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Solid-phase helicase dependent amplification and electrochemical detection of *Salmonella* on highly stable oligonucleotide-modified ITO electrodes†

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An on-surface isothermal helicase-dependent amplification is devised for simple, point-of-need quantification of bacterial genomes. The method relies on the enzyme-extension of a thiol-modified reverse primer anchored to indium tin oxide electrodes, which shows strikingly high thermal and storage stability. Amplification and electrochemical detection of only 10 genomes are thus performed on the same platform without thermal cycling.

Sequence-specific nucleic acid-based sensors hold great economic potential for decentralized genetic testing, a key requirement not only for clinical diagnostics but also in biosafety or food safety control and environmental monitoring. Electrochemical devices are particularly suitable for satisfying most of the characteristics for an ideal point-of-need molecular test.[1,2] Despite very low limits of detection being provided,[3–5] the electrochemical DNA sensors reported until now are most often tested using target short oligonucleotides, or sequences obtained after polymerase chain reaction (PCR) amplification. These hybridization-based platforms for DNA detection are challenging to deploy in genomic DNA where the efficiency of hybridization is hampered by its large size and complexity. Consequently, the development of an integrated and miniaturized platform for genomic DNA quantification usually requires a combination of a sample pretreatment step,[6,7] uncoiling and cutting the genome, and a sequence-specific detection method. One approach is to integrate a nucleic acid-based sensor and an amplification method, which contributes not only to restricting the size but also to multiplying the target genome. PCR is the gold standard amplification technique, although the need for thermal cycling limits its use in easy-to-use devices for on-site detection.[8] Alternative amplification methods that do not require heating and cooling have been developed over the last two decades. In these technologies the amplification reaction takes place at a constant temperature, usually higher than 37 °C, by adapting enzymatic mechanisms from natural biological processes.[8,9] However, to truly integrate isothermal amplification and electrochemical detection there still exist important hurdles to overcome, mainly reproducibility and thermal stability of the sensing phase.

Gold surfaces functionalized with DNA oligonucleotides through the formation of thiol-based self-assembled monolayers (SAMs) are a common approach for electrochemical genosensing, even though the poor stability under dry storage conditions and thermal stability in aqueous solutions limit their widespread commercial application.[10] The use of alternative surfaces for DNA immobilization remains little explored. Indium tin oxide (ITO), a transparent semiconductor material that can be used both as a support for oligonucleotide immobilization and as a resistive heater, has been only scarcely used for DNA immobilization.[11,12] Herein, we demonstrate that a simple and general approach to covalently immobilize thiol-modified oligonucleotides on ITO surfaces provides a robust platform for DNA quantification. Its high thermal and storage stability combined with its good reproducibility opens up a range of applications to develop integrated platforms for both electrochemical and optical detection of isothermal nucleic acid amplification products.

There are several isothermal nucleic acid amplification methods[9] that are more easily adapted to simple instrumentation than PCR. Among them, helicase-dependent amplification (HDA) follows a reaction scheme similar to PCR but taking advantage of the helicases' ability to unwind dsDNA at a constant temperature.[13] Most HDA applications involve the enzymatic extension of primers in solution.[14–16] Although it is known that the immobilization of the primers on a solid surface contributes to the minimization of the typical problems associated with primer–dimer formation, only on one occasion on-chip HDA amplification in combination with fluorescence detection has been described,[17] showing poor sensitivity. Here we present data demonstrating for the first time on-surface HDA amplification with electrochemical detection for *Salmonella* genome quantification with a detection limit that matches the real-time PCR.

The three-step chemical approach used for the covalent attachment of 5′-end thiol-modified oligonucleotides on a glass coated with ITO, pre-treated with an oxidizing solution to increase the

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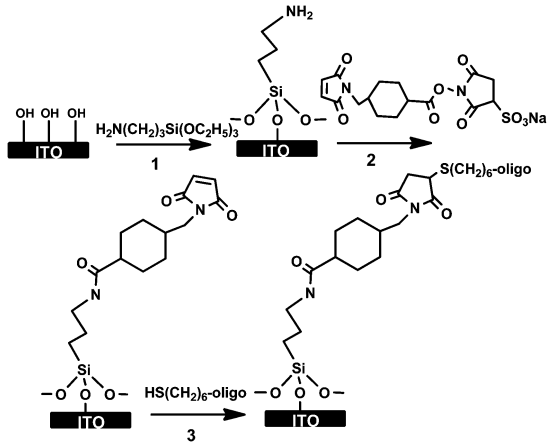

Fig. 1 Steps for ITO modification with thiol-oligonucleotides.

number of free surface hydroxyl groups, is depicted in Fig. 1. First, (3-aminopropyl)triethoxysilane (APTES) reacts with the hydroxylated surface, resulting in an amino-terminated siloxane layer. Subsequently, a heterobifunctional cross-linker, sulfosuccinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC), couples 5′-thiol modified oligonucleotides to the amino derivatized ITO surfaces. The silanization step is crucial as it determines the number of amine groups available for the next step. Improved surface uniformity, leading to higher reproducibility in the final assay, is obtained when this step is performed overnight in 1% APTES/ethanol (Fig. S1, ESI†).

