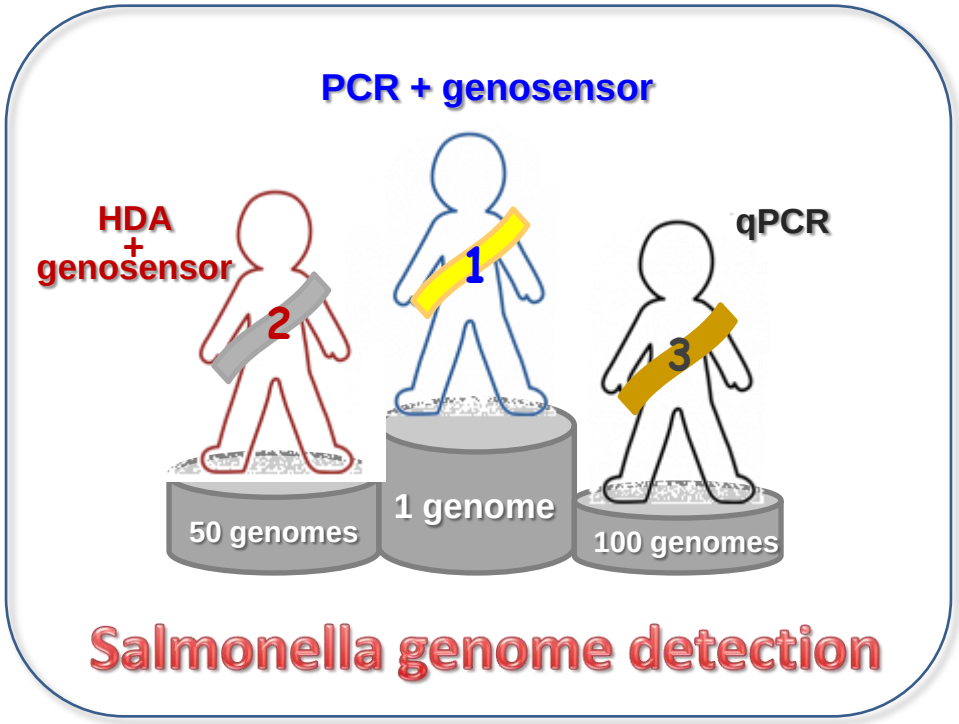
Graphical Abstract (for review)



Highlights

- New insights into rational designing electrochemical genosensors for sequence-specific detection of enzymatic nucleic acid amplification products.
- Rules for the adaptation of PCR primers to isothermal helicase dependent amplification facilitating its adoption for point-of-need applications.
- Electrochemical genosensor paired to PCR achieves single copy detection of *Salmonella* genome
- Under isothermal conditions, helicase dependent amplification-electrochemical genosensor improves in 2-fold the detectability of real-time PCR

Sequence-specific electrochemical detection of enzymatic amplification products of *Salmonella* genome on ITO electrodes improves pathogen detection to the single copy level

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Abstract

Real-time PCR is the gold standard technique for the unambiguous detection of sequence-specific fragments of pathogen DNA in many fields but it not always fulfills the extremely low detectability legislation demands. Herein we demonstrate that rationally designed genosensors can rival or even supersede it when combined with previous amplification strategies even isothermal ones. Among the latter, helicase-dependent amplification (HDA) is a simple and powerful DNA amplification using helicase instead of temperature cycles to unwind DNA; it follows an amplification scheme very similar to PCR, which could facilitate its widespread adoption for point-of-need applications. Unfortunately, the primer design for HDA is more restrictive than for PCR. We show that it is possible to adapt PCR primers to isothermal amplification of a DNA sequence specific for *Salmonella*. We design an electrochemical DNA sensor, based on a sandwich hybridization assay on the surface of indium-tin oxide electrodes, which allows monitoring both PCR and HDA amplicons. The resulting sequence-specific detection strategy not only increased the selectivity of the detection but also its sensitivity, improving real-time PCR in two-fold and a hundred-fold when combined with HDA and PCR, respectively, achieving the single molecule detection.

Keywords

Electrochemical biosensor; Helicase; Isothermal amplification; Salmonella detection

1. Introduction

Foodborne illness outbreaks are reported with increasing frequency, becoming an important health problem worldwide. In this context, the capability to rapidly and reliably detect foodborne pathogens plays a crucial role for safeguarding the food supply and protecting consumers. Traditional microbiological culture methods, which provide adequate target selectivity and sensitivity, are the internationally accepted ones. However, the time to results of these methods is on the order of days. A number of molecular methods based on the detection of intracellular molecules, such as proteins and nucleic acid sequences specific for the target organism, have been reported to reduce the analysis time [1].

Genetic detection of pathogens is mainly accomplished by the polymerase chain reaction (PCR). Taking *Salmonella spp.* as a model, which is one of the leading causes of food-borne infections and outbreaks [2], an increasing number of real-time PCR assays are commercially available for its detection in environmental and food samples [3]. Most of these assays are based on monitoring the amplification of the targeted nucleic acid sequence specific for *Salmonella,* by using a dye that once intercalated with any double-stranded DNA (dsDNA) becomes fluorescent. These methods are able to detect the presence or absence of amplicons in real-time but provide no information about the amplified product. In consequence, they are prone to false positive results, in the sense that undesired products such as primer dimers and other spurious amplicons can increase the signal. To improve detectability, it is desirable to use specific methods for the detection of the amplification products.

On the other hand, new amplification methods, alternative to PCR, are needed to make the routine testing for nucleic acid cost-effective and practical for mainstream applications. Different isothermal nucleic acid amplification methods have been developed for the detection of *Salmonella* such as loop-mediated isothermal amplification (LAMP) [4-7], isothermal target and probe amplification (iTPA) [8], recombinase polymerase amplification (RPA) [9] and helicase-dependent amplification (HDA) [10]. However, these approaches pose a '*de novo'* design of primers, targeting a different region of *Salmonella* specific genes from the well-known PCR assays, and provide no opportunity for adopting sequence-specific detection methods previously developed for PCR amplification.

