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In situ DNA amplification with magnetic primers for the electrochemical detection of food pathogens

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Abstract

A sensitive and selective genomagnetic assay for the electrochemical detection of food pathogens based on *in situ* DNA amplification with magnetic primers has been designed. The performance of the genomagnetic assay was firstly demonstrated for a DNA synthetic target by its double-hybridization with both a digoxigenin probe and a biotinylated capture probe, and further binding to streptavidin-modified magnetic beads. The DNA sandwiched target bound on the magnetic beads is then separated by using a magneto electrode based on graphite–epoxy composite. The electrochemical detection is finally achieved by an enzyme marker, anti-digoxigenin horseradish peroxidase (HRP). The novel strategy was used for the rapid and sensitive detection of polymerase chain reaction (PCR) amplified samples. Promising resultants were also achieved for the DNA amplification directly performed on magnetic beads by using a novel magnetic primer, i.e., the up PCR primer bound to magnetic beads. Moreover, the magneto DNA biosensing assay was able to detect changes at single nucleotide polymorphism (SNP) level, when stringent hybridization conditions were used. The reliability of the assay was tested for *Salmonella* spp., the most important pathogen affecting food safety.

Keywords: Electrochemical DNA biosensor; Magnetic primer; HACCP; Real-time PCR; SNPs; *Salmonella* spp

1. Introduction

Salmonella spp. is one of the most frequently occurring foodborne pathogens affecting food safety. Since food regulatory agencies have established strict control programs in order to avoid food pathogens entering the food supply, official laboratories should be able to process – rapidly and efficiently – a high number of samples. According to these requirements, the development of rapid, inexpensive, sensitive, and high sample throughput and on-site analytical strategies, which can be used as an "alarm" to rapidly detect the risk of contamination by food pathogens in wide variety of food matrixes, is thus a priority, since traditional cultural methods require at least 3–4 days to provide presumptive results and additional 1–2 days for further biochemical confirmation (Tietjen and Fung, 1995).

In order to achieve the rapid detection of *Salmonella*, several methods mainly based on the bacterial genome as well as the antigenic composition of the cell membrane have been developed (Humphrey and Stephens, 2003). Nucleic acid-based detection has shown to be more specific and sensitive than immunological-based detection. Furthermore, the polymerase chain reaction (PCR) can be easily coupled to enhance the sensitivity of nucleic acid-based assays. Nucleic acid-based detection coupled with PCR has distinct advantages over culture and other standard methods for the detection of microbial pathogens such as specificity, sensitivity, rapidity, accuracy and capacity to detect small amounts of target nucleic acid in a sample. The further amplicon detection can be achieved with electrochemical DNA biosensors (Ye et al., 2003; Kara et al., 2004; Del Giallo et al., 2005), reducing the time of the assay and providing results to genetic specificity. The development of new materials such as magnetic beads has also brought unique opportunities to DNA detection strategies (Haukanes and Kvam, 1993). The DNA – as well as other biomaterial such

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as specific cells, antibodies, and enzymes – can be selectively bound to the magnetic beads and then separated from its biological matrix by using a magnetic field. Magnetic beads have been recently used in new strategies for electrochemical DNA genosensing (Wang and Kawde, 2002; Fojta et al., 2003; Palecek et al., 2004; Erdem et al., 2006) and immunosensing (Zacco et al., 2006) with improved sensitivity and selectivity.

In this work, different strategies for the electrochemical genomagnetic detection of DNA based on a graphite–epoxy composite magneto electrode (m-GEC) as electrochemical transducer are reported. Rigid conducting graphite–polymer composites and biocomposites (GECs) have been extensively used in our laboratories for electrochemical biosensing (Alegret, 1996; Pividori and Alegret, 2005), and genosensing (Pividori et al., 2001, 2003) with improved features over other traditional carbon-based materials, such as higher sensitivity, robustness and rigidity.

The novel electrochemical genomagnetic assay for the DNA target detection in a sandwich format performed in one-step relies on its hybridization with a: (i) biotinylated capture probe to achieve the immobilization on the streptavidin-modified magnetic bead and (ii) digoxigenin signaling probe to achieve the further electrochemical detection. The enzymatic labelling is thus achieved in a second step through the DNA probe modified with digoxigenin, by the antiDIG-HRP antibody. The selectivity of this electrochemical assay was studied by using a 'non-specific' target with one mismatch for each probe in order to detect single-point mutations related to SNPs (single nucleotide polymorphisms) (Miyahara et al., 2002; Tolley et al., 2003; Kerman et al., 2004). The utility of this novel electrochemical genomagnetic assay was also illustrated for the specific detection of an amplified sequence – by PCR – related to *Salmonella* spp. The amplicon was obtained from the IS200 insertion sequence (Lam and Roth, 1983), a transposable element of some 700 bp, being present in more than 90% of the pathogenic or food-poisoning isolates of *Salmonella* spp. (Gibert et al., 1990).

