

Electrochemical Platforms for Solid-Phase Isothermal Amplification and Detection of Bacterial Genome [†]

Raquel Sánchez-Salcedo ^{1,2,*}, Rebeca Miranda-Castro ^{1,2}, Noemí de-los-Santos-Álvarez ^{1,2},
María Jesús Lobo-Castañón ^{1,2}

¹ Departamento de Química Física y Analítica, Universidad de Oviedo. Av. Julián Clavería 8, 33006 Oviedo, Spain; mirandarebeca@uniovi.es (R.M.-C.); santosnoemi@uniovi.es (N.d.-l.-S.-Á.); mjlc@uniovi.es (M.J.L.-C.)

² Instituto de Investigación Sanitaria del Principado de Asturias. Av. de Roma, 33011 Oviedo, Spain

* Correspondence: sanchezraquel@uniovi.es

[†] Presented at the 1st International Electronic Conference on Biosensors, 2–17 November 2020; Available online: <https://iecb2020.sciforum.net/>.

Received: date; Accepted: date; Published: date

Abstract: In this work, two sensing approaches for the specific quantification of *Salmonella* genome are comparatively evaluated. These devices successfully integrate solid-phase isothermal HDA or RPA amplification in electrochemical platforms and support efficient detection of small amounts of the bacterial genome without thermal cycling, while using simple equipment. Detectability of the RPA-based sensing platform (10^5 genomes) was surpassed by that of the HDA-based one (10 genomes), though it could be enhanced by primer design optimization. This, together with a low operation temperature compatible with conventional surface chemistry and faster amplification make the RPA-based electrochemical platform a promising device for point-of-need analysis.

Keywords: electrochemical biosensing; bacterial genome detection; on-surface DNA amplification; helicase dependent amplification; recombinase polymerase amplification

1. Introduction

Currently, there is an increasing demand for rapid and sensitive methods for the quantification of bacterial pathogen in very diverse areas, such as clinical diagnosis, environmental monitoring and food safety. Molecular methods based on the amplification of a specific sequence of the bacterium genome by the polymerase chain reaction (PCR) are widely used. Unfortunately, these methods are usually limited to centralized laboratories. Electrochemical DNA genosensors satisfy most of the ASSURED criteria outlined by the WHO [1] for an ideal point-of-need molecular test. Although, they usually require a previous amplification of the target sequence to achieve the extremely low detection limits that are demanded. Thus, the development of on-surface isothermal amplification devices, integrating DNA amplification, hybridization and detection on the same platform would be a very convenient approach. Unlike PCR, isothermal nucleic acid amplification methods occur at a single temperature throughout the entire reaction, thus facilitating the development of simple, easy-to-use, and miniaturized devices for on-site DNA analysis [2].

In this work, we present a comparative evaluation of two different electrochemical platforms for *Salmonella* genome quantification recently developed in our research group. They were fabricated using either indium-tin oxide surfaces in combination with helicase dependent amplification (HDA) [3] or gold surfaces and recombinase polymerase amplification (RPA) [4]. It may be of general utility in the design of genosensors for detecting other bacteria of public health interest.

2. Materials and Methods

2.1. Reagents

RPA TwistAmp® Basic kit and TwistAmp® Liquid Basic kit were purchased from TwistDx Ltd. (Cambridge, United Kingdom). HDA IsoAmp® II kit was obtained from BioHelix (Beverly, CA, USA). *Salmonella enterica* subsp. *enterica* genomic DNA (CECT878) was acquired from Spanish Type Culture Collection (Valencia, Spain).

2.2. Instrumentation

Electrochemical measurements were performed with a three-electrode electrochemical cell driven by an Autolab PGSTAT 12 potentiostat controlled with the software NOVA 2.1 (Eco Chemie B.V., Utrecht, The Netherlands). All the potentials are referred to the Ag|AgCl|KCl reference electrode.

The working electrode in the detection of the HDA products was an ITO surface defined by a polycarbonate mask and a Cu strip for electrical contact. The electrochemical cell is completed with the reference electrode and a stainless-steel counter electrode inside a syringe, placed on the top of the cell.

