately, the electrochemical signal was recorded from the $CoFe_2O_4$ MNPs-assisted non-substrate nanoelectrocatalysis. This strategy exhibited a remarkable sensitivity for miR-21 detection, achieving a low detection limit of 0.3 fM. Overall, the electrochemical methods based on E-RCA allow the detection of various analytes, such as

nucleic acids and proteins, with the advantages of simplicity, rapidness, low cost and high sensitivity and specificity, which have great potential in the applications of clinical diagnostics, food safety and environmental monitoring.

2.2. Loop-Mediated Isothermal Amplification (LAMP)

LAMP is an attractive isothermal exponential amplification technique, which was developed by Notomi et al. in 2000 (Notomi et al., 2000). In a typical LAMP (Fig. 2a), the reaction needs four different specific primers, including a forward inner primer (FIP), backward inner primer (BIP) and two outer primers (F3 and B3) to identify six specific regions of the amplified DNA sequence, and the auto-cycling strand displacement DNA synthesis is performed by DNA polymerase. The first process is the starting material producing step (part 1 in Fig. 2a). In this process, FIP hybridizes with F2c of the template, which thus extends forward by DNA polymerase to initiate the synthesis of complementary strand. Then, F3 hybridizes with F3c and extends forward to initiate strand displacement reaction, releasing a FIP-linked ssDNA, which could form a looped out structure by specific recognition and hybridization of F1 with F1c. This ssDNA provides a template for BIP and B3. These two primers that are similar to FIP and F3 initiate the synthesis process, which thus generates a dumbbell-shaped DNA that quickly converts to a stem-loop DNA through self-primed DNA synthesis, which serves as the starting material for LAMP reaction.

The second process is the cycling amplification step (part 2 in Fig. 2a). In this process, FIP hybridizes to the loop of the stem-loop DNA, which is gained by the extension from F1 of the dumbbell-shaped DNA, and further primes strand displacement reaction to initiate LAMP cycling. This process produces an intermediate one-gapped stem-loop DNA with another loop. Immediately,

self-primed strand displacement mediated DNA synthesis produces a gap repaired stem-loop DNA and a long sequence which is complementary to the original stem-loop DNA. These products are provided as the template for recognition and hybridization of BIP to initiate the new synthesis process, and part of which is pointed to the elongation and recycling step in the subsequent cycles (part 3 in Fig. 2a). The final amplification products are a composite of stem-loop DNAs with different stem lengths and cauliflower-like structures. In comparison with PCR, LAMP achieves exponential amplification in an isothermal manner with a higher sensitivity and amplification efficiency. It has been demonstrated that LAMP can amplify one copy DNA to 10^9 copies within 1 h.

In recent years, LAMP has been combined with electrochemical technology to detect various analytes (Ahmed et al., 2015; Safavieh et al., 2013). The electrochemical detection is carried out by immobilizing redox-labelled LAMP amplicons on the electrode surface or by the detection of redox molecules in the presence or absence of amplicons (Safavieh et al., 2016). The LAMP amplicons can be detected after the amplification reactions or be monitored in real time. The electrochemical signals can be recorded by linear sweep voltammetry (LSV) (Tlili et al., 2013), square wave voltammetry (SWV) (Hsieh et al., 2012), DPV (Xie et al., 2014a), and the change of conductivity (Zhang et al., 2015).

Zourob and colleagues reported a LAMP-based method for electrochemical identification and quantification of *E. coli* Bacteria (Tlili et al., 2013). In this work, the cysteamine was immobilized on the surface of gold electrode and then activated using the cross-linker, 4-dithiocyanate (PDICT). Then, T4-bacteriophage was used to link with PDICT and was blocked by ethanolamine to capture the *E. coli* cells. After disrupting the cells, the LAMP amplification of *E. coli Tuf* gene began. The electrochemical signal was detected by the double responses of

impedance and LSV. This method achieved a detection limit of 10^2 cfu/mL within 40 min, providing a powerful tool for quick, accurate and sensitive detection for *E. coli* Bacteria.

In the above strategy, it requires laborious immobilization on the electrode surface. To make the technique simpler, some immobilization-free LAMP methods have been developed. Zourob and co-workers proposed a real-time electrochemical monitoring method for the detection of pathogen DNA through the measurable current generating by electrostatic interaction between LAMP amplicons with the electroactive molecules [$Ru(NH_3)_6^{3+}$] (Ahmed et al., 2013). In addition, the same group developed a microfluidic electrochemical biosensor for the detection of *E. coli* by performing LAMP (Safavieh et al., 2012). With the amplification of target DNA, numerous redox molecule Hoechst 33258 inserted into the minor groove of LAMP amplicons, which resulted in a significant decrease in anodic oxidation because of the slow diffusion of Hoechst 33258 molecules to the electrode surface after inserting into the LAMP products. The electrochemical signal was detected by LSV, which achieved a detection limit of 48 cfu/mL in 35 min. This microfluidic chip has a potential in clinical applications, which could be used as a point-of-care diagnostic device.

Unfortunately, the electroactive molecule Hoechst 33258 used above may inhibit the activity of DNA polymerase, which thus further limits the efficiency of LAMP and is not suitable for real-time monitoring (Luo et al., 2014; Safavieh et al., 2014). Alternatively, Soh et al. developed a microfluidic electrochemical quantitative loop-mediated isothermal amplification (MEQ-LAMP) for rapid, sensitive and quantitative detection of pathogenic DNA using methylene blue as electroactive molecules (Hsieh et al., 2012). The MEQ-LAMP chip is fabricated as Fig. 2b (A), which was used as the reaction vessel for LAMP and the cell for electrochemical measurement. The LAMP process and the interaction between the LAMP amplicons and the electroactive reagent methylene

blue are illustrated in Fig. 2b (B). Initially, the methylene blue molecules diffused freely in solution, which can encounter the gold electrode and transfer electrons to produce a high current. With the progress of LAMP reaction, various shaped amplicons, containing dumbbell-shaped intermediate DNAs, stem-loop DNAs and cauliflower-like DNAs were produced by primers and DNA polymerase. Meanwhile, the methylene blue molecules intercalated into the LAMP amplicons, which caused the reduction of free methylene blue and thus resulted in the decrease of electrochemical signal. The MEQ-LAMP assay was conducted in a single step by adding the reaction mixture into the microfluidic chip as the device was heated to a constant temperature of 65 ^{o}C [Fig. 2b (C)]. The current was real-time monitored as shown in Fig. 2b (D). This platform can directly detect as few as 16 copies of genomic DNA of *S*. Typhimurium within 1 h. In addition, Nagatani et al. reported a reverse transcription LAMP for the detection of influenza virus RNA using electroactive molecule methylene blue (Nagatani et al., 2011).