Several formats for the detection of the amplicon – all of them based on the electrochemical magneto biosensing assay – were developed in this work. The rapid electrochemical verification of the amplicon coming from the *Salmonella* genome was performed by double-labelling the amplicon during PCR with a set of two labelled PCR primers—one of them with biotin and the other one with digoxigenin. During PCR, not only the amplification of the *Salmonella* genome was achieved, but also the double-labelling of the amplicon ends with: (i) the biotinylated capture primer to achieve the immobilization on the streptavidin-modified magnetic bead and (ii) the digoxigenin signalling primer to achieved the electrochemical detection. Beside this double-labelling PCR strategy, a single labelling PCR strategy with a further confirmation of the amplicon by its hybridization was achieved by performing the PCR with the biotin primer and a further hybridization step with a digoxigenin probe.

Moreover, a PCR reactor for real-time electrochemical detection was also developed. In this case the amplification and double-labelling was directly performed on the streptavidin magnetic beads by using magnetic primers. The features of this new approach are compared with classical DNA analysis and other genosensing strategies.

2. Experimental

2.1. Instrumentation

The instrumentation (i.e. the amperometric controller, the three-electrode set-up, the temperature-controlled incubator, the magnetic separator, the scanning electron microscope) have been detailed described elsewhere (Zacco et al., 2006).

The PCR reaction was carried out in an Eppendorf Mastercycler Personal thermocycler.

2.2. Chemicals and biochemicals

Composite electrodes were prepared using 50 μm particle size graphite powder (BDH, UK) and Epotek H77 epoxy resin and hardener (both from Epoxy Technology, USA). The streptavidin-modified magnetic beads were Dynabeads M-280 Streptavidin Prod. no. 112.05 and were purchased from Dynal Biotech ASA (Oslo, Norway). For the detection of *Salmonella* spp. based on the specific IS200 element (Gene Bank accession AF025380), the oligomer sequences were: (i) target unique to the IS200 element in *Salmonella* spp. (IS200 target, 62 mer): 5′ CAC ACC CGA TGG AAC TGT AAA TAT CAC ATA GTT TTC GCG CCC AAA TAC CGA AGA CAA GCG TT 3′; (ii) biotinylated capture probe: 5′ GTG ATA TTT ACA GTT CCA TCG GG-biotin 3′; (iii) digoxigenin probe: 5′ DIG-CTT GTC TTC GGT ATT TGG GCG CG 3′. The specificity of the assay was shown with a IS200 sequence with one mismatched base for each probe, designed in order to be in the centre of the hybridized sequence, as follows: 5′ CAC ACC CGA TGG AAC T**T**T AAA TAT CAC ATA GTT TTC GCG CCC AAA TA**A** CGA AGA CAA GCG TT 3′.

Two primers, 23 and 21 nucleotides long, were designed for PCR amplification of IS200 sequence, in order to achieved: (i) an amplicon doubly labelled with biotin and digoxigenin at 5′ and 3′ ends, respectively, using a biotinylated and digoxigenin primer; (ii) an amplicon single-labelled with biotin using the biotinylated primer and a 'usual' primer—i.e. without any modification.

The primer sequences were: Biotinylated IS200 up: 5′ bio-ATG GGG GAC GAA AAG AGC TTA GC 3′, whose 5′ end is 194 bp from IS200 5′ end, DIG-IS200 down: 5′ DIG-CTC CAG AAG CAT GTG AAT ATG 3′ and IS200 down: 5′ CTC CAG AAG CAT GTG AAT ATG 3′, whose 5′ ends are 394 bp from IS200 5′ end.

The synthetic sequences were all purchased from TIB-MOLBIOL (Berlin, Germany).

The Expand High Fidelity PCR System Kit (Roche Molecular Biochemicals) was used for performing the PCR.

All other reagents as were of the highest available grade. AntiDigoxigenin-POD Fab fragments, Cat. No. 1207733, used as enzyme label was purchased from Roche Diagnostics GmbH (Mannheim, Germany).

Aqueous solutions were: standard saline citrate (20× SSC) (3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0); hybridization solution 5× SSC (with or without the addition of 0.5% w/v SDS and 50% v/v formamide); Tris Buffer (0.1 M Tris, 0.15 M NaCl, pH 7.5); blocking Tris buffer (2% w/v BSA, 0.1% w/v Tween 20 and 5 mM EDTA, in 0.1 M Tris, 0.15 M NaCl, pH 7.5).

2.3. DNA amplification of the IS200 element specific for Salmonella. The different strategies

Amplification and double labelling. The PCR was performed as explained in the Supporting information section, containing each labelled primer (Biotinylated IS200 up and DIG-IS200 down). As the primers were labelled with biotin and digoxigenin, the amplicon was expected to be labelled by both biotin and digoxigenin in each extreme, respectively (Fig. 1B).

Amplification and single labelling. The PCR was performed in identical conditions as explained for '*Amplification and double labelling*', but in this case, instead of the DIG-IS200 down primer, the IS200 down primer without any modification was used in order to obtain the single-labelled biotinylated amplicon.