Here, we adapt a PCR design to isothermal helicase dependent amplification, providing a benchmark for designing primers for HDA starting from previously developed PCR methods, which could facilitate the widespread adoption of HDA for pathogen detection. Furthermore, we develop a sequence-specific hybridization sensor for monitoring the amplification products. The sensor is constructed on silanized indium-tin oxide (ITO) electrodes modified with thiolated oligonucleotides acting as a capture probe. ITO electrodes provide a conductive and transparent surface that can be easily covalently modified with oligonucleotides. Consequently they may produce an electrochemical an optical output in response to target binding. Compared with gold-surfaces modified with self-assembled monolayers of thiol-modified oligonucleotides, the most tested transducers for hybridization-based sensors [11], they present improved thermal and long-term stability [12]. We prove that these ITO-sensors can be applied to unpurified dsDNA, and allows differentiating the specific amplification products from shorter, spurious amplicons, improving the detectability of enzymatic nucleic acid amplification reactions. Using this method we detected only 1 molecule of *Salmonella* genomic DNA, which we took as a model target.

2. Experimental

2.1. Reagents

(3-aminopropyl)triethoxysilane (APTES), 10× phosphate buffered (10× PBS), 20× saline sodium phosphate-EDTA (20× SSPE) pH 7.4, trizma hydrochloride solution (1 M Tris-HCl) pH 7.4, magnesium chloride solution (1 M), bovine serum albumin (BSA), 1-naphthyl phosphate (1-NPP), 3,3´,5,5´-tetramethylbenzidine (TMB) in a ready-to-use reagent format, hydroquinone, 1-naphthol and hexaammineruthenium (III) chloride were purchased from Sigma-Aldrich (Spain). 1% casein buffer in 1×PBS, and sulfo-succinimidy-l-4-(N-maleimidomethyl)cyclohexane-1-carbonate (Sulfo-SMCC) were obtained from Thermo Scientific (Spain). Anti-fluorescein-alkaline phosphatase Fab fragment (antiFITC-AP) was received from Roche diagnostics (Spain). Absolute ethanol was purchased from Chemlabor (Spain). Water was purified with a Milli-Q system (Millipore, Spain). All other reagents were of analytical grade.

Oligonucleotide sequences were obtained purified by HPLC from Laboratorios Conda (Spain) and their sequences are shown in Table 1. All stock solutions were prepared in Milli-Q water and stored at -20 °C.

2.2. Enzymatic amplification protocols

Genomic DNA of *Salmonella enterica* subsp. *enterica* was obtained from Spanish Type Culture Collection, Valencia, Spain (CECT 878). 5 µg of the lyophilized genome were dissolved in Milli-Q water to obtain 5×10^6 genomic units per microliter (GU/µL), which was aliquoted in small volumes and stored at 4 ºC.

PCR. *Salmonella* genome was amplified using the IMMOLASE™ DNA Polymerase kit (Bioline, Spain). The reaction mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 300 nM forward and reverse primers, 1 U Immolase™ DNA polymerase, 2.5 µL of template DNA (*Salmonella* genome) and nuclease-free water up to a total volume of 20 µL. A negative control containing water instead of DNA was included in each series. PCR amplification conditions were 10 min at 95 °C, then 40 cycles composed by 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, and a last step at 72 °C for 7 min.

To generate the *Salmonella* target used as standard for the electrochemical hybridization assay, the 86 bp PCR product was purified with MinElute PCR purification kit (Qiagen) and quantified spectrophotometrically at 260 nm (GENESYS™ 10S UV-vis Spectrophotometer, ThermoScientific).

For real-time monitoring of the PCR amplicons, reactions were carried using the same protocol. 1.25 µL 20× Evagreen (VWR, Spain) and 0.5 µL 50× ROX (Thermo Scientific) were added instead of an equivalent volume of nuclease-free water and reactions were performed in a HT7900 real-time thermocycler (Applied Biosystems®).

Helicase dependent amplification. *Salmonella* genome was amplified using the IsoAmp® II kit (BioHelix, Beverly, USA) with a two-step protocol. Two different reagents mixtures were employed. The mix A contained 1× annealing buffer, 75 nM forward and reverse primers, 2.5 µL of template DNA (*Salmonella* genome) and nuclease-free water up to 12.5 µL. The mix B contained 1×annealing buffer, 3 mM $MgSO_4$, 30 mM NaCl, 1.75 µL of IsoAmp dNTP solution, 1.75 µL of IsoAmp enzymes mixture and nuclease-free water up to 12.5 µL. Mix A was incubated at 95 ºC for 2 min

and at 65 ºC for another 5 min. Mix B was then added and the final mixture (25 µL) was incubated in an oven at 65 ºC for 75 min (unless otherwise stated).

2.3. Sensing Phase Preparation

Indium tin oxide coated glass slides were obtained from Sigma-Aldrich (Spain). These substrates were cut into pieces of about 8×5 mm and cleaned with acetone, ethanol and water. Then they were immersed in 1 M HCl for 10 min followed by rinsing with water. These pieces were treated with a solution of 1:1:5 (v/v) $H_2O_2/NH_4OH/H_2O$ during 1 h for oxidizing the surface. After washing thoroughly with water and drying under a stream of nitrogen, they were silanized with 1% APTES in absolute ethanol at room temperature (RT) overnight. The modified surfaces (thoroughly rinsed with ethanol and water and dried under a stream of nitrogen) were taped to a copper strip, which provides the electrical contact, defining a 2 mm diameter electrode surface with a Teflon adhesive tape. To form the sensing phase, a thiolated capture probe was bound to the silanized platform with a heterobifunctional reagent, sulfo-SMCC. Thus, silanized ITO surfaces were incubated in 2 mg/mL sulfo-SMCC prepared in crosslinking buffer (CB; 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, 0.15 M sodium chloride and 10 mM EDTA adjusted to pH 7.2) for 1 h at RT and protected from light. After rinsing with CB and drying with nitrogen, the resulting maleimide-modified surface was linked to the oligonucleotide by incubating 10 µM of the thiol-capture probe (CP) in CB for 2 h. Finally, the sensing phase was washed with CB and water.