Recently, Marchal and co-workers established a real-time electrochemical LAMP method for the detection of single DNA copies which used three types of intercalating redox reporters ($[Os(bpy)_2dppz]^{2+}$, methylene blue and PhP) and one nonintercalating reporter ($Ru(NH_3)_6^{3+}$) respectively to investigate the electrochemical performance showed by each of the four reporters compared with the conventional real-time fluorescence LAMP and PCR assays (Martin et al., 2016). With the progress of the reaction, more and more intercalating redox reporters combined with the LAMP amplicons. In addition, in the presence of nonintercalating species $Ru(NH_3)_6^{3+}$, it bound to the by-product pyrophosphate anion which was exponentially released during LAMP. The two different combination assays between intercalating and nonintercalating probes with LAMP products resulted in a decreased current along with the amplification. This analytical method offered

a more reliable way for real-time electrochemical LAMP.

In addition to the above mentioned electrochemical active molecules used in electrochemical LAMP, some insoluble salts and protons can also be used to monitor the LAMP reaction by the change of conductivity. Zhang et al. developed a real-time electrical sensor to detect specific sequence DNA by combining LAMP with the capacitively coupled contactless conductivity detection (C^4D) using insoluble salt, magnesium pyrophosphate precipitate, as signaling medium (Zhang et al., 2015). In the presence of primers, dNTPs and Bst DNA polymerase, LAMP amplification initiated and a mass of amplicons were synthesized. Meanwhile, some insoluble salts, magnesium pyrophosphate precipitate as well as protons, were also produced, which resulted in the decrease of total ionic strength and thus leaded to the decrease of conductivity. A detection limit of 12.5 copy/μL was obtained by this method within 30 min. Furthermore, Yuan and co-workers detected *Nosema bombycis* genomic DNA PTP1 by tracing the phosphate ions produced during LAMP to form molybdophosphate precipitate for signal readout (Xie et al., 2015).

The electrochemical LAMP was further applied to multiplex assay. In 2014, Kong's group developed a microfluidic multiplex electrochemical LAMP (μME-LAMP) system for real-time quantitative differentiation of bacteria (Luo et al., 2014). The equipment of the μME-LAMP chip and the structure of the indium tin oxide electrodes were shown in Fig. 2c (A) and Fig. 2c (B). The multiplex detection was realized by adding various samples into the isolated electrochemical microchambers (1/1', 2/2', 3/3', and 4/4'). After performing the LAMP reaction triggered by a corresponding target DNA [Fig. 2c (C) and Fig. 2c (D)], a lower current was produced than that obtained before LAMP due to the intercalation of methylene blue into the produced dsDNAs and the

slow diffusion of methylene blue to the electrode surface [Fig. 2c (E)]. This μME-LAMP chip achieved the detection limits as low as 28, 17 and 16 copies/μL for *Mycobacterium tuberculosis*, *Haemophilus influenza* and *Klebsiella pneumonia*, respectively.

Moreover, a LAMP-based point-of-care device was established for real-time monitoring of multiplex bacterial pathogens using a novel electrochemical active molecule, Osmium redox, which possesses high binding activity and stability (Safavieh et al., 2014). Before LAMP amplification, the mixture of bacteria, redox molecules and LAMP solution were mixed. After thermal shock lyses of the bacteria, the DNAs were released into solution. Then, the LAMP was initiated, resulting in the binding of redox molecules with the amplicons and the decrease of the current. The electrochemical signal was sensitively recorded by SWV. This assay achieved the detection limits of 30 cfu/mL and 200 cfu/mL for the *Tuf* gene and the *Mcat* gene, respectively, providing an ultrasensitive and high-throughput point-of-care device for pathogen detection.

All the methods mentioned above were in "signal-off" mode. That is, the target leads to the decrease of signal output, which thus suffers from the limited signal capacity and could lead to "false-positive" results (Li et al., 2016a; Wang et al., 2015; Kong et al., 2016). To make LAMP detection more sensitive and reliable, a "signal-on" electrochemical sensor was developed by Chai's group for ultrasensitive detection of Ochratoxin A (OTA) based on LAMP (Xie et al., 2014a). In this method, the capture DNA (cDNA) was first immobilized on the gold nanoparticles modified glassy carbon electrode (GCE) and then hybridized with OTA aptamers. When OTA was added, it bound with aptamers specifically and the formed OTA/aptamer complex was released to solution. Then, the OTA aptamers remaining on the electrode were hybridized to the template DNA to initiate the LAMP reaction. Finally, the electroactive reagent methylene blue inserted into the LAMP amplicons, which

resulted in a decrease of current that was monitored by DPV. This "signal-on" electrochemical LAMP platform achieved a detection limit of 0.3 pM of OTA.

Among isothermal exponential amplification methods, LAMP can be considered as a promising and powerful amplification technique for the detection of pathogens, viruses and bacteria in biological samples (Foudeh et al., 2012). The experimental steps, such as sample purification, nucleic acid extraction and detection, can be integrated into the LAMP-based platform, especially microfluidic chips, to develop point-of-care diagnostic devices (Chang et al., 2013). In addition to electrochemical methods, other detection techniques, such as giant magnetoresistance (Zhi et al., 2014) and bioluminescence (Kiddle et al., 2012), have also been developed for the detection of LAMP-amplified analytes.

Since the temperature significantly effects the polymerase activity in LAMP reaction, accurate temperature control and calibration are required for quantitative analysis. Thus, it is in great demand to develop more stable LAMP enzymes and other LAMP chemical components (Safavieh et al., 2016). Of course, nonenzymatic LAMP strategies may also be realized, which will be more practical for resource-limited settings. As a viable tool, LAMP holds great promise in the development of commercial methods in the near future.

2.3. Helicase-Dependent Amplification (HDA)

HDA as an effective exponential amplification technique for nucleic acid detection was proposed by Kong and co-workers in 2004 (Goldmeyer et al., 2007). HDA mainly contains three steps, which are template separation, primer hybridization and primer extension (Fig. 3a). First, the dsDNAs are separated by DNA helicase and each of them is covered by ssDNA-binding proteins (SSBs) (step 1).

