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Review Article

Enzyme-assisted isothermal amplification of nucleic acids on the electrode surface

Madalin Alexandru Cobzariu[1,2], María Jesús Lobo-Castañón[1,2] and Rebeca Miranda-Castro[1,2]

Abstract

The detection of genetic material (DNA or RNA) is becoming increasingly important not only in the clinical practice but also in food quality control or environmental analysis. Since the amount of the specific nucleic acid sequences targeted is often very low, nucleic acid tests usually involve an amplification step, which makes many copies of the target. Although the polymerase chain reaction (PCR) is the gold standard, nature provides amplification systems that proceed at a constant temperature and are easier to adapt to electrochemical platforms for point-of-need applications. In this short review, we chronicle the evolution of enzyme-assisted isothermal nucleic acid amplification strategies, specifically helicase-dependent amplification (HDA), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA), directly coupled to conductive platforms to facilitate the electrochemical transduction, describing the current state of the art, and identifying some of the challenges to bring these new platforms to the real practice as point-of-need tests.

Addresses

[1] Departamento de Química Física y Analítica. Universidad de Oviedo, Av. Julián Clavería 8, 33006, Oviedo, Spain

[2] Instituto de Investigación Sanitaria del Principado de Asturias, Avenida de Roma, 33011, Oviedo, Spain

Corresponding author: Miranda-Castro, Rebeca (mirandarebeca@uniovi.es)

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Introduction

Simple, decentralized, affordable, and fast quantitation of nucleic acids is demanded in different fields such as clinical diagnosis, food safety, environmental monitoring, or quality control [1,2]. Electrochemical hybridization-based biosensors have the potential to meet these needs since they combine the exquisite specificity of Watson-Crick base pairing with the advantages of electrochemical transducers, including, rapid response, simple instrumentation, compatibility with microfabrication technologies, thus enabling portability and low cost, minimum power consumption, and capability of working with small volumes and cloudy samples. However, a significant hurdle in the development of these affinity sensors is that in most cases the target molecules are relatively long DNA/RNA sequences often at very low levels. In consequence, a previous nucleic acid amplification process is typically required to restrict the size of the oligonucleotide target, thereby facilitating its on-surface hybridization, while improving the method sensitivity.

At present, the polymerase chain reaction (PCR) is the most widely used DNA amplification method, and it only needs the DNA to be amplified (template), a DNA polymerase enzyme, and two primers. Likewise, PCR requires cycles of heating and cooling because the primers cannot bind to the double-stranded DNA template, and hence the DNA polymerase enzyme cannot start their elongation unless the template is thermally denatured [3].

On-surface amplification of nucleic acids allows the integration of an electrochemical genosensor with a target amplification method on the same platform, thus reducing the overall analysis time, the likelihood of contamination, and, ultimately, attaining an actual point-of-need molecular test. To date, however, on-surface nucleic acid amplification with electrochemical detection has seen a slow transition to the practice. Here, we explore the strengths and weaknesses of the latest developments in electrochemical detection of on-surface enzyme-assisted nucleic acid amplifications, focusing on isothermal processes such as helicase-dependent amplification (HDA), loop-mediated isothermal amplification (LAMP), and recombinase polymerase amplification (RPA). To identify the main challenges to be faced, we chronicle the evolution of the different approaches reported to date, using the advances and challenges identified in solid-phase PCR as a starting point.

Solid-phase PCR

Solid-phase polymerase chain reaction (SP-PCR) amplification entails the attachment of one or two primers to the electrode surface through its 5′-end, allowing the extension of the free 3′-end catalyzed by the DNA polymerase [4]. Surface confinement of primers helps to minimize the formation of undesired primer dimers and provides spatial separation of different target-specific amplicons for multiplex detection in the same reaction when using an electrode array.

Nevertheless, solid-phase amplification efficiency is lower than that described for the liquid-phase counterpart. This loss of efficiency is presumably related to the steric hindrance of the solid support that would hamper the capture and enzymatic replication of the target. To address these issues, several points should be considered.