2.3. Electrochemical measurement

PCR purified amplicons, and unpurified HDA and PCR products were analyzed by a sandwich-hybridization assay on the ITO sensing phase. Firstly, 25 µL of hybridization buffer (2× SSPE, pH 7.4; prepared by tenfold dilution of 20× SSPE) containing 2 µM signaling probe (SP) and different concentrations of 86 bp *Salmonella* target were heated at 98 ˚C for 5 min and cooled down in ice-water bath for 5 min. After bringing the mixture to room temperature, 25 µL of 5 % BSA solution in hybridization buffer were added. 6 µL of this mixture were then placed onto the modified ITO electrode for 2 h (RT, in darkness). Once the surface was washed with 1× PBS solution, the electrode was covered with 6 µL of casein buffer (0.5% casein in 1× PBS) containing 0.5 U/mL of the antiFITC-AP conjugate for 30 min (RT, in darkness). Then, the surface was washed with AP buffer (0.5 M Tris-HCl, pH 9.8, 1 mM $MgCl_2$) and the electrode was immersed in a micro-cell containing 400 µL of 4 mM 1-NPP in the AP buffer. After 10 min,

differential pulse voltammetry (DPV) was recorded over a potential range from 0 to 0.8 V (modulation amplitude 50 mV, scan rate 10 mV/s). The measurements were performed at RT using an Autolab potentiostat (EcoChemie, The Netherlands), with a platinum counter electrode and a $Ag \mid AgCl \mid KCl$ saturated reference electrode.

3. Results and discussion

3.1. Target selection and genosensor architecture

The design of a molecular assay based on the detection of a genetic sequence characteristic of the pathogenic species begins with the selection, within its genome, of the DNA sequence that will act as a target. Most PCR- assays for *Salmonella spp.* are directed towards one of the genes involved in the virulence and infectivity of the pathogen, the gene *invA* [13-15]. However, this gene is lacking in some strains, for example in *Salmonella* serovar Saint Paul, thus presenting inclusivity problems. In addition, like other virulence genes, it is affected by strong variability caused by silent mutations [16, 17]. This high number of mutations may be a source of non-specificity that results in false negatives, since primers sequence is constant while target is variable. Regulatory genes are less subject to spontaneous silent mutations [18]. The *bipA* or *typA* gene (GenBank accession number AE006468.2) [19], which is necessary to ensure bacterial survival and successful host invasion [20], is one of these regulatory genes. Specifically, the sequence comprised between bases 4218770 and 4218855 of the *bipA* gene (Table 1), with a practically null variability in the terminal regions [18], was selected as a target, using these regions to define the primers (Table 1), according to a previously developed method [18].

To test the robustness and overall performance of the described system, we evaluated the real-time PCR amplification of the *Salmonella* genome with the defined primers and Evagreen dye as a non-sequence specific DNA intercalator. The experiment was performed in the range from 10^2 to 10^6 GU of *Salmonella*, including a negative control (no bacterial DNA) (Figure S1A). There is a strong correlation between the PCR cycle number needed to achieve an established fluorescence intensity threshold, i.e. the threshold cycle (C_t), and the logarithm of the initial number of genome across the entire range investigated. The efficiency of the PCR amplification, calculated from the slope of this relationship using the expression $(10^{-1/slope} -1) \times 100$, was 93%, which is acceptable [21]. The detection limit was 100 GU, with a linear dynamic range covering five orders of magnitude. The minimum number of DNA copies that can be detected with

reasonable certainty is limited by the appearance of spurious amplicons, which increases the fluorescence at a number of PCR cycles higher than 32 (C_t for the negative control). To obtain information about the differences between these products and the specific ones, we analyzed the corresponding melt curves (Figure S1B). In both cases, we observed a single peak, but the melting temperature for the negative control was lower than that of the specific target, 78 and 86 ˚C respectively, indicating that the non-specific sequence is smaller than the 86 bp expected amplicon. The effect of these template-independent products can be reduced using more specific methods of detection that probe the amplification products, resulting in a more sensitive method.

Towards the goal of sequence specific detection of the amplification products, we have developed an electrochemical sandwich genosensor (Scheme 1). The sensing platform comprises an oligonucleotide capture probe attached to an ITO electrode, and a fluorescein-labelled signaling probe, which are complementary to different fragments of the selected target. Upon the addition of the target, the probes form a rigid duplex structure on the electrode surface, with a terminal fluorescein molecule, which is used for anchoring a Fab fragment of anti-fluorescein conjugated to alkaline phosphatase. The subsequent addition of a suitable enzyme substrate gives rise to a product electrochemically active, which is easily measured on the electrode surface when the sensor is interrogated using differential pulse voltammetry.

Preferred place for **Scheme 1**

When combining nucleic acid amplification systems with hybridization assays, a series of guidelines must be followed in the design of the capture (CP) and signaling (SP) probes to avoid the detection of the amplification byproducts by the genosensor and thus ensure the extra selectivity provided by the hybridization [22]. The primers should not be contained in the specific target sequence recognized by the genosensor, but flank it, especially the primer defining the proximal end to the electrode surface. Moreover, it is favorable to use the capture probe labeled at its 5’ end and the signaling probe at its 3’ end. The *in silico* analysis of the secondary structure of the different sequences, obtained using the Mfold program at 25 ˚C and 0.298 M sodium cation (saline concentration of the hybridization buffer) [23], guided the design. As shown in Figure S2A, the sequence complementary to the target has a very stable secondary structure (Gibbs free energy, ΔG=-11.69 kcal/mol). To reduce this structure and prevent the unconsumed reverse

primer from competing with the capture probe for its binding to the target, we decided to remove the first 15 bases of the 3' end of the target (5’ end of the complementary sequence) from the recognition. The resulting 71 bases sequence (Figure S2B) was then divided into two fragments, one for the capture and one for the signaling probe, attempting to disrupt its strong secondary structure (ΔG=-10.09 kcal/mol). The 25 nucleotides from the 5' end, with only a small loop in the secondary structure (ΔG=-2.06 kcal/mol, Figure S2C) was selected as the capture probe. The secondary structure of the remaining 46 bases is considerable (ΔG=-8.00 kcal/mol) to be used as a signaling probe as it could hinder hybridization (Figure S2D). Therefore, this last oligonucleotide was shortened in seven bases at its 3' end, resulting in a 39 bases long sequence with a twice less stable secondary structure (Figure S2E), which was selected as the signaling probe (Table 1).