Then, two specific primers hybridize to each ssDNA respectively (step 2) and extend the sequence to form two dsDNAs in the presence of DNA polymerase (step 3). Through taking the new produced dsDNAs as the substrates, the next cycle of HDA reaction begins with the help of DNA helicase (step 4). Consequently, the continuous cycle processes result in an exponential amplification of the target.

In 2011, Limoges's group developed a real-time electrochemical method to monitor the HDA process for the detection of nucleic acids (Kivlehan et al., 2011). The dsDNA template was unwound by DNA helicase to promote the combination of primers with each ssDNA. Then, each primer extended forward to generate two dsDNAs in the presence of DNA polymerase. With the exponential accumulation of dsDNAs in solution, more and more redox probes ($Os[(bpy)_2DPPZ]^{2+}$) were intercalated, leading to a decrease of current monitored by SWV. This method was used to quantitatively analyze target DNA with a detection limit of ~10^5 copies within 1 h at a constant temperature of 65 ^oC, which provided a promising alternative isothermal method for nucleic acid detection.

In addition to homogeneous detection, a HDA-based heterogenous-phase strategy has been developed by Lobo-Castañón and colleagues for electrochemical detection of transgene extracted from the Cauliflower Mosaic Virus 35S Promoter (CaMV35S) through analyzing the unpurified HDA amplicons (Fig. 3b) (Moura-Melo et al., 2015). In this assay, the HDA products hybridized with the signaling probes which were labeled with fluorescein isothiocyanate (FITC) and further bound to the thiolated capture probes (P35S-capture probes) which were immobilized on the gold surface. The antiFITC-peroxidase (POD) Fab Fragments were then added to combine with the FITC. The electrochemical signal was detected by catalysing the oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) by H_2O_2, which achieved highly sensitive detection of CaMV35S genomic DNA with a

detection limit of ~30 copies.

Furthermore, Lobo-Castañón's group developed an asymmetric HDA (aHDA)-based method for electrochemical detection of pathogenic genomic DNA (Barreda-García et al., 2015). The genomic DNA was unwound by DNA helicase and the biotin-tagged reverse primer immediately hybridized to one of the ssDNAs to promote the extension of the strand, which thus led to the removal of the other ssDNA. The generated biotin-amplicons were immobilized on the surface of streptavidin-modified magnetic beads, followed by hybridization with FITC-signaling probe. When the antiFITC-POD Fab conjugate was added, it combined with FITC. The bound enzyme activity was immediately quantified by capturing the magnetic beads onto the surface of screen-printed carbon electrodes. The electrochemical signal was detected by catalysing the oxidation of TMB by H_2O_2, which achieved highly sensitive detection of 0.5 aM of synthetic target within 4 h and thus addressed the unspecific amplification limitations.

Despite the advantages of HDA, before HDA optimization of experimental conditions (e.g. buffer composition and primer sets) is often required to ensure the reaction can be carried out successfully. However, the optimization is often carried out through PCR experiments. Consequently, more researches should be done to realize the whole potential of HDA. In addition, future efforts can be focus on the development of helicases and SSBs and the enhancement of the efficiency of the existing helicases and SSBs, which are related to the identification of the rate-limiting step in HDA.

2.4. Recombinase Polymerase Amplification (RPA)

The isothermal exponential amplification method of RPA was developed by Armes's et al. in 2006 for the detection of nucleic acids (Piepenburg et al., 2006). The principle of RPA is

illustrated in Fig. 4a. Briefly, the recombinases combine with the primers to form the protein-DNA complexes, which subsequently scan the template for the homologous sequence. Once recognition and hybridization occur between the complexes and homologous sequence, the strand displacement reaction is initiated to synthesize new DNA sequences. The generated structures combine with SSBs to prohibit the displacement of primer by branch migration. The binding/extension process generates a whole copy of the amplicon which is the same as the initial template. The cyclic repetition of this method leads to the exponential amplification of target DNA.

Recently, Wang et al. developed a method for rapid and sensitive detection of plant pathogen DNA by combining RPA with nanoparticle-electrochemistry (Lau et al., 2017). In this method, the biotin-labeled reverse primer and the forward primer included a 10 nt barcode at the 5' end were employed to perform RPA. After the RPA reaction, the generated amplicons hybridized to the capture probes that were coated on the gold nanoparticles (AuNPs). Then, the streptavidin-coated magnetic beads were applied to enrich the AuNPs/DNA/biotin complexes. Finally, the amplicons were denatured by heating at 95 ^{o}C, resulting in the release of AuNPs. The electrochemical signal generated by the reduction of Au (III) to Au (0) was measured by DPV. This assay was 10000 times more sensitive than traditional PCR/gel electrophoresis assay.

Although RPA could achieve ultrahigh sensitivity, this method still has some disadvantages. For example, since primer-dimers could be formed when target DNA is in low concentration, DNA by-products are existed with random sequences. To solve this problem, O'Sullivan's group reported a solid-phase RPA method for the detection of Francisella tularensis genomic DNA (Fig. 4b) (del Río et al., 2014). Unlike traditional RPA, in the solid-phase RPA the forward primer was

immobilized on the surface of microtiter 96 well plates, providing a support for the reaction. The following amplification processes were the same as the traditional RPA. The proposed solid-phase RPA system was performed by both optical and electrochemical detection, which achieved the rapid and highly specific detection of a trace amount of DNA copies as low as10^4 copies/μL. The above works prove the obvious advantages of RPA compared with PCR, such as the rapid reaction and simplicity, making it a great alternative in the field where accurate control of temperature is often technically challenging.

2.5. Exponential Amplification Reaction (EXPAR)

EXPAR, a unique technique to synthesize short oligonucleotides exponentially by itself as primers for amplification of nucleic acids, was developed by Galas's group in 2003 (Van Ness et al., 2003) (Fig. 5a). The template employed in EXPAR consists of two copies (shown in α) which are complementary to the target DNA (shown in α'). This template is separated by a recognition site for nicking enzyme (shown in light square) and a spacer sequence (shown in green square). Upon the introduction of short target DNA, it hybridizes with the template to form two kinds of transient duplexes at approximately 60 ^{o}C. One kind of the transient duplex as illustrated in Fig. 5a is generated by the hybridization of the target, which can be extended forward in the presence of DNA polymerase. Then, the generated duplex is cleaved by nicking enzyme, resulting in the removal of the newly-synthesized oligonucleotide. The resulting oligonucleotide thus acts as a new primer for the next cycle of the reaction. The cyclic repetition of the process leads to the exponential amplification of the target as high as 10^8-fold in a few minutes. Because of the advantages of high amplification efficiency and high detection sensitivity, EXPAR has been integrated with electrochemical

technology for the detection of various analytes in wide fields.