PCR reactor. Amplification with magnetic primers. The magnetic primers were prepared by full covering of streptavidin-modified magnetic beads with the biotinylated IS200 up primer (Fig. 1C). The immobilization of 50 pmol biotinylated IS200 up primer was performed on variable quantities of magnetic beads (6.4×10^6, 3.2×10^6, 1.6×10^6, 0.7×10^6 and 0.3×10^6). The amount of magnetic beads able to bind 50 pmol of 'biotinylated IS200-up primer', as described by the manufacturer, is 1.6×10^7 streptavidin-modified magnetic beads. The magnetic primers were prepared in a final volume of 140 μl MiliQ water at 42 °C for 15 min with gentle stirring. A separation step was then performed by applying an external magnet in order to

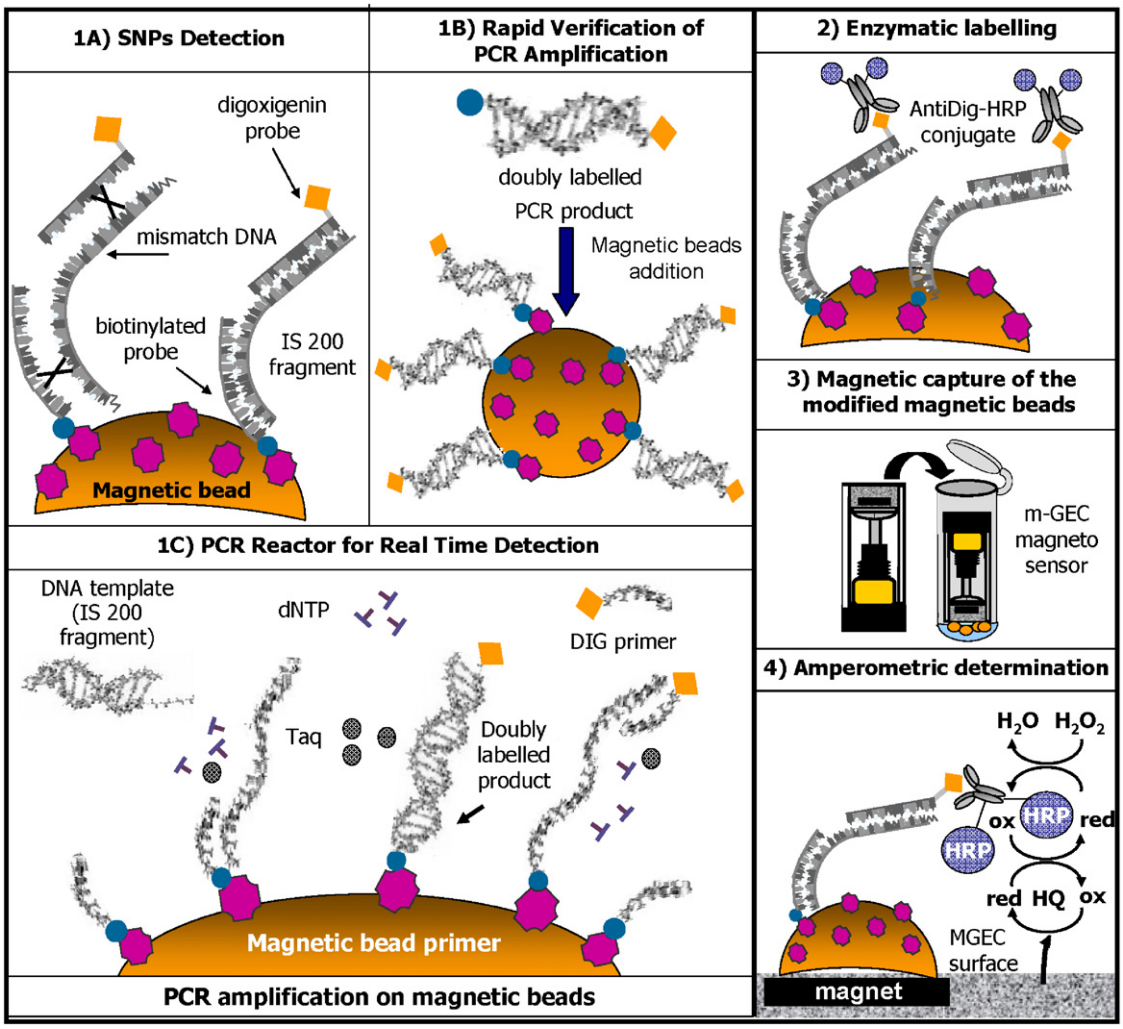

Fig. 1. Schematic representation of the electrochemical strategy for the detection of *Salmonella* spp. (A) One-step procedure based on immobilization of the biotinylated probe onto magnetic beads and hybridization with the ssDNA target, (B) rapid verification of PCR amplification based on the doubly labelled PCR product detection and (C) real-time PCR reactor based on PCR amplification with magnetic primers on streptavidin-modified magnetic beads. Enzymatic labelling (2), magnetic capture of the modified magnetic beads by the magneto electrode (m-GEC) (3), and chronoamperometric determination (4), are common steps for all of these strategies (A–C).

recover the full-covered magnetic beads (the magnetic primer) from the supernatant containing the excess of biotinylated IS200 up primer. Washing steps with 140 μl MiliQ water for 10 min at 42 °C were then performed twice. After that, the different amounts of magnetic primer were introduced into PCR amplification as the IS200 up primer reagent. The PCR reaction was performed as explained in the Supporting information section. The amplicon was expected to be doubly labelled with magnetic beads attached to the biotinylated end (Fig. 1C).