(1) In order to make the surface-tethered primer more accessible to interact with the target, a spacer can be incorporated to its 5′ extreme, thus coming closer to a homogeneous hybridization [5].
(2) Similar to microarrays and traditional DNA/RNA biosensors, the hybridization between the attached primer and the target in solution is highly dependent on the surface density of the former. Thus, a balance between the number of surface-anchored oligonucleotides and their accessibility for hybridization is usually observed as a result of electrostatic repulsion or steric crowding [6].
(3) As far as replication is concerned, an important tip to promote the target amplification on the solid support consists in adding to the solution a small amount of an unlabeled version of the anchored primer (i.e. implementation of an asymmetric ratio of forward and reverse primers in solution). This way, amplification begins in the liquid phase and proceeds until the limiting primer is consumed, resulting in a shorter amplification product. This one hybridizes more easily with the surface-tethered primer than the complete target and becomes the template for on-solid amplification [7]. To thermodynamically favor the surface reaction, the in-solution primer must be shorter at its 3′-terminus than the platform-anchored primer. A difference of approximately 8 °C in the melting temperature is recommended [8].
(4) Furthermore, the selection of appropriate chemistry for primer immobilization becomes critical. As the covalent bond is not significantly affected by repeated thermal cycles during PCR, it is widely used [9*].

Regarding the electrochemical transduction of the on-surface nucleic acids amplification, different strategies are possible, and they can be clustered into label-free and label-based approaches (Figure 1). The study of the electron transfer rate between a redox pair present in the solution and the modified electrode surface by either voltammetry or faradaic impedance spectroscopy (FIS) is framed within the first group. Another alternative is the use of a redox species that binds to the generated dsDNA via intercalation. All of them allow for real-time monitoring, conditioned by sensing layer stability; although, the measurement duration in FIS imposes a higher sampling time (about 10 min). Nevertheless, improved selectivity and sensitivity could be expected with those strategies involving labeled primers for subsequent introduction of a redox enzyme or even deoxyribonucleoside triphosphates (dNTPs) functionalized with an electroactive molecule. The electrochemical quantification of the immobilized enzymatic activity demands end-point measurements, while the detection of amplicons harboring an electroactive molecule attached to their nucleobases requires a denaturation step that the PCR thermal cycling makes compatible with real-time tracking [9*].

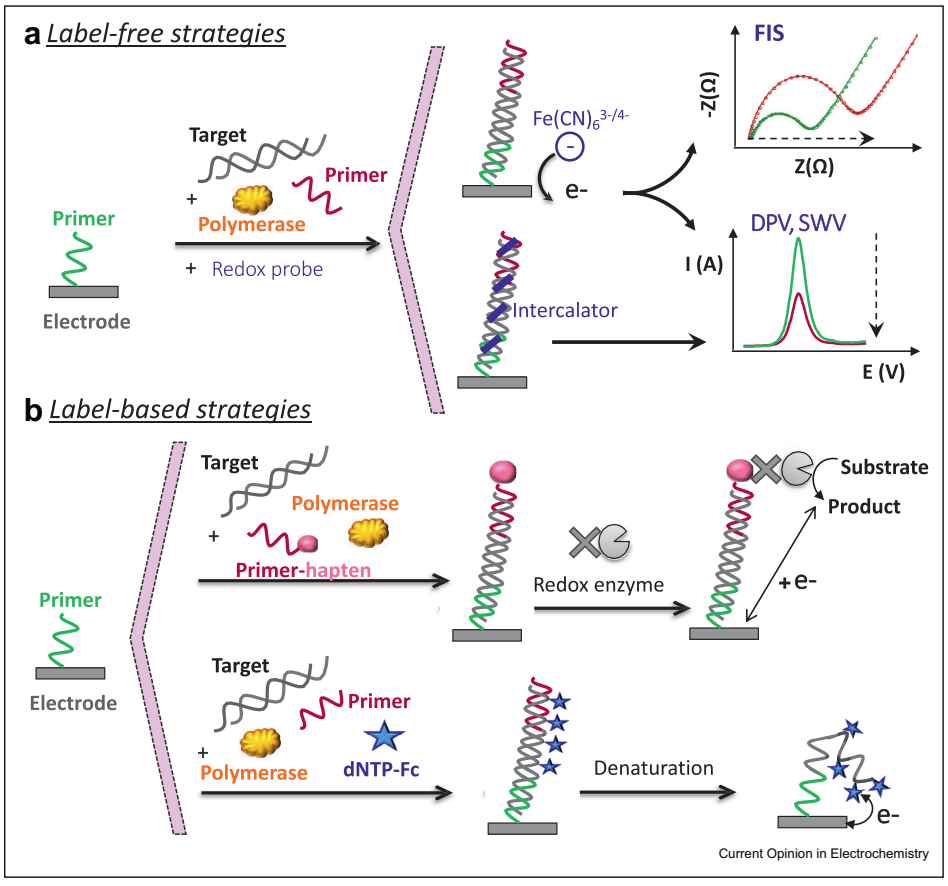
Solid-phase PCR amplification requires a sophisticated thermocycling machine for fast and precise temperature control along with a thermostable DNA polymerase enzyme, thus being constrained to large laboratory infrastructures. Because of these PCR limitations, nucleic acid amplification methods that proceed at constant temperature, i.e. isothermal alternatives to PCR, are of great interest since they do not require complex and specialized equipment or heat-stable enzymes. These methods include, among others, HDA, LAMP, and RPA. They emulate *in vivo* processes of DNA replication and their main differences are the enzymes involved and the mechanism to elude the thermal denaturation of the double-stranded DNA template that enables primer annealing in PCR [10**].