3.2. Optimizing the genosensor performance.

We have previously described DNA-modified ITO electrodes as platforms with strikingly high thermal and storage stability for DNA detection [12]. However, the electrochemical behavior of typical enzymatic products from enzyme labels is not well characterized at these surfaces. To select the most suitable enzyme/substrate couple we explored the voltammetric characteristics of the products enzymatically generated from the most commonly reported substrates for peroxidase (POD) and alkaline phosphatase (AP), which are the enzymes conjugated to the anti-fluorescein Fab fragment commercially available, at silanized ITO electrodes. Cyclic voltammograms (CVs) of tetramethylbenzidine (TMB) and hydroquinone (HQ), substrates for POD, which catalyzes their oxidation by hydrogen peroxide to give products identical to those obtained electrochemically, revealed that the electron transfer is slow at this interface (Figure S3). TMB exhibits an oxidation peak at 0.4 V and the reduction one at 0.2 V, but the cathodic current is especially suppressed, which would reduce the sensitivity of the genosensor. On the other hand, the hydroquinone/1,4-benzoquinone (1,4-BQ) redox process is highly irreversible, with extreme anodic and cathodic peak potentials, higher than 0.6 V and -0.4 V respectively, which are not convenient for genosensor detection. In contrast to the above, 1-naphthol exhibits an irreversible oxidation process at 0.4 V with the highest signal. Therefore, the most suitable enzyme/substrate system for the genosensor design is the one formed by AP/1-naphthylphosphate, which was selected for later work.

The amount of CP on the electrode surface may affect both the sensor response and reproducibility. Therefore, we next investigated the effect of changing the concentration of CP employed during sensor fabrication (1 and 10 μM) and the conditions of the immobilization reaction (2 hours at RT and overnight at 4 °C). The change in the reaction time did not produce any significant change in the sensor response, whereas the increase in the CP concentration improved the reproducibility of the assay. In addition, no significant changes were obtained in either the signal or the blank by blocking with mercaptohexanol the maleimide groups of the crosslinking reagent that remained free after immobilizing the CP, so it was decided not to block the modified surface.

Under the optimized conditions, the surface concentration of CP-immobilized on the ITO electrodes was quantified by chronocoulometry in the presence of hexaammineruthenium (III) (Figure S4). This trivalent cation electrostatically interacted with the negatively charged DNA phosphate groups in the ratio 1:3, and its amount on the surface was electrochemically measured [24], thus providing an additional evidence of the immobilization of the CP. The estimated DNA surface coverage, 2.5×10^{12} molecules/cm^2, is about 2-fold higher than that achieved with the conventional mixed self-assembled monolayers of mercaptohexanol and thiol-oligonucleotides on gold electrodes [25].

Using dsDNA as the target, the extent to which the SP displaces the target complementary sequence in the first hybridization step is critical. To achieve this, two different strategies have been explored: 1) the addition of 2.5% of BSA to the hybridization buffer, performing the reaction at room temperature for 30 min [22, 25], and 2) a thermal treatment of the hybridization mixture at 98 ° C for 5 min and then cooling it rapidly on ice [26, 27]. We find that the first of these strategies, the incorporation of BSA in the hybridization medium, does not lead to signals different from the negative control, even at concentrations 100 nM of target and 1 μM of signaling probe. In contrast, the thermal shock leads to a signal to blank ratio of 21 for the same target and SP concentrations. This presumably occurs because of the self-hybridization of the *Salmonella* target involves the region at which the signaling probe hybridizes (Figure S5), whereas in previously described systems [22] the signaling probe interacts with a large number of bases that are not part of the secondary structure of the target, facilitating the process.

Heterogeneous hybridization requires longer reaction times compared to the homogeneous one because the kinetics of hybridization on solid surfaces is slower since the accessibility to the immobilized DNA probes is smaller [28]. This is seen by comparing the signal from the negative control and that obtained for 100 nM of target at different hybridization times. Whereas the negative control was not affected by the time of heterogeneous hybridization, the current intensity obtained for 100 nM of target was 154 ± 42 and 207 ± 28 nA for 1 and 2 hours, respectively. Therefore, higher signal and better reproducibility were obtained for the longest incubation period. We presume this effect occurs for two reasons. First, as we used dsDNA as a target, hybridization with SP and CP on surface requires opening the dsDNA target, which is unfavorable because the double-stranded sequence of the amplicon has 86 base pairs and the surface ternary duplex only 64. In order to avoid as far as possible the zipper effect, both probes are in excess [22]. Second, the strong secondary structure of the target makes the surface reaction more difficult, forcing hybridization times to be extended as recently demonstrated [26].

Finally, another critical step in the operation with the sensor is the development of the enzymatic reaction as a previous step to the well-established voltammetric detection of 1-naphthol. The time and temperature of the enzymatic reaction affect the sensor response. We observed that increasing the reaction time (from 2 to 10 min), both the negative control and the signal increased at either 30 or 37 °C. However, the effect is not the same on signal and blank, and the signal-to-blank ratio reaches a maximum value at 30 °C and 5 min of reaction (Figure S6), which were selected thereafter.

3.3. Analytical performance of the genosensor

We verified the use of the peak current of the differential pulse voltammograms as a quantitative signal of the target DNA by evaluating the sensor response, under the selected optimum conditions, to increasing *Salmonella* dsDNA concentrations obtained by serial dilution of PCR amplification products (Figure 1). The calibration revealed a linear relationship across the entire concentration range tested (from 5 to 250 nM):

i_{net}/ nA = (2.72 ± 0.05) × ([*Salmonella* DNA]/ nM) + (41 ± 6), R = 0.9994

where i_{net} represents the difference between the peak current measured for each target concentration and that obtained for the negative control. The sensor showed good reproducibility, with an average relative standard deviation (RSD) for three independent

measurements of 17%. The detection limit, estimated as $3S_b/ m$ (where S_b is the standard deviation of the blank and m is the slope of the calibration curve) was 2.4 nM.

Preferred place for **Figure 1**

The detection limit obtained is relatively high compared to other described genosensors, presumably owing to the strong secondary structure of the target DNA sequence, which slows the kinetics of the hybridization event [29]. A similar effect has been described by Martín-Fernández *et al.* for an electrochemical genosenor on gold electrodes using a target with a particularly stable secondary structure [26].