The characterization of the surface by X-ray photoelectron spectroscopy (XPS) following each functionalization step reveals a N/Si ratio of one after silanization, the theoretical value according to the APTES structure. After DNA modification, the peaks corresponding to tin and indium decrease, accompanied by an increase in the signal of C as well as in the ratio N/Si, indicating that the modification has taken place. However, quantification of the amount of the cross-linker and DNA immobilized on ITO is not possible using this technique. The surface coverage of the heterobifunctional reagent, which indicates the total number of active groups for the anchoring of DNA, has been electrochemically estimated by linking 6-(ferrocenyl)hexanethiol moieties, and quantifying the amount of charge passed to oxidize the ferrocene sites by cyclic voltammetry. The surface density of active sites obtained *via* this procedure is 1.7×10^{14} molecules cm^{-2}, which is close to the maximum expected coverage (2.7×10^{14} molecules cm^{-2})[18] assuming a hexagonally close-packed layer of ferrocene molecules, pointing to a high efficiency for the attachment of the coupling reagent.

The surface density of the immobilized ssDNA probes was estimated by using a cationic redox marker (hexaammineruthenium(III), RuHex) at pH 6 and the classical Tarlov method.[19] The density of ssDNA, 2.5×10^{12} molecules cm^{-2} as obtained using chronocoulometry, corresponds to only 1.5% of the number of active sites estimated with ferrocene. However, this medium surface density is highly favourable and provides adequate DNA spacing for hybridization considering that a maximum packed dsDNA of $\sim 2 \times 10^{13}$ molecules cm^{-2} is expected.[20]

To evaluate the suitability of this platform for DNA sensing applications, we measured the hybridization efficiency of ITO surfaces modified with a 25-mer probe complementary to the gene *Typ*A of *Salmonella* (GenBank accession no. AE006468.2, bases 4218770 to 4218855). Using a well-established sandwich assay (the details of which are included as the ESI†), in combination with PicoGreen® as a specific dsDNA dye, fluorescence spectroscopy measurements showed a hybridization efficiency of ~50% after 2 h of reaction. This efficiency is acceptable for a bulky 86-base long target sequence.

The development of a marketable platform that integrates amplification and detection requires both thermal and storage stability of the sensing phase. For thermal stability assessment, we simulated HDA conditions by exposing the capture probe layer to a drop of buffer at 65 °C for 1 hour. After this, we performed an electrochemical sandwich assay using a fluorescein-labelled signalling probe. Following hybridization, the surface bound dsDNA is labelled with a Fab fragment of antifluorescein conjugated to alkaline phosphatase (AP), thus providing an additional amplification route for the primary recognition event. The results obtained were comparable with the results of the same assay at 25 °C within the experimental error (Fig. S2, ESI†), so it is anticipated that the amplification process could be performed on the platform. Regarding sensor stability, the sensing surface was dry-stored at 4 °C after washing with a solution containing 2.5% of both BSA and glucose, which provides desiccation protection.[21] As shown in Fig. 2, no significant loss of the signal was observed after 9 months, provided a rehydration step for 2 h in phosphate buffer is performed before its use, indicating a remarkably improved storage stability when compared with the most stable ternary SAMs on Au.[22] This kind of SAM shows a loss in its analytical performance after 3 month storage, probably due to the displacement of the capture probe by the blocking components of the sensing layer.

Having established that HDA conditions are compatible with the ITO platform, we next sought to extend one of the primers, directly on the sensing surface. With that aim, we envisioned the isothermal amplification strategy shown in Fig. 3, in which the

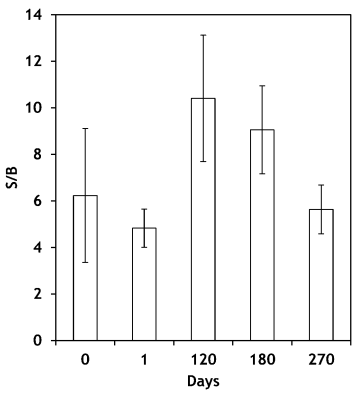

Fig. 2 Signal to blank ratio obtained for a concentration of DNA amplicons 2.5×10^{-8} M with ITO sensors washed with 2.5% glucose and BSA, after dry storage at different point times. Values represent the average and standard deviation of measurements performed with three different sensors.

reverse primer, HS-T_{10}-RP_2, is covalently bound to the ITO surface at the 5′-end. The forward fluorescein-labelled primer, 6-FAM-FP_2, and the *Salmonella* genome are incorporated in the solution as well as helicase and polymerase enzymes. We took some measures to promote the somehow impeded on-surface amplification. A T_{10} spacer in the anchored primer was used and a small amount of a shortened version of the reverse primer (trimmed at 3′ end, RP) was added to the solution. This is expected to strongly shift the amplification toward the solid phase due to favourable thermodynamics.[23]

Under these conditions, we should distinguish two different stages for the enzymatic amplification reaction. In the first one (Fig. 3A), the liquid-phase amplification process shortens the *Salmonella* genome, giving rise to 86-base long 6-FAM labelled amplicons. This process could take place until the reverse primer in the solution is depleted. After that, the second stage begins (Fig. 3B), in which the generated 6-FAM-strands, unwound by the helicase and stabilized by the single-strand DNA-binding protein (SSB), predominantly anneal to the solid-phase anchored reverse primers and are extended by the polymerase, whereas in the solution the asymmetric amplification provides additional 6-FAM-ssDNA, the template for surface amplification. Following amplification, the on-surface synthesized fluorescein-modified dsDNA is detected after enzyme labelling.

