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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).