In 2014, Chen and co-workers established an ultrasensitive label-free electrochemical biosensor for the detection of microRNA (miRNA) based on arched probe mediated EXPAR (Yu et al., 2014). As shown in Fig. 5b, the arched probe was immobilized on the electrode surface. Upon target miRNA hybridized to the purple region of the arched probe, the newly formed DNA/RNA complex was released into solution and the free strand on the electrode was immediately combined with hemin, which thus formed G-quadruplex-hemin complex to catalyse the generation of electrochemical signal with the help of K^+. Moreover, a primer was bound to the DNA/RNA duplex, which thus extended forward by DNA polymerase and launched the strand displacement reaction. This process resulted in the release of target miRNA, which thus initiated a new cycle reaction and generated new DNA complex. In particular, the resultant DNA complex including a nicking site can be cleaved by nicking enzyme, and the primer extended the strand again. Meanwhile, part sequence of the resulting ssDNA (target DNA) was the same as the target miRNA, which thus can initiate a new cycle of the reaction. As a result, the process of nicking, extension, and strand displacement repeated continuously, leading to the accumulation of target DNA and an enhanced electrochemical signal, achieving a low detection limit of 5.36 fM miRNA-21 with excellent specificity.

Furthermore, an ultrasensitive label-free electrochemical biosensor was developed by the same group by combining EXPAR with hybridization chain reaction (HCR) (Yu et al., 2015). The probe consisting of two complementary strands (S1 and S2) was immobilized on the surface of gold electrode. The target hybridized with S1, resulting in the formation of S1/target complex and the release of S2. The primer was hybridized with the single-stranded domain of the S1/target complex and the strand was extended forward in the presence of DNA polymerase. During this process, the

target is released to initiate a new cycle reaction. Meanwhile, the resultant DNA duplex containing a recognition site was nicked by nickase, and the primer was extended forward again. Finally, the released single-stranded S2 on the electrode surface was used to initiate the HCR with the continuous hybridization of molecular beacons (MB1 and MB2), resulting in the formation of DNA polymer nanowires consisting of numerous G-quadruplex units, which were further assembled into hemin/G-quadruplex HRP-mimicking DNAzymes when hemin was added. The electrochemical signal was detected by catalysing the oxidation of TMB by H_2O_2, which achieved highly sensitive detection of avian influenza A (H7N9) virus DNA sequence with a low detection limit of 9.4 fM. A similar electrochemical biosensor through combining EXPAR with HCR has also been reported for thrombin detection with a detection limit as low as 33 fM (Xie et al., 2014b).

Although the above mentioned EXPAR-based strategies could achieve ultrahigh sensitivity, they required laborious probe immobilization on the electrode surface, which makes the reaction complicated. Alternatively, immobilization-free electrochemical EXPAR strategies have been developed (Nie et al., 2014). As shown in Fig. 5c, in this system, "Y" DNA hybridized with the template Y'-Y' to form two kinds of transient duplexes, and the following reaction was the same as EXPAR, which thus leaded to the exponential amplification of "Y". As a result, a large amount of "Y" DNAs were generated, which further interacted with hemin to form the HRP-mimicking DNAzymes, catalysing the oxidation of hydroquinone (HQ) in the presence of H_2O_2 and producing electrochemical signals. This method achieved a detection limit as low as 1 pM for target DNA.

Furthermore, an ultrasensitive and flexible biphasic electrochemical biosensor was demonstrated by integrating the immobilization-free programmable hairpin probe with EXPAR for the detection of DNA (Zhuang et al., 2014) (Fig. 5d). The target DNA hybridized with region I of ferrocene-labeled

hairpin, which extended forward in the presence of DNA polymerase and opened the hairpin structure. The polymerization reaction was terminated until the recognition of the encoded alkane spacer at the end of region II, resulting in the formation of anti-streptavidin aptamer (probe A). The resultant duplex DNA containing a nicking site was then cleaved by nicking enzyme. Therefore, the processes of extension, strand displacement as well as nicking repeated continuously, generating a large amount of initiator strands. The initiator strands further hybridized with region II of the hairpin probes, resulting in the conformation change of region III to activate the anti-streptavidin aptamers (probe B). After the reaction, the probes A and B were immobilized on the electrode surface via the interaction between the anti-streptavidin aptamer and the streptavidin. This behaviour activated the connection between ferrocene and Au electrode to produce an electrochemical signal, achieving a detection limit as low as 2.56 fM for target DNA. The advantages of simplicity and high adaptability make EXPAR methods more and more attractive in the fields of biochemical analysis and clinical diagnosis.

3. Enzyme-free Exponential Amplification

Toehold-mediated strand displacement (TMSD) has demonstrated to be a powerful tool in the construction of enzyme-free and isothermal autonomous systems for ultrasensitive and selective detection of various analytes (Zhang and Seelig, 2011). In a typical TMSD, a long ssDNA firstly hybridizes to the “toehold” region of a DNA duplex, and the strand displacement is immediately initiated, leading to the generation of a dsDNA with more complementary base pairs (Genot et al., 2011; Seelig et al., 2006; Yurke et al., 2000). The prominent example of TMSD is HCR. In a typical HCR, an initiator triggers a cascade of hybridization events between alternating

two hairpin structures, resulting in the formation of a long nicked double-helix (Dirks and Pierce, 2004). In addition to linear growth kinetics, branched HCR strategies have been designed for exponential amplification (Bi et al., 2015; Bi et al., 2016; Bi et al., 2017). Particularly, Hsing and co-workers proposed a TMSD-based nonlinear HCR system which grows in an exponential manner (Fig. 6a) (Xuan and Hsing, 2014). When trigger is introduced into the system, it hybridizes with the toehold of Substrate-A, initiating the first TMSD reaction and thus displacing the black sequence to open the first loop. Then, the Assistant-A binds with the new exposed toehold of the displaced strand and extends forward to open the second loop, resulting in the release of Byproduct-A. The two exposed sequences of Substrate-A further hybridize with the toeholds of Substrate-B, which allows the hybridization of Assistant-B and the dissociation of Byproduct-B. As a result, the product containing two single-stranded sequences that are the same as the trigger DNA are exposed and continually initiate TMSD reactions, which thus results in the exponentially growth of the branched DNA nanostructure.