The non-specific amplification reaction was performed – if any – by processing as negative control the PCR reaction performed with all the reagents except the DNA *Salmonella* template in the PCR mixture. Those blank controls were used in order to evaluate the non-specific adsorption in the electrochemical magneto biosensing assay.

2.4. Construction of the magneto graphite–epoxy composite (m-GEC) transducer and SEM characterization

The construction of the Magneto Graphite–epoxy composite (m-GEC) transducer is explained in detail in the Supporting Information section. The scanning electron microscopy (SEM) technique was used to evaluate the distribution of the magnetic beads on the surface of the m-GEC. The SEM was performed as explained in the Supporting information section.

2.5. Electrochemical DNA sequence-specific detection based on m-GEC and enzyme labelling

The protocol consists briefly of the following steps (Fig. 1A). (i) *One-step immobilization/hybridization procedure* in which the biotin-labelled capture probe is immobilized on the streptavidin magnetic beads, while the hybridization with the target and with a second complementary probe – in this case labelled with digoxigenin – is occurring at the same time; (ii) enzymatic labelling using as enzyme label the antibody anti–Dig–HRP; (iii) magnetic capture of the modified magnetic beads; (iv) amperometric determination based on the enzyme activity by adding H_2O_2 and using hydroquinone as a mediator (Fig. 1, steps 1A, 2, 3 and 4).

The detailed experimental procedure is described in the Supporting information section. After the final washing steps, the modified magnetic beads were captured by dipping the electrode inside the reaction tube.

The evaluation of the non-specific adsorption was performed by adding all reagents except the DNA target.

2.6. SNPs electrochemical detection based on m-GEC and enzyme labelling

The selectivity of the new electrochemical genomagnetic assay was evaluated by comparing the signal obtained with an interfering probe consisting of the IS200 sequence with one mismatch for each probe (Fig. 1, steps 1A, 2, 3 and 4). In this case, the electrochemical magneto biosensing strategy was performed with both the target and the mismatch target, with 5× SSC as hybridization buffer and under stringent conditions in order to improve selectivity (Pividori et al., 2001; Hernández-Santos et al., 2004). To achieve this purpose, 50% formamide in the hybridization buffer was used.

2.7. Rapid electrochemical verification of PCR amplification of Salmonella

The protocol consists briefly of the following steps: (i) DNA amplification and double-labelling of Salmonella IS200 insertion sequence; (ii) immobilization of the doubly labelled amplicon in which the biotin extreme of the ds-DNA amplicon was immobilized on the streptavidin magnetic beads; (iii) enzymatic labelling using as enzyme label the antibody anti-Dig-HRP able to bond the other labelled end of the ds-DNA amplicon; (iv) magnetic capture of the modified magnetic beads; (v) amperometric determination (Fig. 1, steps 1B, 2, 3 and 4).

After the DNA Amplification and Double Labelling of the IS200 element specific for *Salmonella* spp., a dilution of the amplified product coming directly from the PCR was prepared in 5× SSC. The Immobilization of the doubly labelled amplicon was achieved by adding the amplicon to 6.2×10^6 streptavidin magnetic beads. The immobilization was performed in 5× SSC solution at a final volume of 140 μl for 30 min at 42 °C. All the experimental conditions for the following steps were performed as detailed explained in the Supporting information section.

2.8. Specific electrochemical detection of the Salmonella PCR amplification product

In this case, the protocol consists briefly of the following steps: (i) DNA amplification and single-labelling of Salmonella IS200 insertion sequence; (ii) immobilization of the single-labelled amplicon in which the biotin extreme of the ds-DNA amplicon was immobilized on the streptavidin magnetic beads; (iii) amplicon denaturation by increasing the temperature at 95 °C; (iv) hybridization with a second DIG-IS200 probe; (v) enzymatic labelling using as enzyme label the antibody anti-Dig-HRP able to bond the other labelled extreme of the ds-DNA amplicon; (vi) magnetic capture of the modified magnetic beads; (vii) amperometric determination.

The detailed experimental procedure is described in the Supporting information section. After the final washing steps, the modified magnetic beads were captured by dipping the electrode inside the reaction tube.

A variation of this protocol was performed by changing the order of the steps. In this case, the protocol was performed as follows: (i) DNA amplification and single-labelling of Salmonella IS200 insertion sequence; (ii) amplicon denaturation by increasing the temperature at 95 °C; (iii) hybridization with a second DIG-IS200 probe; (iv) immobilization of the single-labelled amplicon in which the biotin extreme of the ds-DNA amplicon was immobilize on the streptavidin magnetic beads; (v) enzymatic labelling using as enzyme label the antibody anti-Dig-HRP able to bond the other labelled extreme of the ds-DNA amplicon; (vi) magnetic capture of the modified magnetic beads; (vii) amperometric determination.