Helicase dependent amplification

In HDA, a helicase disrupts the hydrogen bonds between the complementary strands that make up the DNA duplexes, and a single-strand DNA-binding (SSB) protein stabilizes the resulting ssDNA. Afterwards, primer annealing and extension occur at a fixed temperature of 65 °C [11] (Figure 2a). This amplification technique was first developed in solution with real-time [12] and end-point [13] electrochemical detection. Although slightly slower than PCR [14], the combination of liquid-phase HDA with an electrochemical hybridization-based biosensor led to increased selectivity and sensitivity with respect to qPCR [15].

Solid-phase HDA was first developed in combination with fluorescent detection, although low analytical sensitivity was reported [16]. Some years later, HDA integration on conductive indium tin oxide (ITO) surfaces was successfully carried out achieving, after 90 min

Figure 1



Electrochemical transduction of nucleic acid amplification. (**a**) Label-free strategies: signal-on impedimetric (upper scheme), and signal-off voltammetric measurements (bottom scheme). (**b**) Label-based strategies: primer tagged with a hapten, e.g., biotin or fluorescein for incorporation of a redox enzyme (upper scheme), use of dNTPs modified with a redox probe (bottom scheme). Abbreviations: FIS faradaic impedance spectroscopy, DPV differential pulse voltammetry, SWV square wave voltammetry.

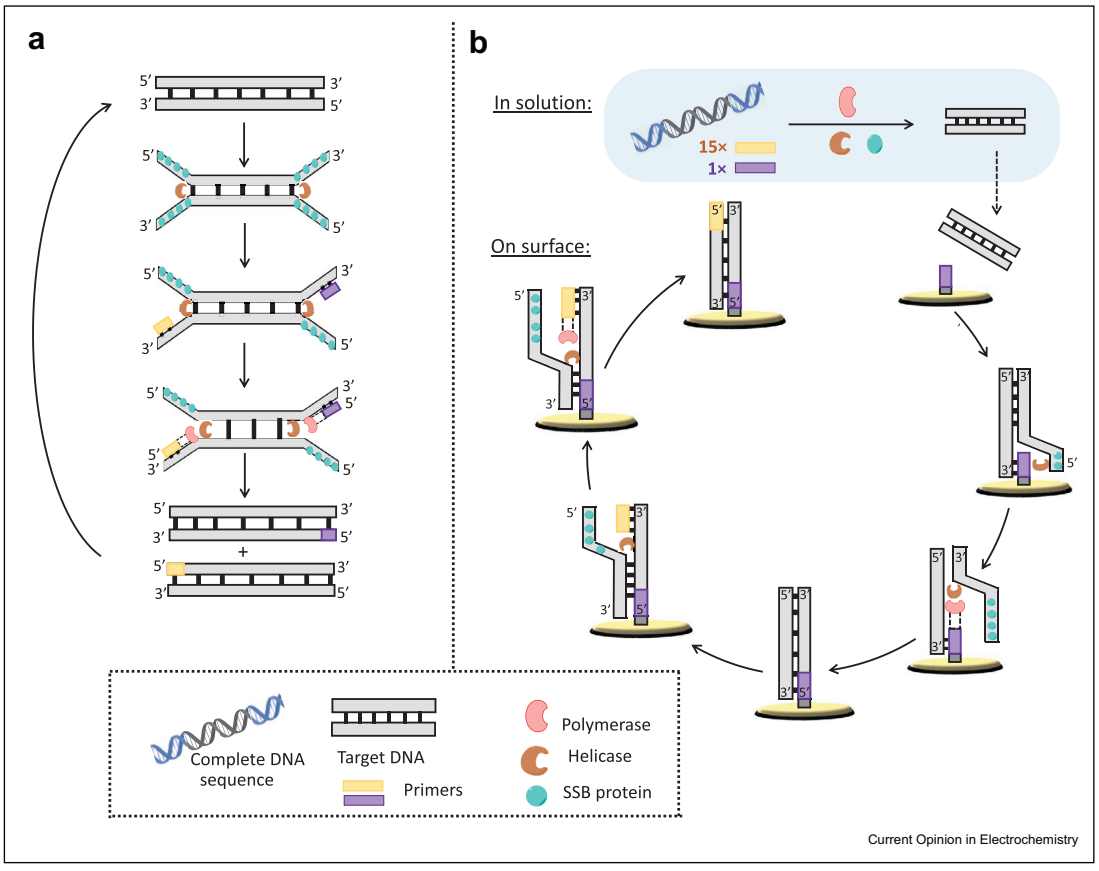
of amplification, a detectability in line with that of real-time PCR [17*]. An electrode-bound reverse primer was combined with a labeled forward primer in solution, such that amplification is detectable as an increase in the activity of alkaline phosphatase on the surface, electrochemically measured using 1-naphthyl phosphate as substrate. The use of asymmetric ratio for the primers in solution led to a two-stage mechanism (Figure 2b) supported by the presence of two different regions in the response curve. No further attempts to implement and/or improve this methodology have been reported, despite the conductive and transparent support allows both electrochemical and optical detection. The cumbersome multi-step process required for primer immobilization and the length of the amplification step (90 min) represent a practical limitation.