On ITO electrodes some electrochemical processes otherwise reversible become irreversible. Unfortunately this is the case for tetramethylbenzidine (TMB), the typical substrate of peroxidase (POD), which cannot be sensitively detected at usual potentials.

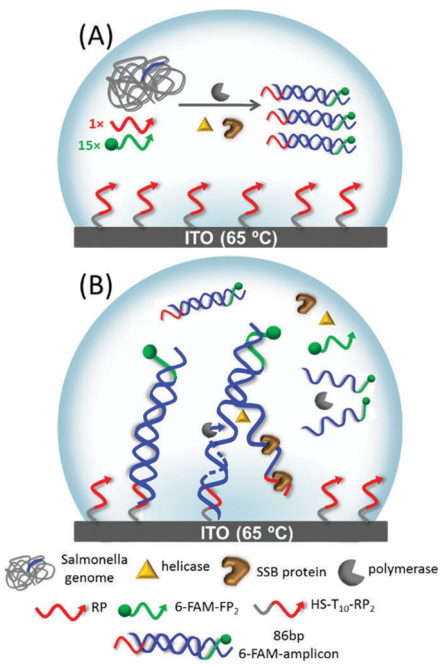

Fig. 3 Overview of the on-surface helicase-dependent amplification. (A) The first stage involves amplification in solution, in the presence of lower amounts of RP than FP, until RP is depleted. At this stage the target genome is shortened. (B) The second stage involves the elongation of anchored RP taking as a target the 6-FAM-labelled strands obtained as copies in the liquid-phase amplification.

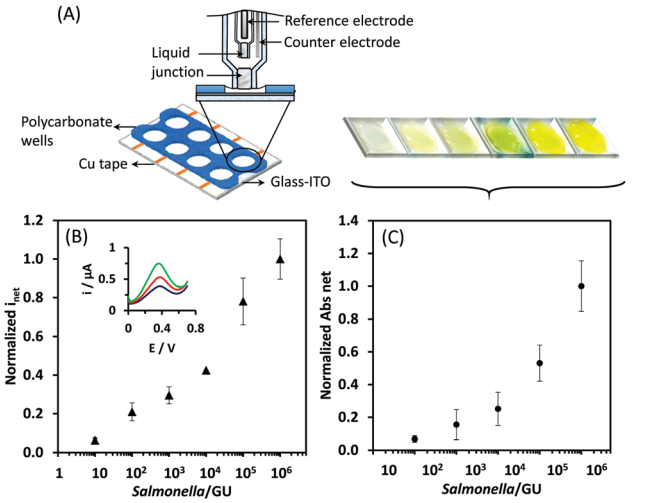

Fig. 4 (A) Overview of the ITO platform used for on-surface HDA amplification, and the electrochemical cell for quantification. Results for *Salmonella* genome quantification with on-surface helicase-dependent amplification and (B) electrochemical detection (inset: representative voltammograms for blank, 10 and 10^3 GU) or (C) optical detection. Each value represents the average (and standard deviation) of three independent measurements.

For this reason, AP was used instead of POD for the electrochemical transduction. AP transforms 1-naphthyl phosphate into 1-naphthol that can be measured by DPV. Fig. 4A shows the experimental setup used for the measurement. The amplification is performed within a well, defined on the ITO surface by a polycarbonate mask. The electrical contact of the ITO working electrode is established through a copper tape, and the electrochemical cell is completed with an Ag|AgCl|KCl reference electrode and a stainless-steel counter electrode in a syringe.

Achieving the greatest sensitivity with negligible non-specific amplification due to mispriming or primer–dimers, which can lead to false positive results, requires a high asymmetric ratio for the primers in solution.[14] To see how the amount of liquid-phase primers influences both the signal and the blank, we fixed the direct primer at 75 nM, and monitored the on-surface amplification reaction for 10^4 units of *Salmonella* genomes in the presence of 8-times and 15-times lower amounts of the reverse primer in the solution. The background amplification after 90 min of reaction is significantly reduced (from 1.8 μA to 0.4 μA) for the highest asymmetry ratio while the signals are similar, indicating an effective removal of primer–primer interactions. Additionally, when the amplification time is reduced to 75 min using a reverse to forward primer ratio of 1 : 15, no differences between the blank and the signal are obtained, pointing to a delay time between reactant mixing and the appearance of on-surface amplified products according to the proposed scheme of amplification.