The detection of RPA products was carried out on chips with 16 independent electrochemical cells defined by a polycarbonate mask. Each cell consists of a gold working electrode surrounded by a gold counter electrode. The reference electrode is disposed on top of each cell. Moreover, a MUX.MULTI4 multiplexor module (Metrohm Autolab B.V., Madrid, Spain) along with a DRP-CAC8X connector, (Metrohm-DropSens, Oviedo, Spain) allowed conducting eight electrochemical measurements sequentially.

Isothermal amplifications were conducted in an oven Heraus VT6025 (± 0.1 °C).

2.3. Sensing Surface Construction onto ITO

Indium-tin oxide coated glass slides were initially subjected to an oxidation treatment with a solution of 1:1:5 (*v/v*) H₂O₂/NH₄OH/H₂O for 1 h. The resulting hydroxylated surfaces were then silanized with 1% (3-aminopropyl)triethoxysilane (APTES) in absolute ethanol at RT overnight. Subsequently, a heterobifunctional reagent, sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) was used to connect the amine-modified platform with the thiolated oligonucleotide (i.e.: the reverse primer). For that, the silanized ITO surfaces were incubated with 2 mg/dL sulfo-SMCC protected from light for 1 h. Finally, the thiolated reverse primer was linked to the maleimide-functionalized surface by incubation with 10 μ M oligonucleotide for 2 h.

2.4. Sensing Surface Construction onto Gold

A binary thiol-based self-assembled monolayer was built onto the gold surfaces in two steps. First, a 1 μ M solution of the thiolated reverse primer was incubated at 4 °C overnight. Then, the free gold surface was blocked with the aromatic thiol *p*-aminothiophenol (1 mM) for 50 min at RT.

3. Results and Discussion

For the detection of human pathogenic *Salmonella* spp. through its genetic material, an 86-mer specific sequence of the regulatory gene *bipA* (GenBank AD006468.2) was selected as the target [5]. To achieve the sensitivity levels demanded, two different nucleic acid amplification techniques were evaluated as isothermal alternatives to PCR: helicase dependent amplification (HDA) and recombinase polymerase amplification (RPA). Both techniques require a set of primers that flank the intended target and are elongated by a suitable polymerase following exponential kinetics. The main difference lies on the strategy to circumvent the thermal denaturation of the DNA duplexes performed in PCR for subsequent annealing of the primers. Specifically, HDA uses a helicase for dsDNA unwinding along with a single-strand DNA-binding (SSB) protein to stabilize the resulting

ssDNA. Then, primers hybridization and elongation proceed at a fixed temperature of 65 °C [6]. For its part, RPA entails the formation of complexes between the primers and the recombinase that scan for homologous sequences. Once these are identified, a strand displacement occurs with the assistance of the SSB protein, and the elongation of the inserted primers proceeds optimally at 37 °C [7].

For the implementation of isothermal *Salmonella* DNA amplification onto the electrode surface, the thiolated reverse primer was immobilized through its 5'-end, because polymerases incorporate the nucleotides to the 3'-OH group via a phosphodiester bond, including a poly T spacer to minimize steric hindrance of the surface. In the case of HDA, the primer was covalently attached to an activated ITO surface, while the RPA was conducted with the primer chemisorbed onto gold chips. The on-surface generated amplicons were subsequently labeled with a redox enzyme that catalyzes the formation of a redox species that is electrochemically detected and whose concentration is directly proportional to the genome copies present in the sample.

3.1. On-surface HDA

Successful solid-phase HDA of *Salmonella* genome was accomplished when including in the solution a small quantity of a trimmed version of the immobilized reverse primer along with the forward primer modified with 6-carboxyfluorescein (6-FAM) in a 1:15 molar ratio. This way, amplification would start in solution and occur until the limiting primer is consumed, giving rise to an 86 bp product. This one would then serve as the template for heterogeneous amplification boosted by the longer immobilized primer. The 6-FAM duplexes generated onto the ITO surface were electrochemically detected by differential pulse voltammetry (DPV) after tagging with the enzyme alkaline phosphatase. Under these conditions, 10 *Salmonella* genomes were reliably detected, which is in line with the detectability of real-time PCR approaches, with a reproducibility of 20%. The mechanistic proposal for on-surface HDA (**Error! Reference source not found.**A) is underpinned by an observed delay in the amplicon detection (a minimum of 90 min was required) as well as the presence of two different regions in the response curve.