Loop-mediated isothermal amplification

The widely used loop-mediated isothermal amplification (LAMP) of DNA operates at a single temperature of 60–65 °C and it requires four primers (forward inner primer FIP, forward outer primer F3, backward inner primer BIP, and backward outer primer B3) that recognize six different regions in the sense and antisense strands of the target as well as a polymerase enzyme with strand displacement activity, thus circumventing the thermal denaturation. LAMP follows a two-step mechanism in which a symmetrical single-stranded dumbbell structure with two terminal loops is first generated that serves as the initiator of a cyclic amplification step. The four primers participate in the first step, while in the second one, only the inner primers are involved. Self-priming and elongation of the FIP and BIP 3′-ends induce strand displacement, unfolding of the hairpin and subsequent folding of the new strand. By repeating this process, long structured amplicons are obtained [18] (Figure 3a).

Besides its tolerance of intercalating and non-intercalating redox probes [19], LAMP is also capable of incorporating modified deoxyribonucleoside

Figure 2



Mechanism of the helicase-dependent amplification (HDA): (**a**) in solution, (**b**) on surface.

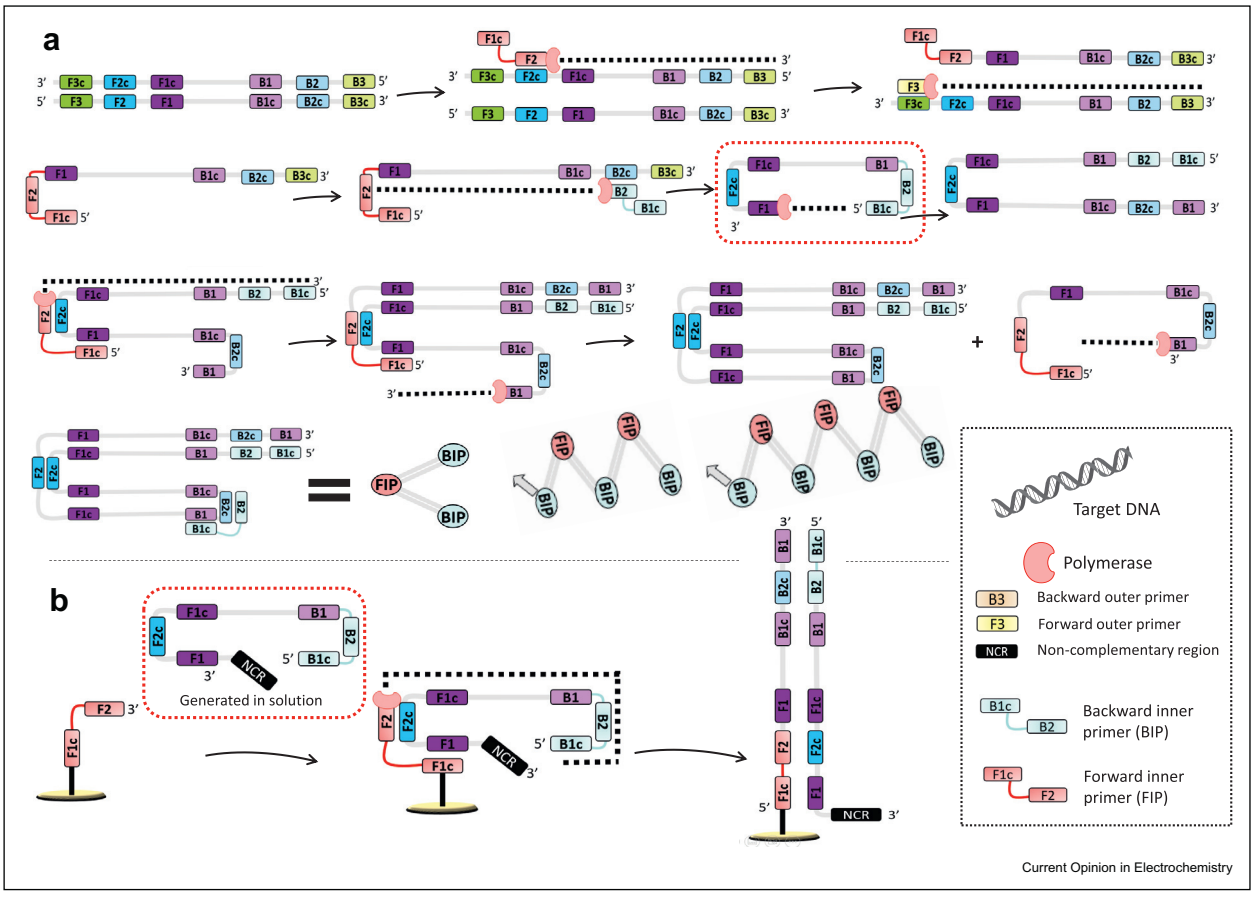
triphosphates [20*,21] that could be harnessed for electrochemical monitoring. LAMP products have been detected on an electrode surface by capturing the amplicons once the reaction has finished [22] or even as they are formed in solution [23,24], but solid–phase LAMP on electrochemical platforms has not reported so far. Conversely, on-surface LAMP has been very recently developed in combination with optical detection [25]. To surmount the challenges posed by its complex mechanism, two extra primers resulting from