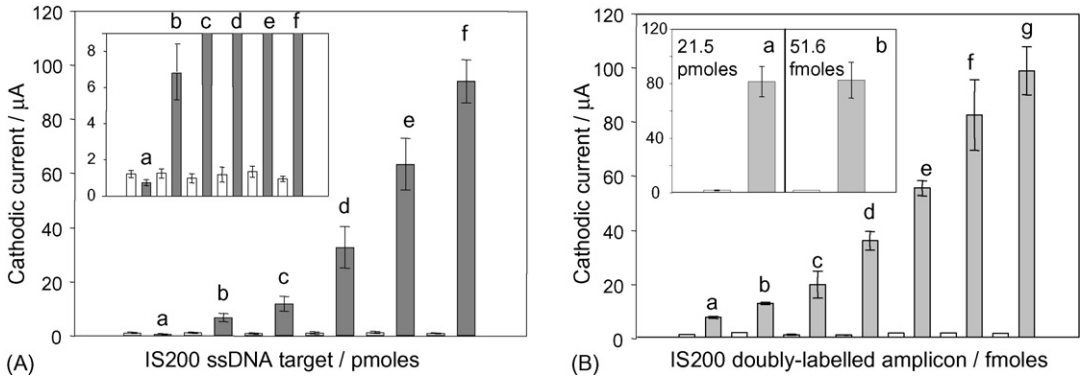

Fig. 2. (A) Electrochemical DNA sequence-specific detection with increasing amounts of IS200 sequence specific of *Salmonella*. Gray bars show the increasing amount of DNA IS200 target specific for *Salmonella* (a: 0.9 pmol; b: 2.1 pmol; c: 3.8 pmol; d: 9.0 pmol; e: 16.6 pmol; f: 45 pmol), while white bars show the corresponding non-specific adsorption. In all cases, 205 pmol biotinylated probe, 225 pmol digoxigenin probe, 60 μg AntiDig-HRP and 1.6×10^6 magnetic beads were used. (B) Rapid electrochemical verification of PCR amplification of *Salmonella enterica serovar Typhimurium* ATCC 14028 with the doubly labelled IS200 primer set. Gray bars show the signal by increasing the amount of IS200 doubly labelled amplicon (from 2.8 to 75.4 fmol), performed with the following dilution of the doubly labelled PCR final product (a: 1/400; b: 1/200; c: 1/100; d: 1/50; e: 1/30; f: 1/20; g: 1/15) simulating a increasing number of PCR cycles while white bars show the total electrochemical signal processing the negative PCR control. The frame within (B), right, shows the electrochemical signal achieved by 51.6 fmol of *Salmonella* amplicon following the rapid electrochemical verification of PCR amplification and, left, by 21.5 pmol of DNA target following the DNA sequence-specific detection. In all cases 60 μg antiDig-HRP and 6.2×10^6 magnetic beads were used. Other experimental details are: phosphate buffer 0.1 mol L^{-1}, KCl 0.1 mol L^{-1}, pH 7.0; mediator, hydroquinone 1.81 mmol L^{-1}; substrate, H_2O_2 4.90 mmol L^{-1}; applied potential = −0.1 V (vs. Ag/AgCl). All data are given as average ± S.D. In all cases, $n = 3$.

2.9. PCR reactor for the real-time electrochemical detection of Salmonella

The protocol consists briefly on the following steps: (i) *in situ* DNA amplification and double-labelling of Salmonella IS200 insertion sequence on streptavidin-modified magnetic beads by using a magnetic primer; (ii) enzymatic labelling using as enzyme label the antibody anti-Dig-HRP able to bond the other labelled extreme of the ds-DNA amplicon; (iii) magnetic capture of the modified magnetic bead; (iv) amperometric determination (Fig. 1, steps 1C, 2, 3 and 4). The detailed experimental procedure is described in the Supporting information section.

2.10. Amperometric determination

The 'Amperometric determination' is based on the activity of the HRP enzyme of the marker, as previously reported (Pividori et al., 2001, 2003), by using hydroquinone as a mediator and H_2O_2 (the HRP substrate), which was added to a final concentration of 4.90 mmol L^{-1}. Fig. 1, step 4 represents the reactions that occur at the m-GEC sensors surface polarized at −0.100 V (versus Ag/AgCl) upon H_2O_2 addition in the presence of hydroquinone.

3. Results and discussion

The results for SEM characterization of m-GEC modified with the magnetic beads are shown in the Supporting information section.

3.1. Electrochemical DNA sequence-specific detection based on m-GEC and enzyme labelling

The chronoamperometric response of the magneto DNA biosensors with increasing amounts of DNA target (from 0.9 to 45 pmol) is shown in Fig. 2A, while the concentration of biotinylated probe, digoxigenin probe, AntiDig-HRP and magnetic beads remaining constant. The signal clearly increases when increasing the amount of IS200 sequence related to *Salmonella* spp. (gray bars). Moreover, the contribution of the non-specific adsorption was constant and almost negligible (white bars). The non-specific adsorption is mostly related to the adsorption of the enzyme conjugate. The inset in Fig. 2A, shows detailed the limit of detection region. The lowest amount of analyte producing a useful analytical signal was found to be 2.1 pmol.