The described isothermal on-surface amplification with electrochemical detection showed excellent performance (Fig. 4B). As few as 10 *Salmonella* genomes give a signal significantly different from that for the negative control (blank experiment), with a reproducibility (RSD for three different measurements) of 20%.

Two different regions may be distinguished in the response curve, *i.e.* a log-linear relationship between initial units of *Salmonella* genomes and the normalized net signal that spans four orders of magnitude (from 10 to 10^4 units of the starting genome), and a second region with a greater slope for higher target concentrations. The increase in the slope for the second region presumably occurs because the time needed for RP depletion for target concentrations higher than 10^4 genomes is shorter than the 90 min used as a fixed time for amplification. In consequence, the template for on-surface amplification, the 86-base 6-FAM-ssDNA, is preferentially synthesized in solution boosting the kinetics of the on-surface amplification reaction.

To investigate whether the use of anchored-primers affects the HDA amplification efficiency, we also compared the on-surface amplification *versus* liquid-phase amplification followed by electrochemical detection of the amplicons using a sandwich hybridization assay similar to that described for the stability assessment. The increase in the slope of the response curve while performing the amplification in solution, 45 times higher relative to the on-surface amplification (Fig. S3, ESI†), confirms that the former exhibits the highest efficiency. Despite that, our on-surface amplification platform displays detectability comparable to the real-time PCR.

ITO is not only a conductive surface but also transparent, so this isothermal DNA amplification platform is convenient for both electrochemical detection and optical detection. Consequently, we also evaluated the optical response of the solid-phase amplification system for different concentrations of the *Salmonella* genome, using Fab fragments of an anti-fluorescein-peroxidase (POD) conjugate as a label of the anchored amplified strands, and TMB as a substrate. The absorbance measured at 450 nm after stopping the enzymatic reaction with sulfuric acid increases with the concentration of genomes (Fig. 4C). After 90 min of amplification at 65 °C, we are able to distinguish from the negative control 10^2 units of the *Salmonella* genome with a reproducibility of 30%. The response curve revealed a similar log-linear relationship between the initial target copy number and the normalized signal. The lower detectability of the optical system presumably occurs because of the higher unspecific adsorption of the enzyme-conjugates or its higher activity, as the signal for the negative control is in this case higher than that obtained for the electrochemical measurement.

In summary, using ITO surfaces for the covalent anchoring of thiol-oligonucleotides, we have shown for the first time that on-surface helicase-dependent amplification is useful to quantitatively detect very small amounts of genomic DNA. The proposed electrochemical platform reduces typical HDA artefacts as primer–dimers, and is potentially extended to other pathogens, constituting an excellent platform for genetic detection at the point-of-need even after 9 month storage.

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Supporting information for:

Solid-phase helicase dependent amplification and electrochemical detection of *Salmonella* on highly stable oligonucleotide-modified ITO electrodes.

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SUPPORTING METHODS

Materials and instrumentation

1-Naphthyl phosphate (α-NPP), 3,3´,5,5´-tetramethylbenzidine (TMB) containing hydrogen peroxide as substrates of peroxidase (POD), bovine serum albumin (BSA), phosphate buffered saline (10× PBS; 0.1 M phosphate, 1.54 M NaCl pH 7.4), 20× saline sodium phosphate (20× SSPE; 200 mM sodium phosphate, 3 M NaCl, 20 mM EDTA pH 7.4), (3-aminopropyl)triethoxysilane (APTES), 6-(ferrocenyl)hexanethiol, hexaammineruthenium (III) chloride, D-(+) Glucose, and indium tin oxide coated glass slide (square, surface resistivity 8-12 Ω/sq) were purchased from Sigma-Aldrich (Spain). Blocking casein (1% casein in PBS), sulfo-succinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carbonate (Sulfo-SMCC) and PicoGreen® Assay for dsDNA were obtained from Thermo Scientific (Spain) and the enzyme conjugates anti-fluorescein-alkaline phosphatase (antiFITC-AP) and anti-fluorescein-POD Fab Fragments from Roche diagnostics (Spain).

Four different buffers were used: crosslinking buffer (CB; 50 mM sodium hydrogen phosphate (Na_2HPO_4) and sodium dihydrogen phosphate (NaH_2PO_4), 0.15 M sodium chloride and 10 mM EDTA kept at pH 7.2), hybridization buffer (2× SSPE, pH 7.4; prepared by tenfold dilution of 20× SSPE), casein blocking buffer (0.5% casein in 1× PBS), and AP buffer (0.5 M Tris-HCl, pH 9.8, 1 mM $MgCl_2$).

Synthetic oligonucleotide sequences were obtained from Laboratorios Conda (Spain) purified by HPLC; and their sequences are listed in Table S1. All oligonucleotides stock

solutions were prepared in Milli-Q water and stored at -20° C. The thiolated oligos were commercially supplied as the respective disulfides. Prior to use, these products were treated with dithiothreitol (DTT, Sigma-Aldrich, Spain) and then purified by elution through a Sephadex G25 column (NAP-10, Amersham Biosciences) with Milli-Q water[1].

Table S1: Synthetic oligonucleotides used in this work.