inner primer modification are incorporated, ModFIP and SSFIP. ModFIP possesses an oligonucleotide tail at its 3′-end that prevents self-priming and, in turn, blocks its extension. As a result, a primary product of shorter and defined size is formed in solution, and captured by SSFIP, the primer attached to the surface, for further elongation and detection (Figure 3b).

This scheme could be easily integrated into an electrochemical platform by, for example, replacing the fluorescent label incorporated at the 5′-terminus of conventional BIP by a redox compound, without excluding the possibility of monitoring the amplification by recording the changes in the electron-transfer rate of a redox probe in solution. In any case, similar to HDA, enzyme-extension of the LAMP primer anchored to the electrode demands a thermal stable surface chemistry.

Recombinase polymerase amplification

The RPA mechanism is inspired by the homologous recombination occurring in cells to repair mismatches or double-strand breaks in the DNA double helix [26,27]. In this method, thermal denaturation of the DNA duplexes is replaced by a DNA strand invasion mechanism catalyzed by a recombinase enzyme. This protein binds to the primers (ssDNA) and forms a complex that scans for the homologous sequence in the duplex DNA (template). Once the homology is located, the complex invades the dsDNA and triggers a strand exchange reaction assisted by a single-strand DNA-binding (SSB) protein that stabilizes the displaced DNA strand. Subsequently, the recombinase dissociates from the complex and the hybridized primers are elongated by a strand-displacing polymerase at 37 °C. The repetition of this process results in the exponential amplification of

Figure 3



Mechanism of the loop-mediated isothermal amplification (LAMP): (**a**) in solution, (**b**) on surface.