Recently, an immobilization-free and enzyme-free electrochemical nucleic acid sensing method has been developed based on this nonlinear HCR system (Fig. 6b) (Xuan et al., 2015). In the absence of target, the ferrocene-labeled PNA probes (Fc-PNAs) dispersed freely in solution, which can bind with the negatively charged indium tin oxide (ITO) electrode and produce a measurable current [Fig. 6b (B)]. When the target was added, the nonlinear HCR was initiated, resulting in the exponentially growth of the branched DNA/PNA nanostructures that were hardly in close to the surface of ITO electrode, which thus led to the amplified reduction of the electrochemical signal (“signal-off” mode) [Fig. 6b (C)]. It should be noted that in this assay Fc-PNA was employed to hybridize onto the dendritic DNA nanostructures with high

hybridization kinetics efficiency and strong binding strength between PNA and DNA. This method achieved an ultrahigh sensitivity with a detection limit of 100 fM target.

Unlike the immobilization-free method mentioned above, Ding and co-workers proposed an electrochemical biosensor by immobilizing the capture probe onto the gold electrode surface for "signal-on" detection of ATP (Fig. 6c) (Ding et al., 2017). When ATP was added, it bound with the aptamer, which thus led to the release of trigger DNA. This trigger DNA then hybridized with the toehold of Substrate 1, initiating the first TMSD reaction which thus displaced region iii of Substrate 1-B to open the first loop. Then, Helper 1 hybridized with the region iii of Substrate 1-B and extended forward to open the second loop, resulting in the release of Byproduct-1. The two identical sequences of Substrate 1-A strand combined with the two toeholds of Substrates 2 respectively, and then Helper 2 was used to dissociate Byproduct 2. Finally, the product containing two target sequences was generated, which could initiate another cycle of reaction and result in the exponentially growth of DNA nanostructures. The resultant DNA dendrimers contained considerable terminal biotin molecules were immobilized on the electrode through capture probe, which further linked to ST-AP to generate a high electrochemical signal by the irreversible conversion of α-NP to electroactive products catalyzed by AP. This method achieved a detection limit of 5.8 nM ATP.

In comparison with enzyme-assisted isothermal exponential amplification techniques, enzyme-free strategies have the advantage of simplicity owing to the inherent non-enzyme nature. In addition, enzyme-free methods possess controllable kinetics, high amplification efficiency and structural versatility, which thus hold great potential in the application fields of biosensing, bioimaging and biomedicine.

4. Conclusion and Perspectives

In this review, we have introduced a series of isothermal exponential amplification techniques and summarized their applications in electrochemical biosensors during the past five years. The comparison of the enzyme-based isothermal exponential amplification techniques is depicted in Table 1. Given the remarkable advantages of ultrahigh amplification efficiency and detection sensitivity, isothermal exponential amplification has demonstrated as a powerful tool for bioanalytical applications. With the development of molecular biology, such as aptamers and DNAzymes, isothermal exponential amplification techniques have been expanded to detect a wide range of analytes, including nucleic acids, proteins, and biological small molecules. Moreover, the integration of isothermal exponential amplification with microsystems or portable devices has promoted the development of point-of-care diagnosis with ultrahigh sensitivity. Although isothermal exponential amplification strategies have achieved considerable progress, there are still some challenges. The first is the nonspecific amplification and the amplification bias inherent in the exponential amplification techniques. The development of novel tool enzymes, such as polymerases and nicking enzymes, may help to improve the detection specificity to some degree. In addition, integration of different assays such as nanomaterials, droplet microfluidic chips and nonexponential preamplification into one multifunctional system is expected to minimize the amplification bias.

Another challenge is how to apply these techniques to intracellular and in vivo analysis. Up to now, some exponential amplification techniques have been applied to intracellular nucleic acid detection, such as miRNA and mRNA. Nevertheless, it is rarely reported the intracellular and in

vivo exponential amplification analysis of non-nucleic acid bioanalytes, such as proteins and small biomolecules. The delivery of additional recognition components (e.g., antibodies) and amplified components into live cells or in vivo may transform the non-nucleic acid analytes detection into nucleic acid amplification events (Zhao et al., 2015). What's more, another promising solution is the application of intracellular enzymes (e.g., polymerases) to carry out the exponential amplification reactions (Ono et al., 2012; Qian et al., 2014). Furthermore, there still exists vast improvement space to develop enzyme-free exponential amplification techniques and further apply these methods into the field of electrochemistry. Although there are many challenges ahead of us, it is hopeful to believe that with the development of isothermal amplification techniques, significant breakthroughs and widespread applications could be achieved in the fields of biosensing, biomedicine, biotechnology, and so on.

Acknowledgement

This work was supported by the National Natural Science Foundation of China (21722505, 21535002 and 21375056), the Special Funds of the Taishan Scholar Program of Shandong Province (tsqn20161028), the Natural Science Foundation of Shandong Province (ZR2017JL009), and Foundation of Key Laboratory of Sensor Analysis of Tumor Marker, Ministry of Education, Qingdao University of Science and Technology (SATM201803).