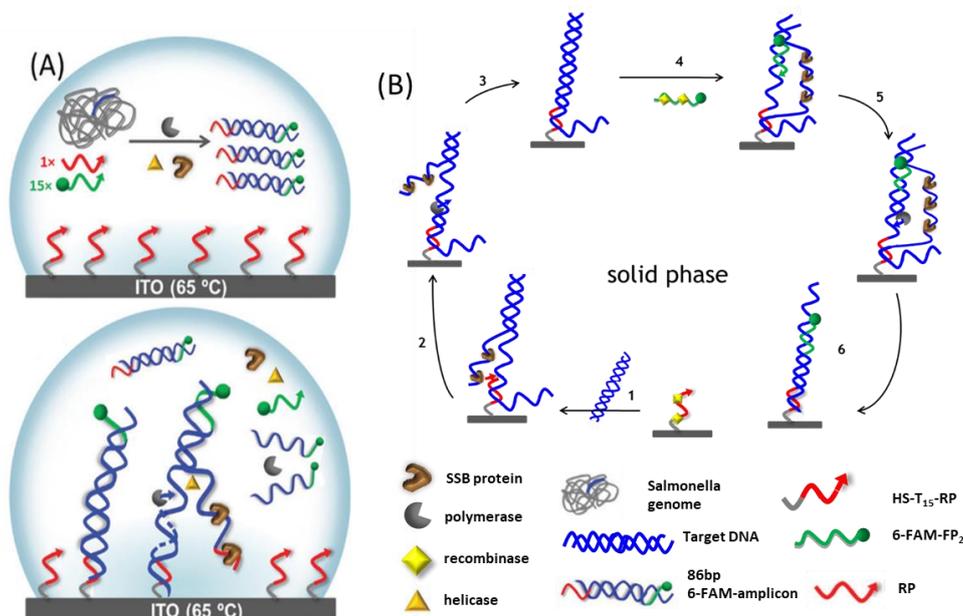


Figure 1. (A) Mechanism of the on-surface helicase-dependent amplification. Top: the first stage involves amplification in solution, the target genome is shortened. Bottom: the second stage is the elongation of the anchored primer. Reprinted with permission from [3]. (B) Overview of the solid-phase RPA on gold surface. Reprinted with permission from [4].

3.2. On-Surface RPA

The solid-phase RPA of *Salmonella* DNA was completed in a totally asymmetric way, meaning that solely the 6-FAM modified forward primer is present in solution. The proposed mechanism involves the hybridization between the anchored reverse primer and the pathogen genome, facilitated by the recombinase, for subsequent extension of the primer by the polymerase. The resulting product then acts as the template for on-surface elongation of the 6-FAM forward primer (**Error! Reference source not found.**B). Finally, upon labeling with the enzyme peroxidase, the immobilized 6-FAM amplicons were indirectly quantified by chronoamperometry. The possibility of a mere entrapment of the amplicons generated in solution onto the sensing surface was excluded since the substitution of the anchored reverse primer by its counterpart blocked at the 3'-end (i.e., unable to be elongated) gave rise to signals equivalent to those recorded in the absence of *Salmonella* genome.

This method led to a detection limit of 10^5 genomes after 30 min of reaction time. Likewise, it exhibited an excellent selectivity when challenging with *Legionella pneumophila*, another human pathogenic bacterium.

3.3. Comparative Evaluation of the Performance of the Sensing Platforms

We have designed and developed two hybridization-based platforms for electrochemical detection of *Salmonella* DNA that integrate an amplification reaction, either HDA or RPA, occurring at a constant temperature. A set of primers designed for PCR amplification [5] were slightly modified for on-solid HDA, to satisfy primer length recommendations, while directly used for solid-phase RPA.

In the case of on-solid HDA amplification, the reverse primer was immobilized onto ITO surfaces through a covalent bond that is stable at 65 °C, the optimal temperature of HDA. Although, this attachment entails a tedious multiple-step process. Conversely, RPA takes place at 37 °C, therefore compatible with reverse primer chemisorption onto gold surfaces, favoring an easier process to fabricate the sensing layer.