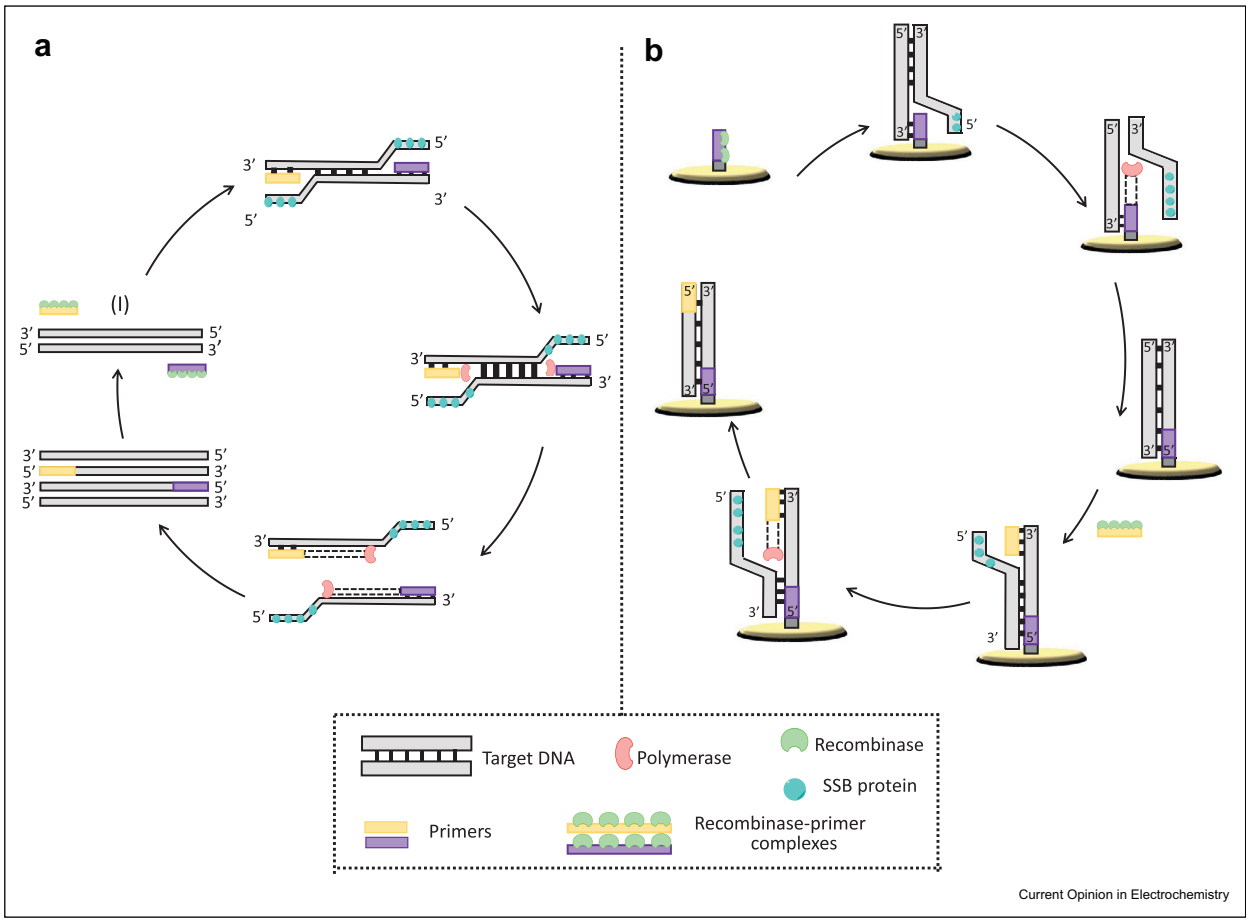
the sequence flanked by the pair of primers (Figure 4a). To get the maximum efficiency, primers of 30–35 nucleotides in length are recommended, although successful RPA amplification has been informed when using shorter primers.

Electrochemical transduction has been applied to the detection of RPA amplicons produced in solution and selectively captured with a complementary probe anchored to the electrode [28–30]. However, with the aim of attaining a molecular decentralized test, the implementation of RPA on a conductive surface results more appealing. The most common approach entails the immobilization of one primer onto the electrode, while the other primer is free in solution with the rest of the reagents. The mechanism of this totally asymmetric amplification strategy was elucidated (Figure 4b), excluding a simple entrapment onto the sensing surface of the products previously amplified in solution [31]. Unlike solid-phase PCR and HDA, the incorporation of both primers in the solution to shorten the template has been scarcely explored in RPA [32], presumably due to a higher prevalence of primer-dimer artifacts, as a consequence of a lower amplification temperature and longer primers.