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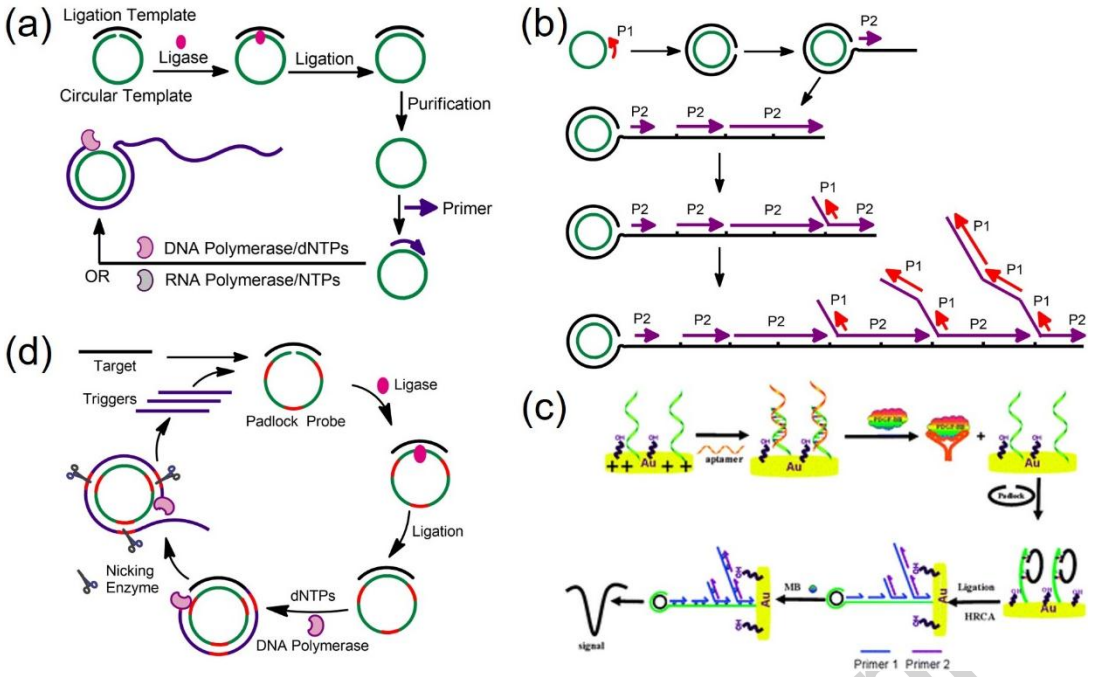

Fig. 1 (a) Principle of RCA. (b) Fundamental of HRCA. (c) HRCA-based electrochemical biosensor for PDGF-BB detection. Reprinted with permission from (Wang et al. 2013). Copyright (2013) Royal Society of Chemistry. (d) Reaction mechanism of P-ERCA.

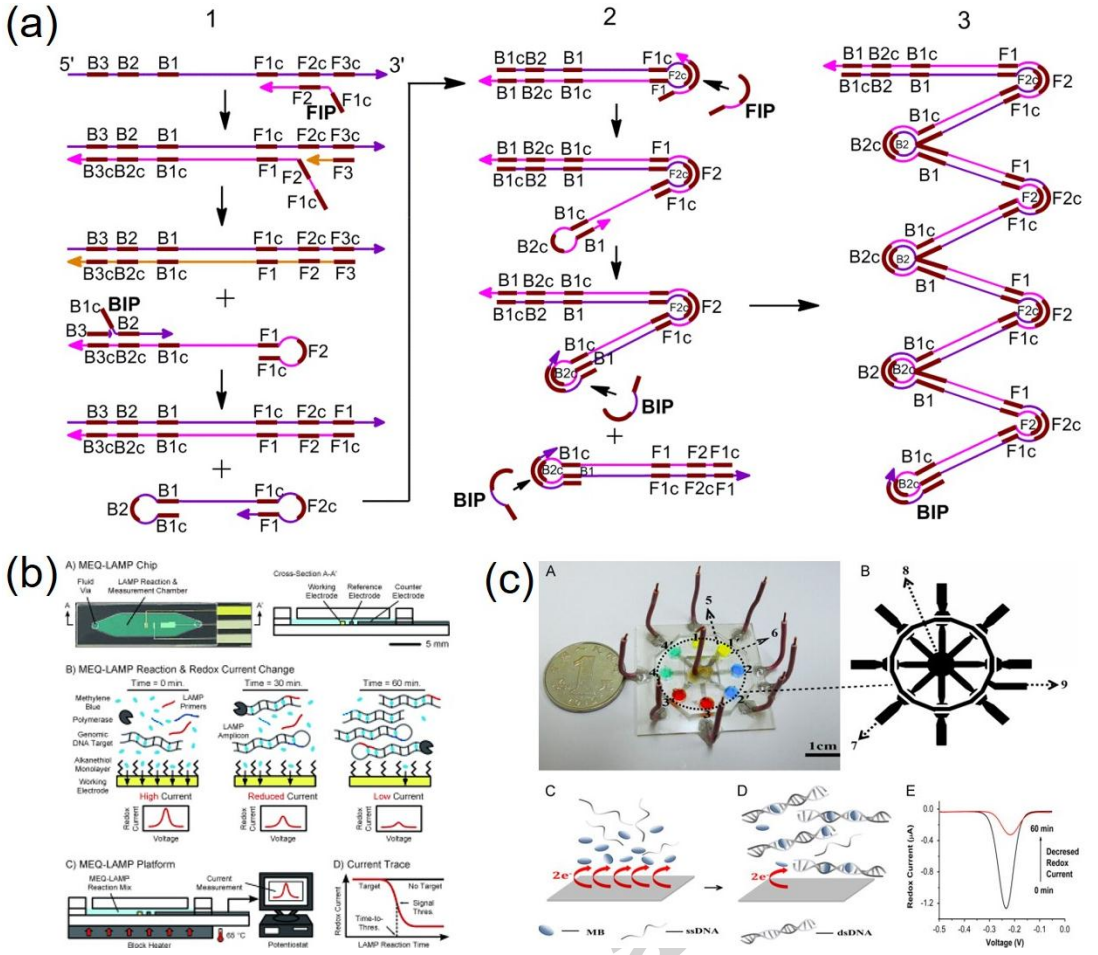

Fig. 2 (a) Schematic representation of LAMP. (b) MEQ-LAMP system for pathogenic DNA detection. Reprinted with permission from (Hsieh et al. 2012). Copyright (2012) John Wiley & Sons. (c) Immobilization-free μME-LAMP system for bacteria detection. Reprinted with permission from (Luo et al. 2014). Copyright (2014) Elsevier.