3.2. SNPs electrochemical detection based on m-GEC and enzyme labelling

The selectivity of the new electrochemical magneto biosensing assay was evaluated by comparing the signal obtained with an interfering probe consisting of the IS200 sequence with one mismatch for each probe. As shown in Fig. 3A, similar results were obtained for the two sequences when using 5× SSC as hybridization buffer. When adding 50% (v/v) formamide to the buffer, it was possible to clearly distinguish the two mismatches bases, as shown in Fig. 3B. The mismatch sequence produced an 80% lower analytical signal in comparison with the full matching nucleotide. The use of stringent experimental conditions decreased the analytical signal up to 10 times, obtaining less sensibility with the assay but distinguishing a single-point mutation in DNA.

3.3. Rapid electrochemical verification of PCR amplification of Salmonella

The electrochemical magneto biosensing strategy was applied for the rapid detection of PCR amplification of *Salmonella* spp., using a previously amplified doubly labelled

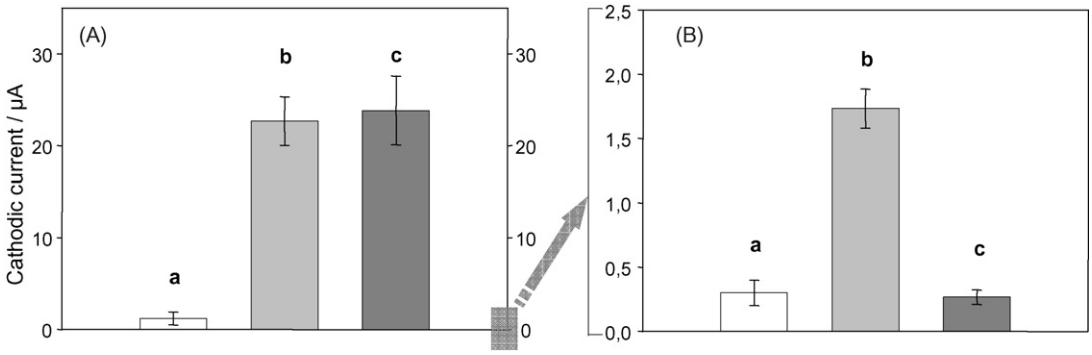

Fig. 3. Selectivity study performed with (A) 5× SSC as hybridization solution and with (B) 50% (v/v) formamide in 5× SSC as hybridization solution. Schematic representation: (a) non-specific adsorption (omitting addition of the DNA target), (b) 22.1 pmol of IS200 DNA target and (c) 22.1 pmol two-bases mismatch IS200 sequence. In all cases: 205 pmol biotinylated probe, 225 pmol digoxigenin probe, 60 μg antiDig-HRP and 1.6×10^6 magnetic beads. Other experimental details as Fig. 2.

PCR product with biotin and digoxigenin in each DNA end.

This primer set amplified exclusively the IS200 insertion sequence, producing only the expected 201 pb fragment according to the agarose gel electrophoresis (shown in the Supporting information section). The amplicon was immobilized on the streptavidin-modified magnetic beads and then labelled with the antibody AntiDig-HRP by means of the double-labelling with biotin and digoxigenin, respectively. The amperometric response of the doubly labelled product was then evaluated. Fig. 2B shows the calibration plot obtained with different dilutions of the doubly labelled PCR final product (1/400, 1/200, 1/100, 1/50, 1/30, 1/20 and 1/15) simulating an increasing number of PCR cycles. The plot is represented against the PCR amplicon concentration determined spectrophotometrically at 260 nm. A linear range for the quantification of the PCR amplicon is observed from 2.8 to 75.4 fmol giving a sensitivity of 1315 μA per decade and a regression coefficient of 0.980. The non-specific adsorption, performed with a PCR blank control omitting the addition of *Salmonella* template during the amplification, was negligible for all the evaluated concentration range. This kind of assay is useful when the set of primers for PCR amplifies specifically the sequence of interest. In this case, the PCR reaction produces only the expected 201 pb fragment according to the agarose gel electrophoresis. The amplified product can be rapidly and sensitively detected by this strategy. The inset Fig. 2B, shows comparatively that an amperometric signal of almost 80 μA was obtained with 51.6 fmol of doubly labelled PCR product with the 'Rapid Electrochemical Verification Strategy', while the same signal was achieved with 21.5 pmol of IS200 target by the 'Electrochemical DNA sequence-specific detection based on m-GEC Strategy'. In both cases, R.S.D. of 16% and 14%, respectively, were obtained. The latter is a sandwich format whereon both a biotinylated and digoxigenin probes detected the synthetic DNA target by its double hybridization, as previously explained. The loss in sensitivity up to 1000 times for *Salmonella* by using the sandwich format can be explained because two hybridizations were performed during the assay, with both the biotin and digoxigenin probes whereas higher signals were obtained when no hybridization steps were performed in PCR amplicon detection. However, regarding the selectivity, the sandwich format is able to detect a single mismatch, as shown in Fig. 3.