Despite the above-mentioned drawback, the low operation temperature of RPA greatly expands the possibilities to construct the sensing layer. In particular, solid-phase RPA is compatible with the primer chemisorption onto gold electrodes, one of the easiest processes to fabricate biosensing platforms. When using this immobilization chemistry, the spacer incorporated in the grafted primer is generally a tail of thymines [33], the nucleobase with less affinity for gold.

Recently, in order to maximize the number of immobilized primer molecules with appropriate orientation and accessibility, 3D architectures as poly(amidoamine) dendrimers (PAMAM) have been evaluated as scaffolds for oligonucleotide coupling [34]. The typical approach based on the dendrimer film formation onto the solid support, followed by the covalent attachment of oligonucleotides was replaced by the immobilization of preformed dendrimer-primer hybrids, thus circumventing dendrimer aggregation [35]. Even though

Figure 4



Mechanism of the recombinase polymerase amplification (RPA): (**a**) in solution, (**b**) on surface.

developed on thermoplastic materials employed in microfluidic chips, this surface chemistry could be implemented in conductive surfaces [36].

Different strategies have been proposed for the electrochemical detection of the on-surface generated amplicons. The simplest one exploits the impedance changes in the electrode-solution interface in the presence of ferri/ferrocyanide added to the solution, thus resulting in a label-free strategy [37]. This method involves two impedance readouts, before and after DNA amplification. To reduce unspecific adsorption of RPA reagents onto the binary self-assembled monolayer (thiolated primer + 6-mercapto-1-hexanol onto AuNP-modified SPCEs), a washing step including Tween detergent is carried out. Upon 1 h of RPA at 25 °C, the detection of a DNA sequence specific of a plant virus at 1 pg/mL level (ca. 4×10^7 molecules in 15 µL) is achieved. An alternative label-free approach allows continuous monitoring (sampling time 5 min) of on-chip amplicon elongation by recording the decrease in the current intensity of ferricyanide free in solution arisen from electrostatic repulsions with DNA. This way, after 40 min of amplification at 37 °C, was possible to detect about 10^3 copies of a SARS-CoV-2 gene [38]. However, it is unclear if the reverse-thiolated immobilized primer is also added to the solution, and a second concern is that the approach has only been tested in synthetic DNA sequences.

Label-based strategies, however, have been more widely explored. The typical approach for amplicon labeling consists of using hapten-modified primers for subsequent incorporation of a redox enzyme. Because of its high turnover number, the enzyme peroxidase is particularly convenient, and generally attached through biotin-(strept)avidin interaction [31]. Alternatively, DT-diaphorase-tagged amplicons were generated on an ITO surface and electrochemically detected employing 1,4-naphthoquinone as the electron mediator [32].

Nevertheless, in order to incorporate multiple labels per amplicon molecule, thus enhancing its detectability, the use of modified nucleotides results more convenient. The RPA reaction accepts deoxyribonucleotides functionalized with haptens [39,40], redox-active molecules

Table 1

Main features of the enzyme-assisted nucleic acid amplification methods.

Parameter	PCR	HDA	LAMP	RPA
Operation temperature	Thermal cycling	65 °C	60–65 °C	37–41 °C
Number of primers	2	2	4 (at least)	2
Primer length	17–28	24–33	20–45	30–35
Software for primer design	PrimerBLAST and Primer3	Primer3	PrimerExplorer V5	PrimedRPA[a]
Main enzymes	Polymerase	Polymerase, helicase	Polymerase	Polymerase, recombinase
Amplicon size	variable	80–120	Concatenated DNA	Up to 1,5 kbp (ideally 100–200 bp)
Availability	Free of patent	Partially under patent	Free of patent	Partially under patent
Tolerance to inhibitors	Low	Moderate	Moderate	Moderate
Initial thermal denaturation	yes	yes	yes	no
Kinetics in solution	Exponential	Exponential	Exponential	Exponential
Strategy to avoid thermal cycling	none	Strand unwinding mechanism	Isothermal strand displacement	Strand invasion mechanism
Original reference	[47]	[48]	[49]	[50]

[a] PrimedRPA was implemented in Python 3 and supported on Linux and MacOS and is freely available from http://pathogenseq.lshtm.ac.uk/PrimedRPA.html [46].