Moreover, the analytical performance of the *Salmonella* genosensor constructed on ITO electrodes was compared with a similar design on gold screen-printed electrodes. A mixed self-assembled monolayer (see protocol in Supporting Information), composed of the same thiolated CP and *p*-aminothiophenol as diluent acted as the sensing platform [25], using antifluorescein-POD as a reporting molecule and TMB as a substrate. In this case, a linear response was obtained within the range of 5 and 300 nM:

$i_{net}/\mu A = (0.0087 \pm 0.0006) \times ([\textit{Salmonella}\ \text{DNA}] / \text{nM}) + (0.2 \pm 0.2),\ R = 0.998$

with a detection limit of 4 nM. This similar analytical performance on both platforms, even using different enzymes, indicates that the sensor response is limited by the hybridization reaction rather than by the enzymatic amplification step subsequent to the recognition step. However the ITO platform showed improved storage stability. The sensing surfaces are single-use, but after modification and washing with a solution containing 2.5 % of BSA and glucose, they can be dry-stored at 4 °C for 9 months without significant loss in response [12].

3.4. Sequence-specific detection of PCR amplicons

Given the good stability and performance of the ITO-sensing platform, we next tested its ability to perform the sequence-specific detection of the PCR amplicons generated from *Salmonella* genome. To obtain quantitative information, it is important to select the number of PCR cycles that, providing the desired sensitivity, ensure that the plateau phase of the amplification reaction is not reached [30]. We find that for the largest number of cycles investigated (36 cycles) the combination of PCR and electrochemical genosensor allows to differentiate only 1 GU of *Salmonella* DNA from

the negative control, which gives a signal seven times higher than that obtained for the negative control (Figure 2A); this is one order of magnitude more sensitive than real-time PCR.

Preferred place for **Figure 2**

There is a linear relationship between the net peak current obtained in the PCR-genosensor system and the logarithm of the initial target copy number

i_{net}/ nA = (140 ± 8) × log (*Salmonella* DNA / GU) + (112 ± 15), R = 0.997

in the range from 1 to 1000 GU (Figure 2B), which can be used for quantification purposes.

3.5. Adaptation of PCR primers for combining HDA and genosensor

Helicase dependent amplification (HDA) is a more tractable amplification technique than PCR for point-of-need applications. The adaptation of PCR protocols to HDA amplification would facilitate its widespread adoption for pathogen detection. However, the design conditions of the HDA primers are more restrictive than those of PCR, especially in terms of length, since the number of bases should be between 24 and 33 nucleotides. Other considerations are: 1) near melting temperature (T_m), with differences of ± 5 °C, for both primers; 2) to avoid sequences of three or more cytosines or guanines in the 3' end regions; 3) the 3' ends of both primers should not be complementary to limit the formation of dimers; and 4) primers with a minimal secondary structure are preferable [31].

As a first step, we investigated whether the PCR primers could be elongated towards the center of the target sequence to fulfill the above-mentioned characteristics and amplifying the same target sequence of 86 bp. Two set of primers were designed, F_{24}/R_{24} (set 1) and F_{27}/R_{28} (set 2) (Table 1). We tested the suitability of the different primers by performing HDA amplifications of 10^3 GU of *Salmonella*, followed by quantification with the designed genosensor (Figure 3). We also verified the formation of the appropriate amplification product via agarose gel electrophoresis (Figure S7). As anticipated, PCR primers gave no target signal after HDA. Whereas both sets of adapted primers gave significant amount of amplification products, only the first one allowed to clearly discriminate the *Salmonella* template from reactions lacking a template. With set

2, the accumulation of parasitic amplicons yielded false positive signals in the negative control. This is presumably related to the similar size of the specific and non-specific products originated from the longest primers, which could hybridize with both CP and SP. This is confirmed by the gel electrophoresis results, where we observed strong bands, corresponding to very similar sizes, for both blank and sample. As general rule, primers in the low range of the interval recommended for HDA are preferable to avoid confusion between the specific amplicons and the erroneous products due to the similar size between both, which would result in false positives [32].

Preferred place for **Figure 3**

The next step was the selection of the amplification time that leads to the best analytical performance. With that aim, HDA reactions with primers defined in set 1 and different amplification times were monitored for increasing initial *Salmonella* GU (Figure 4A). After 60 min, low signals were obtained for the hybridization assay. On the contrary, for the longest amplification time (90 min), starting target concentrations differing by tenfold serial dilutions gave similar signals, with signal to blank ratio about 3. These may be related to the onset of the plateau phase of the amplification reaction. Finally, after 75 minutes of amplification, the sensor response was linearly related with the logarithm of the starting *Salmonella* units in the range between 50 and 10^3 GU

i_{net}/ nA = (454 ± 26) × log (*Salmonella* DNA / GU) + (755 ± 62), R = 0.995)

with a detection limit of 50 GU (Figure 4B).

Preferred place for **Figure 4**

The selectivity of the assay was tested by evaluating its response to the genome of *Mycobacterium bovis*, microorganism that can be found in raw milk like *Salmonella*. Genomic DNA extracted from a culture of this pathogen was supplied by the Regional Reference Unit of Mycobacteria in Asturias. We evaluated the sensor response to 10^4 GU of *M. bovis* after HDA amplification with *Salmonella* primers, and we found that the signal is indistinguishable from the negative control (S/B =2). To rule out potential false negatives arising from inhibition of the amplification due to other matrix components,

we also compared the signal obtained from the same *M. bovis* sample spiked with 10^3 GU of *Salmonella* as an internal control and another sample containing only 10^3 GU of *Salmonella*. The signal to blank ratio obtained for *Salmonella* (S/B = 14) is not affected by the presence of *M. bovis (*Figure S8), which clearly supports the selectivity of the assay.

4. Conclusions

By combining PCR amplification with a sandwich type genosensor with electrochemical detection on indium tin oxide (ITO) surfaces, we show that sequence-specific detection of amplification products improves the detection limit of the standard real-time PCR in two orders of magnitude, achieving the single molecule detection. In addition, we have successfully adapted PCR primers to isothermal helicase dependent amplification for the detection of the same 86 bp sequence specific for the *Salmonella bipA* gene with the ITO sensing platform. In this way, we preserve the best features of isothermal amplification for molecular detection, providing suppression of parasitic amplification, thus achieving a detectability and selectivity similar to real-time PCR. The results strongly support the feasibility of rationally adapting PCR protocols for the detection of other pathogens to HDA amplification, facilitating their adoption for point-of-need applications.