3.4. Specific electrochemical detection of the Salmonella PCR amplification product

A specific detection of the PCR product was achieved by a further hybridization step with a signalling probe. In this case, the PCR was performed with a set of primer being the 'up' primer modified with biotin, in order to obtain a biotinylated amplicon in the 5′ end. The single-labelled and double-stranded amplicon was then submitted to high temperature (95 °C) to produce the double strand denaturalization. After this step, the biotinylated strand of the amplicon remained bonded to the magnetic bead and was able to be hybridized with the digoxigenin-modified signalling probe, as shown in Fig. 4B(i). The corresponding electrochemical signal is shown in Fig. 4A(i), which shows a big signal due to the non-specific adsorption. This higher level of non-specific adsorption was suspected to be produced by a change in the protein conformation of the streptavidin magnetic bead due to the denaturing step at 95 °C, which produce different interactions with the reagents. To confirm that issue, the order of the steps in this assay was changed. Firstly, the single-labelled PCR product was submitted to a denaturalization step, and then to the hybridization with the DIG-IS200 probe in order to avoid the heating of the magnetic beads. Finally the magnetic beads were added in order to attach the amplicon (Fig. 4B(ii)). As can been seen in Fig. 4A(ii), the new assay permits an improved identification between the negative PCR control and the amplified product. The signal-to-non-specific adsorption ratio increase from 1.36 (i) to 4.00 (ii).

This strategy is useful when the set of primers for PCR amplifies not only the sequence of interest but also other non-specific fragments, which are detected by agarose gel electrophoresis as a line with several bands, i.e., several products with different MW. In this case, it is necessary to confirm that the expected fragment was amplified. This confirmation is normally performed by means of a second hybridization in order to determine the internal sequence of the amplicon.

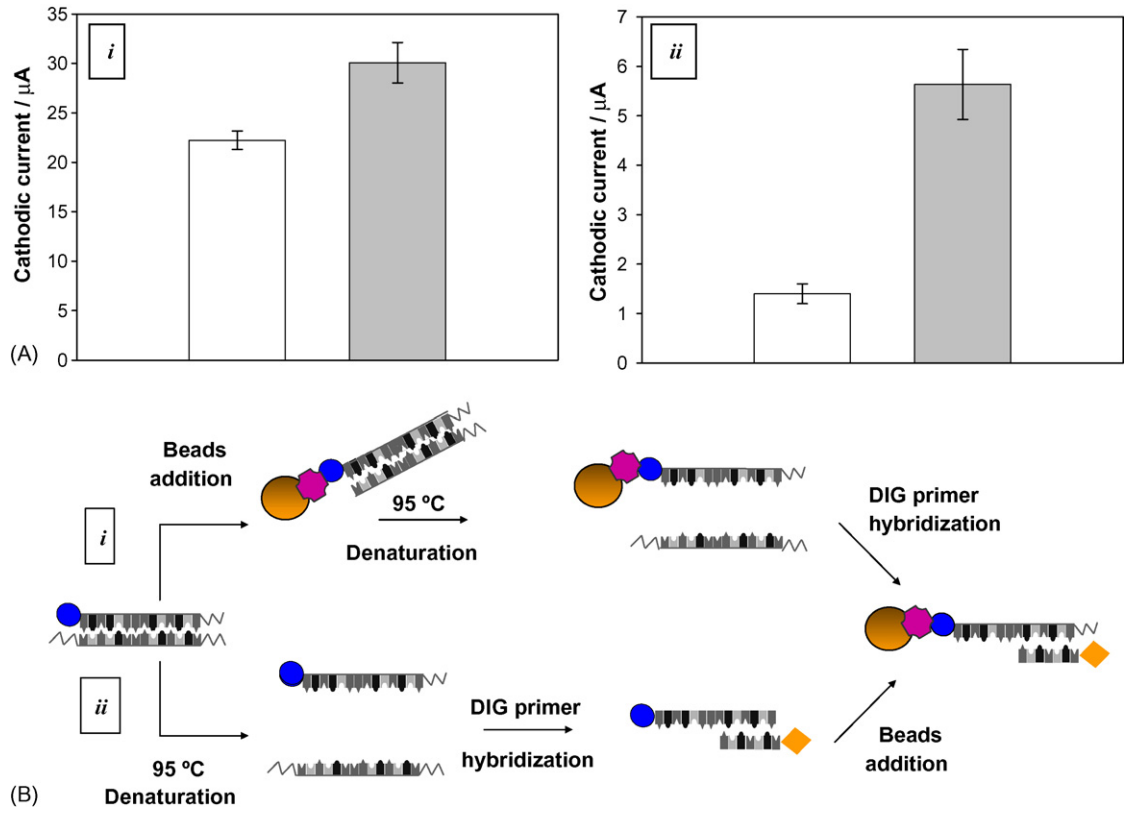

Fig. 4. (A) Specific electrochemical signal based on the single-labelled PCR amplification of *Salmonella enterica serovar Typhimurium ATCC 14028*, performed by: (i) heating magnetic beads during the denaturation step and (ii) addition of magnetic beads after the denaturation step. In both cases white bars show the non-specific signal coming from the negative PCR control, while gray bars show the signal of the single-labelled amplified product hybridized with a second DIG-IS200 probe. 225 pmol digoxigenin probe, 60 μg AntiDig-HRP and 6.2×10^6 magnetic beads were used. Other experimental details as in Fig. 2. (B) Schematic representation of the specific electrochemical detection of the *Salmonella* PCR single-labelled amplification product.