	Function	**Name**	**Sequence (5'→3')**
Hybridization assay	Capture Probe	HS-CP	HS-C_6 CCGTTCTGACGCTGGCCCACTTCAC
	Signaling Probe	SP-6FAM	CCGGACGAATATCGTCGTAATG GCTGAAGGTGGAGTACA-6FAM
86bp Target Generation	Forward Primer	FP	GGTCTGCTGTACTCCACCTTCAG
	Reverse Primer	RP	TTGGAGATCAGTACGCCGTTCT
Solid Phase Amplification	Forward Primer (*in solution*)	6-FAM-FP_2	6FAM-GGTCTGCTGTACTCCACCTTCAGC
	Reverse Primer (*immobilized*)	HS-T_{10}-RP_2	HS-C_6-$(T)_{10}$TTGGAGATCAGTACGCCGTTCTGACGCT
	Reverse Primer (*in solution*)	RP	TTGGAGATCAGTACGCCGTTCT

All other reagents were of analytical grade. Unless otherwise indicated, double-deionized water (Milli-Q Millipore Corporation) was employed to prepare all aqueous solutions.

Electrochemical measurements of the hybridization assay were carried out with a conventional three-electrode electrochemical cell driven by a computer-controlled μ-Autolab type II potentiostat with GPES 4.9 software (Ecochemie, The Netherlands). A homemade platinum wire electrode acted as auxiliary electrode. All the potentials are referred to Ag | AgCl | KCl saturated reference electrode. The working electrode was kept in a micro-cell designed by our group and immersed in a regular cell maintaining the electrical contact through a salt bridge at the bottom. The measurements that required temperature control were carried out in a thermostatized cell by a HAAKE DC1 circulation thermostat.

For the integrated electrochemical solid phase HDA, measurements were carried out on ITO wells, each one connected through a copper strip and using external auxiliary and reference electrodes placed into a syringe (Figure 3 main text).

Optical measurements were performed with an Eon microplate spectrophotometer (BioTek Instruments, Inc.).

Sensing Surface Construction

For the hybridization assay, indium tin oxide coated glass slides were cut into pieces of about 8 × 5 mm and modified as follows. Each ITO surface was cleaned with acetone, ethanol and water and then treated with 1 M HCl for 10 min. Afterwards an oxidizing step was carried out by immersing the pieces into a solution of 1:1:5 (v/v) $H_2O_2/NH_4OH/H_2O$ during 1 h followed by rinsing with water and drying under a stream of nitrogen. These treated ITO was silanized with 1 % (3-aminopropyl)triethoxysilane (APTES) in absolute ethanol at room temperature overnight. After this reaction, the modified substrates were rinsed with ethanol and water to remove the physically adsorbed silanes and dried under a stream of nitrogen. To provide the electrical contact, a copper strip was coupled to each ITO fragment. Then, a 2 mm in diameter electrode surface was defined with an adhesive tape. Finally, lateral sides were protected from the solution with Teflon[2]. Sulfo-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carbonate (Sulfo-SMCC) was used as a heterobifunctional reagent with an amine-reactive group at one end and a thiol-reactive group at the other end that connects the silanized platform with the thiolated capture probe to form the sensing phase. Thus, amine modified ITO surfaces were incubated in 2 mg/mL sulfo-SMCC prepared in CB for an hour at room temperature and protected from light. The resulting maleimide-modified surface was rinsed with CB and dried with nitrogen. Finally, oligonucleotides were linked to the surface by incubation with 10 μM thiol-capture probe (HS-CP) in CB for 2 hours. The resulting surface was then washed with CB followed by water.

For the solid-phase HDA, ITO coated glass slides were scratched with a diamond to short-circuited the different cells. After silanization, different wells, each 8 mm in diameter, were defined by sticking a polycarbonate piece onto the modified ITO slide. The thiolated-reverse primer, $HS\text{-}T_{10}\text{-}RP_2$, was anchored to the surface as previously described for the thiolated capture probe, HS-CP.

Measurement of active groups for the DNA anchoring

Accessible on-surface maleimide groups, serving as attachment sites for thiolated DNA, were estimated as follows. ITO surfaces, functionalized with sulfo-SMCC and transformed into individual electrodes, were incubated with 1 mM of 6-(ferrocenyl)hexanethiol in CB for 2 h. Afterwards, the electrodes were thoroughly washed with CB and water, and the potential was scanned in 0.1 M $HClO_4$ by cyclic voltammetry (scan rate 100 mV/s). The anodic peak area (i.e: the quantity of charge consumed during the oxidation of the surface-confined ferrocene) was used to calculate the surface coverage of ferrocene, Γ_{Fc}.

Generation of Salmonella amplicons used as a target in the hybridization only assay

Genomic DNA of *Salmonella enterica* subsp. *enterica* was purchased from Spanish Type Culture Collection, Valencia, Spain (CECT 878) in lyophilized form. It was dissolved in Milli-Q water, aliquoted (5×10^6 genomic units per microliter, GU/µL), and stored at -20ºC.