The enzyme selected for labeling the RPA products was peroxidase, whose enzymatic product, the oxidized TMB, can be easily detected by chronoamperometry at 0 V, thus resulting in a simple and fast detection step. This strategy could not be applied to detect HDA products generated onto ITO surface because the reduction of the enzymatic product onto ITO is very suppressed and exhibits a large overpotential. Consequently, alkaline phosphate was selected instead, and its enzymatic product 1-naphthol was detected by DPV. However, a particular strength of ITO surfaces is their optical transparency that allows for optical detection as well.

Under optimized conditions, the HDA-based platform is able to detect as few as 10 *Salmonella* genomes in 90 min, while the RPA-based platform distinguishes down to 10^5 genomic units in 30 min. This modest detectability could be attributed to the employment of suboptimal primers from a PCR design, consequently, better sensitivity could be gained with an improved primer design. Additionally, intrinsic characteristics of RPA such as its low operation temperature and fast amplification kinetics make RPA particularly suitable for point-of-need analysis.

Regarding storage stability of the sensing surfaces, the modified ITO platforms were stable for 9 months under dry storage conditions when, prior to storage, a washing step with a solution containing glucose and BSA was performed. The same protocol applied to the modified gold surfaces showed a good stability at least during one month [8].

Funding: This research was funded by the Spanish Government (grant number RTI-2018-095756-B-I00) and Principado de Asturias Government (grant number IDI/2018/000217) co-financed by FEDER funds.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wu, G.; Zaman, M.H. Low-cost tools for diagnosing and monitoring HIV infection in low-resource settings. *Bull. World Health Organ.* **2012**, *90*, 914–920.
2. Obande, G.A.; Singh, K.K.B. Current and future perspectives on isothermal nucleic acid amplification technologies for diagnosing infections. *Infect. Drug Resist.* **2020**, *13*, 455–483, doi:10.2147/IDR.S217571.
3. Barreda-García, S.; Miranda-Castro, R.; de-los-Santos-Álvarez, N.; Miranda-Ordieres, A.J.; Lobo-Castañón, M.J. Solid-phase helicase dependent amplification and electrochemical detection of: Salmonella on highly stable oligonucleotide-modified ITO electrodes. *Chem. Commun.* **2017**, *53*, 9721–9724, doi:10.1039/c7cc05128j.
4. Sánchez-Salcedo, R.; Miranda-Castro, R.; de-los-Santos-Álvarez, N.; Lobo-Castañón, M.J. On-gold recombinase polymerase primer elongation for electrochemical detection of bacterial genome: Mechanism insights and influencing factors. *ChemElectroChem* **2019**, *6*, 793–800, doi:10.1002/celec.201801208.
5. Calvó, L.; Martínez-Planells, A.; Pardos-Bosch, J.; Garcia-Gil, L.J. A new Real-Time PCR assay for the specific detection of Salmonella spp. Targeting the bipA gene. *Food Anal. Methods* **2008**, *1*, 236–242, doi:10.1007/s12161-007-9008-x.
6. Vincent, M.; Xu, Y.; Kong, H. Helicase-dependent isothermal DNA amplification. *EMBO Rep.* **2004**, *5*, 795–800, doi:10.1038/sj.embor.7400200.
7. Piepenburg, O.; Williams, C.H.; Stemple, D.L.; Armes, N.A. DNA detection using recombination proteins. *PLoS Biol.* **2006**, *4*, 1115–1121, doi:10.1371/journal.pbio.0040204.
8. Miranda-Castro, R.; Sánchez-Salcedo, R.; Suárez-Álvarez, B.; de-los-Santos-Álvarez, N.; Miranda-Ordieres, A.J.; Lobo-Castañón, M.J. Thioaromatic DNA monolayers for target-amplification-free electrochemical sensing of environmental pathogenic bacteria. *Biosens. Bioelectron.* **2017**, *92*, 162–170, doi:10.1016/j.bios.2017.02.017.

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



© 2020 by the authors. Submitted for possible open access publication under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).