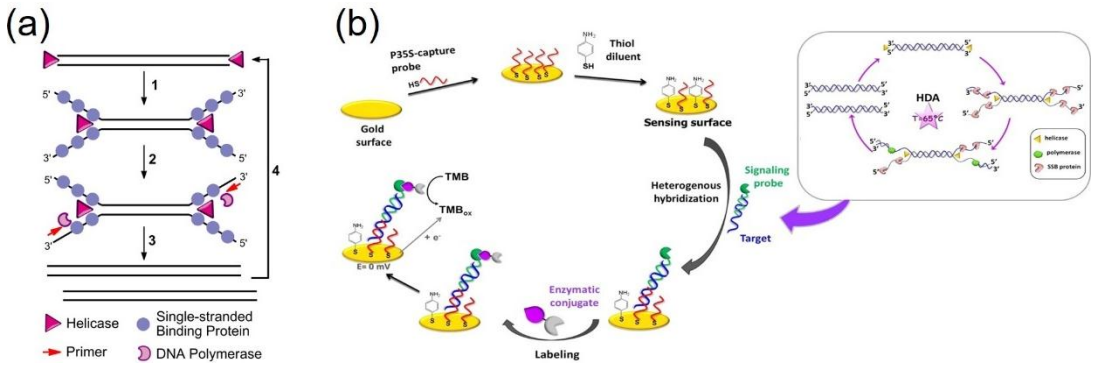

Fig. 3 (a) Principle of HDA. (b) HDA-based heterogenous-phase electrochemical biosensor for transgene detection. Reprinted with permission from (Moura-Melo et al. 2015). Copyright (2015) American Chemical Society.

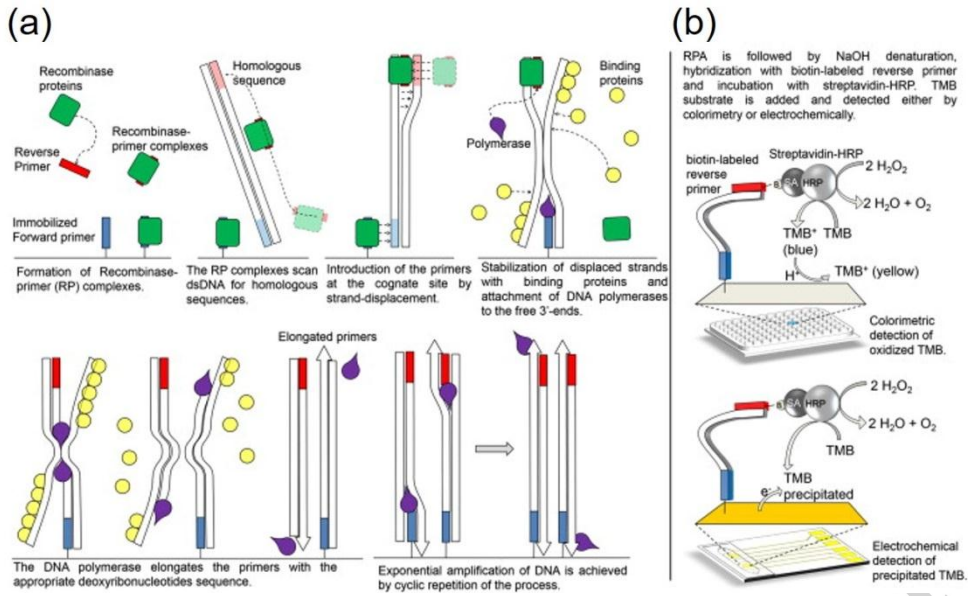

Fig. 4 (a) Principle of RPA. (b) The solid-phase RPA method for electrochemical detection of Francisella tularensis genomic DNA. Reprinted with permission from (del Río et al. 2014). Copyright (2014) Elsevier.

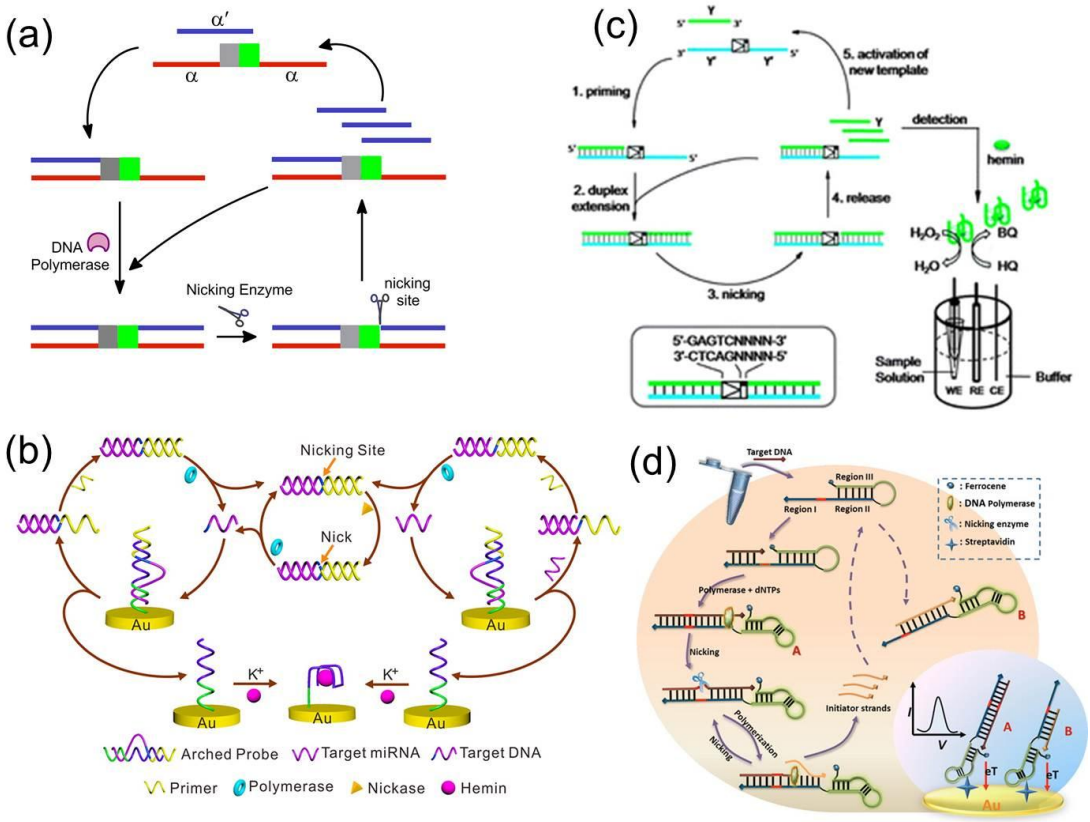

Fig. 5 (a) Mechanism of EXPAR. (b) An arched probe mediated EXPAR method for electrochemical detection of miRNA. Reprinted with permission from (Yu et al. 2014). Copyright (2014) American Chemical Society. (c) Immobilization-free EXPAR strategy for electrochemical detection of target DNA. Reprinted with permission from (Nie et al. 2014). Copyright (2014) Royal Society of Chemistry. (d) Immobilization-free hairpin probe-mediated EXPAR for target DNA detection. Reprinted with permission from (Zhuang et al. 2014). Copyright (2014) American Chemical Society.