Acknowledgments

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Figure Captions

Scheme 1. Steps involved in the operation with the electrochemical genosensor for *Salmonella*. Step 1: homogenous hybridization. Step 2: heterogeneous hybridization. Step 3: enzymatic labeling. Step 4: electrochemical measurement.

Figure 1. *Salmonella* DNA quantification with the genosensor. (A) Voltamperograms for different initial dsDNA concentration. (B) Calibration curve relating the net peak current, once the negative control signal is substracted (i_{net}) and the concentration of *Salmonella* DNA.

Figure 2. (A) Effect of the number of cycles of PCR on the peak current obtained with the genosensor for increasing amounts of *Salmonella* DNA. (B) Relationship between the analytical response and the initial units of *Salmonella* genome after 36 cycles of PCR amplification.

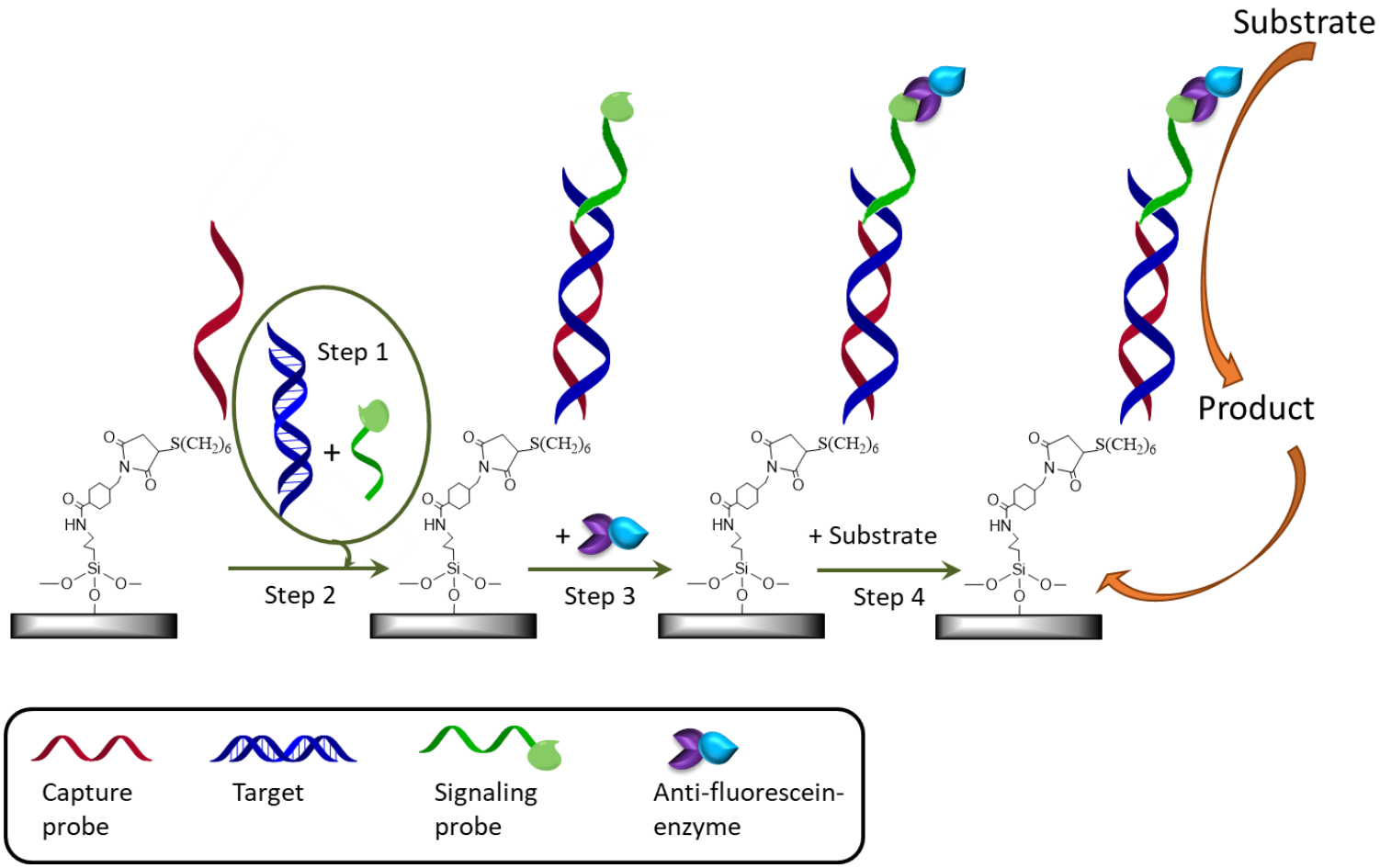
Figure 3. Peak current obtained with the genosensor for the products generated from HDA reactions of a negative control (blue) and a sample containing 10^3 GU of *Salmonella* (green) with three different primer pairs.

Figure 4. (A) Genosensor signal for HDA products generated for increasing amounts of *Salmonella* DNA after different amplification times. (B) Relationship between i_{net} and the initial amount of *Salmonella* genome obtained after 75 min of HDA amplification.