For the electrochemical hybridization assay, an 86 bp DNA sequence specific of pathogenic *Salmonella* was used as a target and obtained by PCR amplification of *Salmonella* genome, using the IMMOLASE™ DNA Polymerase kit (Bioline, Spain), as well as a set of primers, FP and RP (see Table S1), previously described[3]. The reaction mixtures contained 1× PCR buffer, 2 mM $MgCl_2$, 0.25 mM dNTPs, 1 µM each primer, 1 U Immolase™ DNA polymerase, 2.5 µL of 10^6 GU of *Salmonella* genome and water to a total reaction volume of 25 µL. The amplification reactions were performed in a thermal cycler (GeneAmp® PCR System 2700 thermocycler (Applied Biosystems, Spain)). 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. A final step was carried out at 72 °C for 7 min to extend any incomplete product. The 86 bp PCR product was purified with MinElute PCR purification kit (Qiagen) and quantified spectrophotometrically at 260 nm (GENESIS™ 10S UV-vis Spectrophotometer, ThermoScientific).

Hybridization assay

Hybridization experiments were carried out in a sandwich-like format. First, the homogenous hybridization between the target (86 bp *Salmonella* amplicon) and the signaling probe (SP-6FAM) takes place. 25 µL of solution containing 2 µM SP-6FAM and varying concentrations of 86 bp *Salmonella* amplicons in the hybridization buffer (2× SSPE; pH 7.4) 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 (30 min), 25 µL of 5 % BSA solution in 2× SSPE were added. 6 µL of this mixture was placed onto the sensing phase for 2 h (RT, in darkness). Then, the surface was washed with 1× PBS solution and 6 µL 0.5 U/mL of the antiFITC-AP conjugate in the casein blocking buffer was added to the electrode for 30 min (RT, in darkness). After a washing step with AP buffer, the electrode was placed in a micro-cell with 400 µL of AP buffer containing 4 mM of α-NPP and, after 10 min the current due to the oxidation of α-naphthol was measured by DPV from 0 to 0.8 V (modulation amplitude 50 mV, scan rate 10 mV s^{-1}).

Hybridization efficiency

Hybridization efficiency was estimated by measuring the fluorescence of a dsDNA-binding dye, PicoGreen®, with a Bio-tek FL600 fluorescence reader. With this aim, upon performing the sandwich hybridization assay onto sensing surface-modified ITO wells, these were filled with 50 µL of 0.5×PicoGreen® in 1×TBE buffer (10-times diluted form 10×TBE buffer (Tris-borate-EDTA), ThermoFisher Scientific) and the fluorescence emission intensity was recorded at 525 nm (excitation wavelength: 490 nm, optical path length was defined by the total volume in the well). The recorded signal was translated into dsDNA by means of a calibration curve carried out in solution with 0.5× PicoGreen® in 1× TBE buffer and dsDNA concentrations ranging from 0 to 500 ng/mL, using ITO wells.

Electrochemical/optical solid-phase HDA

The set of primers for isothermal solid-phase HDA amplification was designed starting from that described for PCR (FP and RP, Table S1), with minor modifications to bring them closer to the specific guidelines for HDA, i.e. primer size within the interval 24-33 nt, melting temperature (Tm) ranging from 60 to 74 °C, and GC% 35-60 %; considering specificity (Primer Blast[4]) and lack of complementary as well. As a result, FP_2 (24mer, Tm=68.5 °C, GC% of 58) and RP_2 (28mer, Tm=71.8 °C, GC% of 54) were selected, the latter being attached to the solid support through its 5' thiolated terminus with 10 thymines as spacer. Likewise, a shorter reverse primer, RP (22mer, Tm=62.1 °C, GC% of 50), was added to the solution.

Salmonella genome was amplified using the IsoAmp® II kit (BioHelix, Beverly, USA). The reactions were carried out in 50 µL total volume. Two separate reaction mixtures, A and B, were prepared. Mix A (25 µL) contained 11.25 µL of nuclease-free water, 2.5 µL of 10× annealing buffer, 3.75 µL 1 µM of forward primer, 2 µL 125 nM of reverse primer and 5 µL of *Salmonella* genome at several levels to obtain a final concentration between 10 and 10^6 GU. Mix B (25 µL), contained 6 µL of nuclease-free water, 2.5 µL of 10× annealing buffer, 1.5 µL of 100 mM $MgSO_4$, 3 µL of 500 mM NaCl, 3.5 µL of IsoAmp dNTP solution, 3.5 µL of IsoAmp enzymes mixture and 5 µL of 2.5% BSA in water. 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 new mixture was incorporated to each well. A transparent adhesive film, devised for real-time PCR measurements, was used to seal the wells, reducing the possibility of cross-contamination between thereof and the evaporation of samples. This amplification reaction was conducted in an oven at 65 ºC for 90 min.