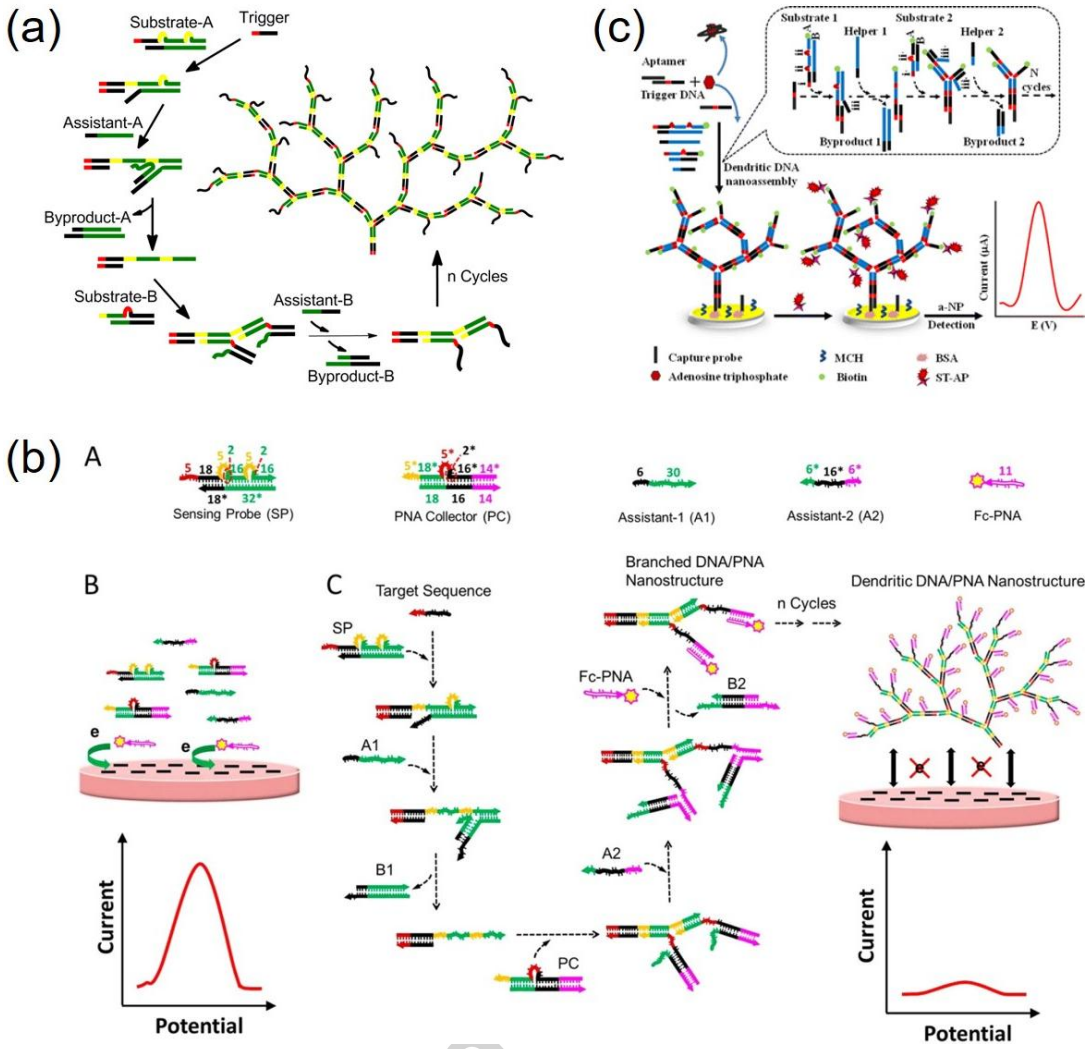

Fig. 6 (a) Principle of enzyme-free exponential amplification system based on TMSD-mediated nonlinear HCR. (b) Immobilization-free and enzyme-free exponential amplification for electrochemical detection of nucleic acids based on nonlinear HCR. Reprinted with permission from (Xuan et al. 2015). Copyright (2015) American Chemical Society. (c) A solid-phrase and enzyme-free exponential amplification assay for ATP detection based on nonlinear HCR. Reprinted with permission from (Ding et al. 2017). Copyright (2017) Springer-Verlag Wien.

Table 1. Comparison of different enzyme-based isothermal exponential amplification strategies.

Methods	Substrates	Temperature (°C)	Time (h)	Analytes	Efficiency	Refs.
HRCA	ligase, DNA polymerase, dNTPs, 2 primers	~ 60	1-1.5	DNA, RNA, protein	10^9	(Lizardi et al., 1998; Wang et al., 2013; Li et al., 2016b)
P-ERCA	ligase, DNA polymerase, dNTPs, nicking enzyme	30	6	miRNA, DNA	10^6-10^8	(Liu et al., 2013; Yu et al., 2017)
LAMP	DNA polymerase, dNTPs, 4 primers	60-65	< 1	DNA, RNA, small molecule	10^9	(Notomi et al., 2000; Hsieh et al., 2012; Nagatani et al., 2011)
HDA	helicase, DNA polymerase, dNTPs, 2 primers	37-65	0.5-2	DNA, protein	10^7	(Goldmeyer et al., 2007; Kivlehan et al., 2011)
RPA	recombinase, DNA polymerase dNTPs, 2 primers	37-42	~ 1	DNA, RNA, protein	10 copies of genomic DNA	(Piepenburg et al., 2006; del Río et al., 2014; Lau et al., 2017)
EXPAR	DNA polymerase, dNTPs, nicking enzyme	~ 60	< 0.5	DNA, RNA, protein	> 10^6	(Van Ness et al., 2003; Xie et al., 2014b; Zhuang et al., 2014)

Highlights

- Isothermal exponential amplification has demonstrated as a powerful tool for bioanalytical applications
- Basic principles of isothermal exponential amplification techniques are introduced.
- Their applications in electrochemical biosensors during the past five years are summarized.
- The challenges and perspectives of isothermal exponential amplification-based electrochemical biosensors are highlighted.