[41], and even enzymes [42] whose activity is not affected at 37 °C, but the modified dNTPs/natural dNTPs ratio should be properly optimized.

RPA-based solid-phase primer elongation using ferrocene-label nucleotides (Fc-dNTPs) has been recently applied to the electrochemical detection of single-point mutations [41*]. In particular, four 5'thiol-modified primers whose base sequence differs only in the 3'terminal nucleobase, the mutation site, were chemisorbed onto different electrodes of an array, and subsequently incubated with the target DNA containing the mutation. This one hybridizes with the four surface-confined primers, but the extension only takes place on the electrode surface modified with the primer totally complementary. Prior to Fc detection by square wave voltammetry, an acidic treatment with glycine-HCl at pH 3 was carried out to, according to the authors, remove RPA reagents nonspecifically adhered, but probably equally important, to denature the rigid duplex and bring Fc moieties closer to the electrode surface. This methodology allowed the identification of single-point mutations at femtomolar level (13.3 fM or about $8x10^5$ copies of mutant DNA) in fingerpick blood samples after thermal lysis and minimum dilution. It implies an improvement of almost three orders of magnitude with respect to just on-solid primer elongation without RPA amplification [43].

Not yet implemented on conductive surfaces, alternative RPA-based approaches to detect single-point mutations have recently been reported. They explore different strategies to suppress replication of the wild-type allele, while promoting amplification of the mutant allele by DNA polymerase. One option is the so-called blocked-RPA, an adaptation of the blocked-PCR at lower and constant temperature. More specifically, a 3′-blocked oligonucleotide complementary to the wild-type sequence competes favorably with the primer, inhibiting its amplification [44]. Another alternative makes use of nuclease-dead forms of clustered regularly interspaced short palindromic repeats (CRISPR) ribonucleoprotein complexes as sequence-specific blockers, whose heat sensitivity makes them incompatible with PCR [45].

Partial substitution of dTTP by HRP-dTTP in the RPA amplification mixture was performed to obtain, tethered to a screen-printed gold electrode, a dsDNA product harboring several redox enzyme molecules. The expected signal amplification would offset the extra step of adding the enzyme substrates. This approach was applied to the detection of an antibiotic resistance gene in *E. coli*, and preliminary results revealed significant nonspecific interactions that render difficult the quantification [42].

Conclusion and outlook

Nucleic acid amplification is an essential tool in numerous fields, being PCR the leading option. Nevertheless, alternatives running at a constant temperature such as HDA, LAMP, and RPA can be more easily integrated into electrochemical platforms for the development of cost-effective and low-power handheld biosensing devices. In Table 1, a summary of the main features of the isothermal alternatives to standard PCR

discussed here is shown. All of them combine, at least, a DNA polymerase enzyme and a set of primers to initiate the replication that proceeds exponentially in a liquid phase. Unlike PCR, kinetics is not determined by thermal cycling but by the efficiency and processivity of the enzymes. In this regard, RPA is the fastest of the three [10], with LAMP and HDA showing similar speed. And although the criteria for primer design are dissimilar, there are dedicated software to aid in this task. In a strict sense, only RPA is a true isothermal method because HDA and LAMP require initial thermal denaturation. Recently, however, a new helicase with enhanced processivity and speed has been engineered, leading to an improved version of HDA named SHARP (SSB-helicase assisted rapid PCR) [51].

The integration onto an electrode surface has been accomplished in the case of HDA and RPA but, considering the recent findings [25], we envision that solid-phase electrochemical LAMP will be reported in the near future. On-surface isothermal amplification, although not necessarily exponential, is still competitive, being able to detect few copies of DNA in 40–90 min and move the multiplex detection closer to the point of need. In this context, the low working temperature makes RPA especially attractive, even for developing wearable tests [52]. However, it is also more prone to generating spurious nonspecific products, issue addressed in its variant SIBA (Strand Invasion-Based Amplification) by incorporation of an invasion oligonucleotide [53]. A general pending task is the integration of sample preparation and DNA extraction that depend on the matrix and the specific aim of the analysis. Notably, the evaluated isothermal amplification methods seem to be less affected by inhibitors than PCR.

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.

Data availability

Data will be made available on request.

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