After two washing steps with 1× PBS buffer and additional one with casein blocking buffer, the enzymatic labeling was performed in 50 µL of the blocking solution containing 2 U/mL of anti-fluorescein-AP conjugate for the electrochemical detection or 0.5 U/mL of anti-fluorescein-POD conjugate for the optical detection. After 30 min of incubation time, the surface was washed three times with 1× PBS buffer. For the electrochemical detection, the cell was covered

with 100 µL of 4 mM α-NPP in measuring buffer. After 10 min of enzymatic reaction, a DPV voltammogram from 0 to 0.8 V was recorded (modulation amplitude 50 mV, scan rate 10 mV/s). For the optical detection, the enzymatic reaction was started by adding 50 µL of TMB + H_2O_2 solution and stopped, after 5 minutes, with 50 µL 1 M of sulfuric acid to measure the absorbance at 450 nm.

SUPPORTING FIGURES

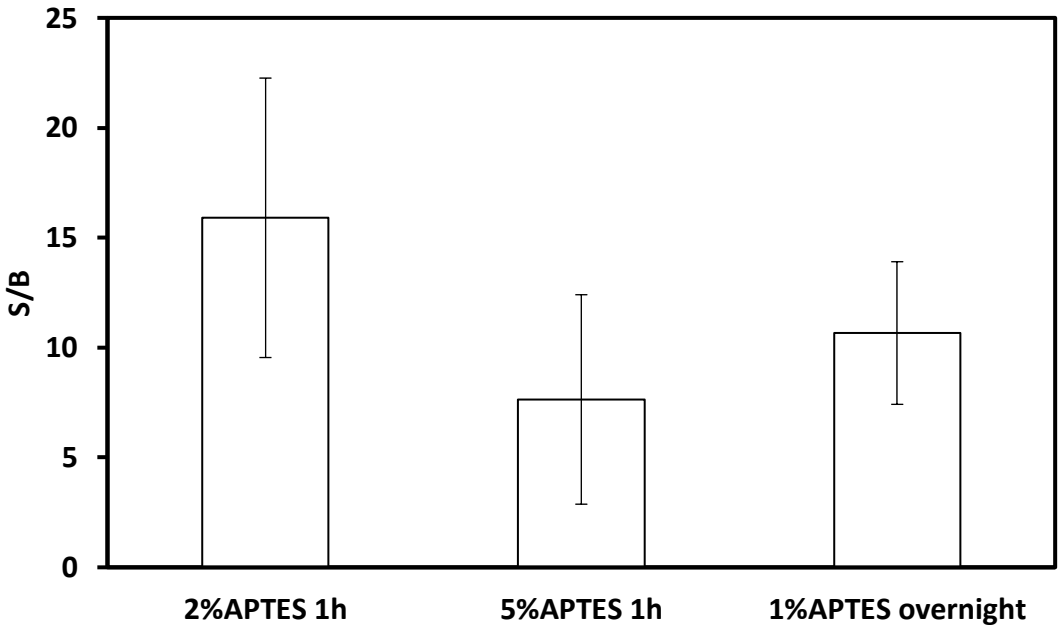

Figure S1: Silanization step optimization. Three different conditions for the silanization step with the amino silane compound were evaluated, modifying the concentration of silane and the reaction time. The surfaces were then modified with 10 µM capture probe and tested with and without 100 nM of 86 bp target. Though the highest signal/blank ratio was obtained with 2% APTES for 1 h, improved reproducibility was achieved with the lowest APTES concentration overnight.