3.5. PCR reactor for the real-time electrochemical detection of Salmonella

A novel PCR assay was done by adding the streptavidin-modified magnetic beads fully covered by the biotinylated IS200 up primer (the 'magnetic primer'), to the PCR reaction, as shown in Fig. 1C. A doubly labelled PCR product attached to the magnetic beads was thus achieved by *in situ* amplification of the DNA template on the magnetic beads. Fig. 5 shows the amperometric response achieved by the doubly labelled amplicon on the magnetic beads. Each point of the plot corresponds to different PCR amplification assays, using increasing amount of magnetic beads (2.5, 5.0, 12.0, 25.0 and 49.0 μg), respectively. As can be seen, the enzymatic signal increases with an increment of the amount of magnetic beads. An increasing signal is observed up to 12.0 μg of magnetic beads reaching a plateau with higher amount of magnetic beads. The non-specific adsorption increases linearly with the amount of magnetic beads (Fig. 5, white dots). Nevertheless, there is a clear difference in the electrochemical signal between the PCR amplification of the sample and the negative PCR control (omitting the addition of DNA template). The linear increment in the non-specific adsorption signal with the amount of magnetic beads can be explained by a modification in the structure of the magnetic beads, when submitted to high temperatures during the PCR cycles, as previously explained.

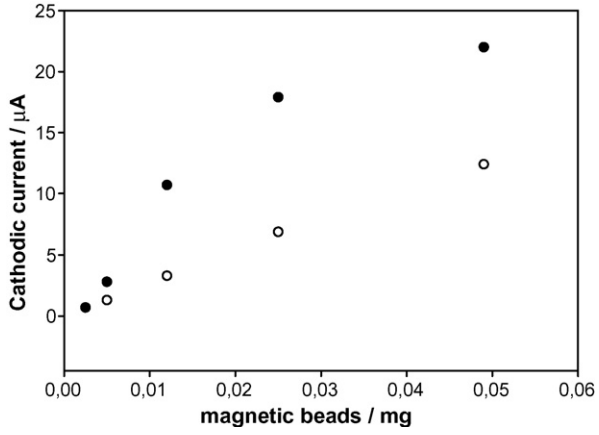

Fig. 5. PCR reactor for the real-time electrochemical detection of *Salmonella enterica serovar Typhimurium ATCC 14028* based on the doubly labelled PCR amplification performed with the magnetic primer. White dots show the non-specific electrochemical signal processing the negative PCR control, while the black dots show the increasing signal of DNA IS200 doubly labelled amplicon onto magnetic beads. Sixty micrograms AntiDig-HRP was used. Other experimental details as Fig. 2.

4. Conclusions

A novel electrochemical genomagnetic strategy for the DNA detection has been developed. The utility of this novel strat-

egy was demonstrated for the detection of a PCR amplicon of the IS200 insertion sequence coming from real samples of *Salmonella* spp. in different PCR formats.

The use of magnetic beads provided improved features regarding sensitivity and selectivity of the assay. Streptavidin-modified magnetic beads easily immobilized the DNA hybrid on the m-GEC transducer, avoiding difficult and time-consuming immobilization procedures as those previously reported. Moreover, the magnetic beads provided an easy way for completely removing the non-specific adsorption.

On the other hand, the magneto biosensing strategy in a sandwich format performed under stringent conditions allowed an efficient way to detect a single mismatch for each probe. Therefore the assay can be used for the easy, cheap and rapid detection of SNPs.

The rapid and very sensitive verification of the PCR amplicon related to *Salmonella* was achieved with 2.8 fmol of amplified product. This strategy can be used for the electrochemical real-time quantification of amplicon since a linear relationship with the amount of amplified product was obtained. This strategy is only useful when a unique and specific band is observed by gel electrophoresis, because of the high specificity of the set of primers being used in the PCR for the amplification of the *Salmonella* genome. On the contrary, if the set of primers amplifies not only the sequence of interest but also other non-specific fragments, it is necessary to confirm the internal sequence of the amplicon by a second hybridization with a digoxigenin signalling probe. In this case, a single labelling with biotin during PCR was performed followed by a further selective hybridization with a digoxigenin signalling probe.

Moreover, magnetic primers were used for *in situ* amplification on magnetic beads of the *Salmonella* genome and the further electrochemical detection of the amplified product. The DNA amplification on magnetic beads by using the magnetic primer with electrochemical detection using m-GEC electrodes results in a rapid, cheap, robust and environmentally friendly device which would allow the detection of pathogenic species on food samples for *Hazard Analysis Critical Control Point* (HACCP) programs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2006.08.048.

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