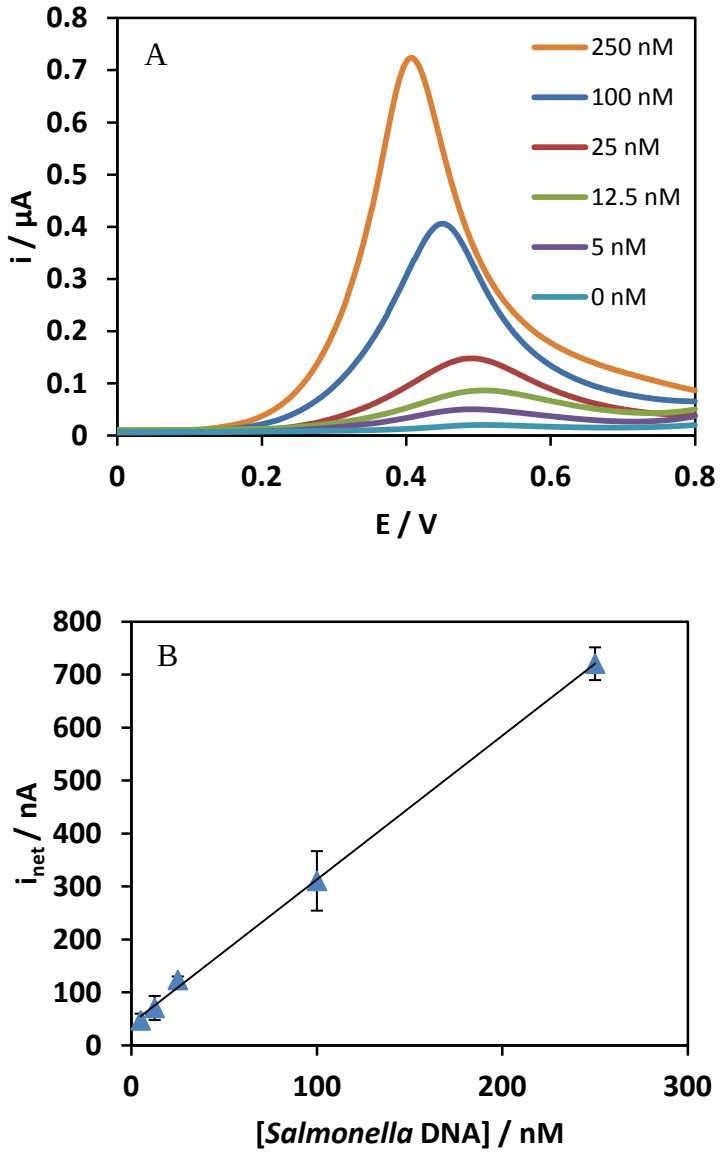
Table 1. Oligonucleotide sequences used in this work.

	Function	Name	Oligonucleotide Sequences 5'→3'	T_m (°C) Salt adjusted	%G+C
Hybridization assay	Capture Probe	CP	HS-C_6 CCG TTC TGA CGC TGG CCC ACT TCA C	72.3	64
	Signaling Probe	SP	CCG GAC GAA TAT CGT CGT AAT GGC TGA AGG TGG AGT ACA-6FAM	78.8	51.3
Target sequence	Target	T	GGT CTG CTG TAC TCC ACC TTC AGC CAT TAC GAC GAT ATT CGT CCG GGT GAA GTG GGC CAG CGT CAG AAC GGC GTA CTG ATC TCC AA	73.3	56
PCR primers	Forward Primer23	FP_{23}	GGT CTG CTG TAC TCC ACC TTC AG	66.6	56.5
	Reverse Primer22	RP_{22}	TTG GAG ATC AGT ACG CCG TTC T	62.1	50
HDA primers Set 1	Forward Primer24	FP_{24}	GGT CTG CTG TAC TCC ACC TTC AGC	68.5	58.3
	Reverse Primer24	RP_{24}	TTG GAG ATC AGT ACG CCG TTC TGA	65.2	50
HDA primers Set 2	Forward Primer27	FP_{27}	GGT CTG CTG TAC TCC ACC TTC AGC CAT	71.5	55.6
	Reverse Primer28	RP_{28}	TTG GAG ATC AGT ACG CCG TTC TGA CGC T	71.8	53.6

Scheme 1



Scheme 1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100 nM
25 nM
12.5 nM
5 nM
0 nM
i / µA
E / V
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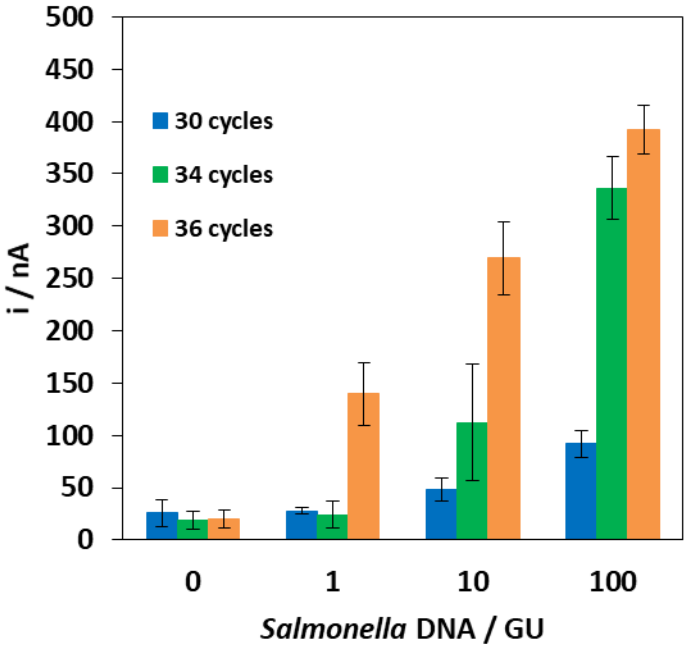
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.2
0.4
0.6
0.8
B
i_net / nA
[Salmonella DNA] / nM
800
700
600
500
400
300
200
100
0
0
100
200
300

Figure 1


500
450
400
350
300
250
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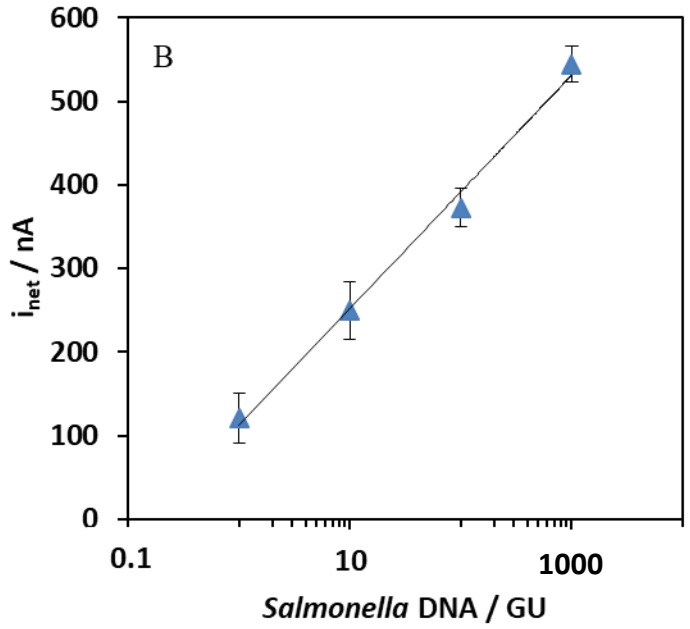
150
100
50
0
i / nA
30 cycles
34 cycles
36 cycles
0
1
10
100
Salmonella DNA / GU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400
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200
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0
i net / nA
0.1
10
1000
Salmonella DNA / GU