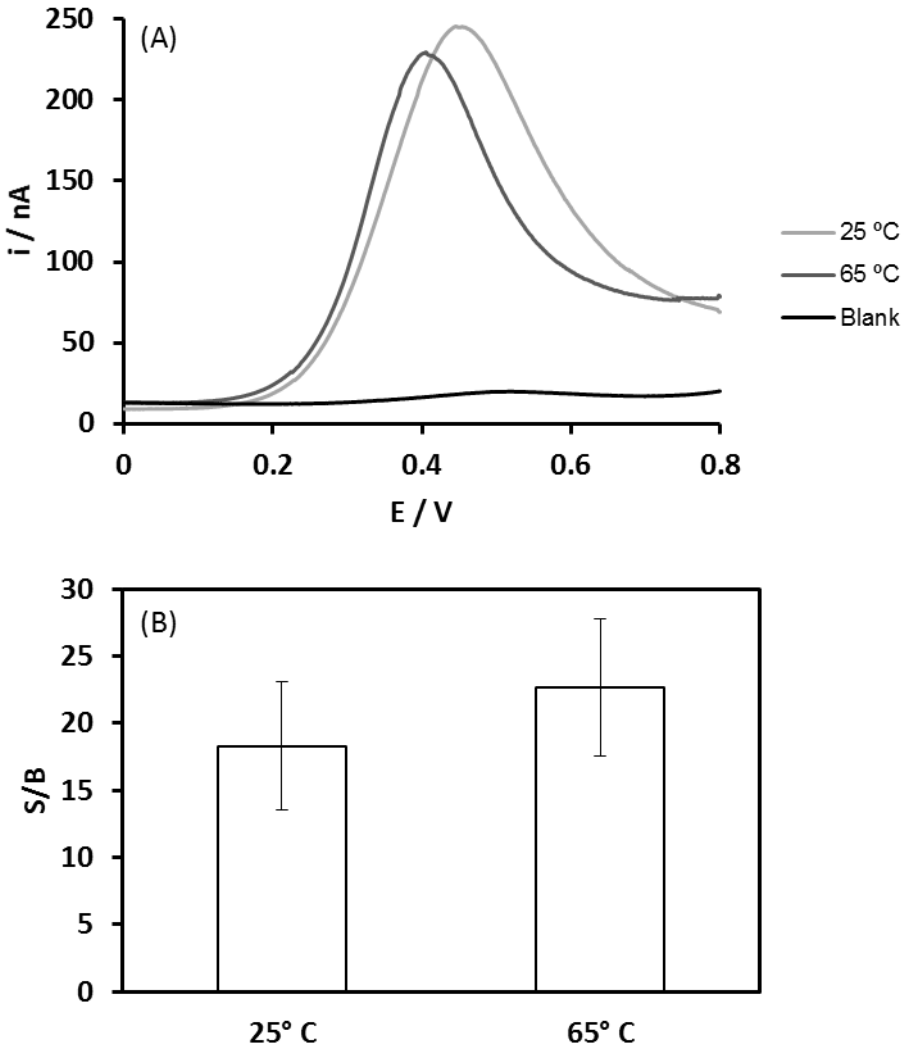

Figure S2: Thermal stability. (A) Differential pulse voltammograms and (B) signal to blank ratio obtained with the electrochemical sandwich assay in the presence of 100 nM 86 bp dsDNA target. Sensing surfaces built onto ITO slides were subjected to room temperature (25 ºC) or HDA temperature conditions (65 ºC) for 1 h.

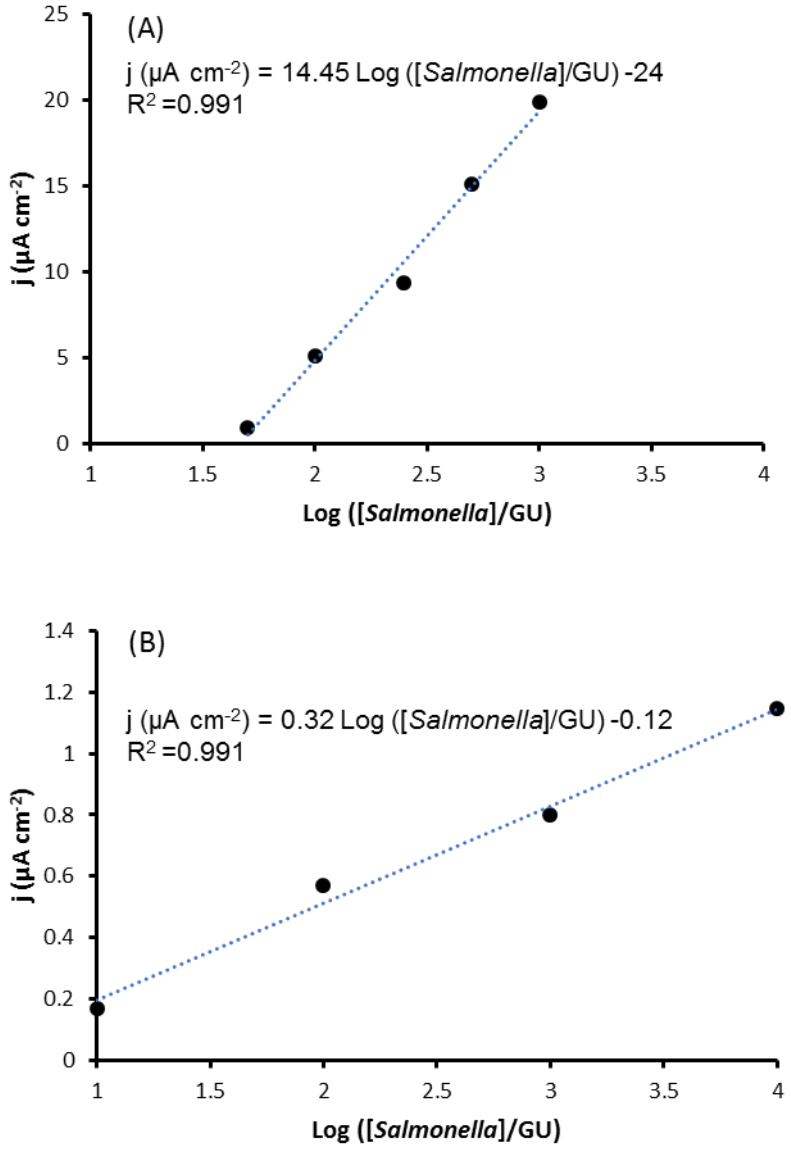

Figure S3: Electrochemical monitoring of HDA amplification for increasing amounts of *Salmonella* genome (A) liquid phase amplification with 75 nM of both reverse and forward primer, followed by the detection of amplicons by a sandwich assay on ITO electrodes (B) on-surface amplification with anchored RP_2 primer and a RP to FP_2 ratio 1:15 in solution.

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