Figure 2

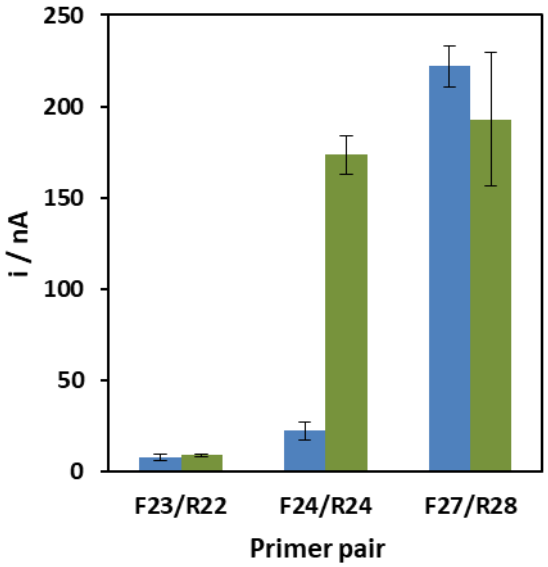

Figure 3

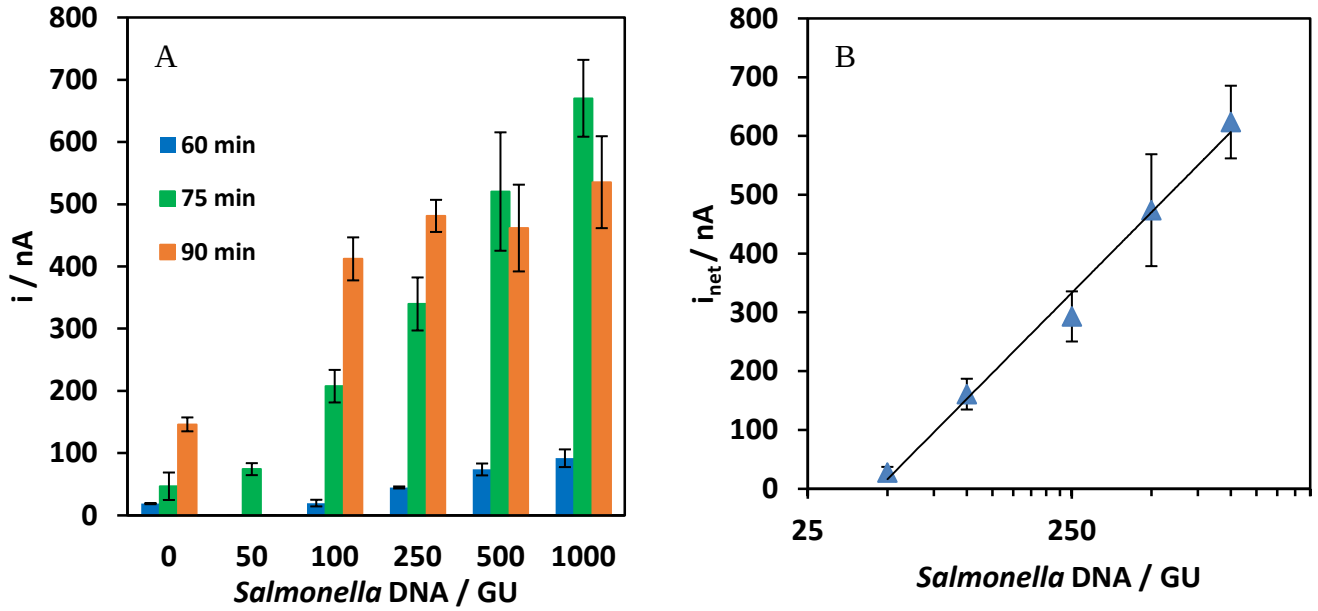
A
60 min
75 min
90 min
i / nA
800
700
600
500
400
300
200
100
0
0
50
100
250
500
1000
Salmonella DNA / GU
B
i net / nA
800
700
600
500
400
300
200
100
0
25
250
Salmonella DNA / GU

Figure 4

Author Biographies

Susana Barreda-García received her BS in chemistry in 2012 and her PhD in analytical and bioanalytical sciences in 2017 from Oviedo University. She is now a Postdoctoral researcher in the Electroanalysis research group headed by Prof. M.J. Lobo-Castañón. Her main areas of research are isothermal nucleic acid amplification coupled to electrochemical detection and aptamer-based biosensor fabrication.

Rebeca Miranda-Castro is a Postdoctoral Researcher in the Electroanalysis Research Group headed by Prof. M.J. Lobo-Castañón. Her research interests focus on the development of electrochemical sensors for clinical diagnosis and food analysis using molecular recognition elements based on nucleic acids (genosensors and aptasensors).

Noemí de-los–Santos-Álvarez is an Associate Professor at Universidad de Oviedo (Spain). Her field of expertise is Electroanalysis. Her first studies were conducted on electrocatalytic processes and characterization of intermetallic materials for fuel cells (during her postdoc stage at Cornell University). Her current interests are focused on genosensing and aptamer selection (allergens, viruses and cancer biomarkers) to be used in biosensors for diagnostics.

María Jesús Lobo-Castañón is a Professor at University of Oviedo, Department of Physical and Analytical Chemistry, Spain, where she leads the Electroanalysis research group. Her research interests focus on the development of electrochemical sensors for clinical diagnosis and food analysis, using different molecular recognition elements, such as enzymes, DNA, aptamers, and molecularly imprinted polymers. She is the author or co-author of over 100 scientific articles and several book chapters in the field.

Supplementary Material

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