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Disposable Magnetic DNA Sensors for the Determination at the Attomolar Level of a Specific *Enterobacteriaceae* Family Gene

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Disposable magnetic DNA sensors using an enzyme-amplified strategy for the specific detection of a gene related to the *Enterobacteriaceae* bacterial family, based on the coupling of streptavidin-peroxidase to biotinylated *lacZ* gene target sequences, has been developed. A biotinylated 25-mer capture probe was attached to streptavidin-modified magnetic beads and hybridization with the biotinylated target was allowed to proceed. Then, a streptavidin-peroxidase polymer was attached to the biotinylated target, and the resulting modified magnetic beads were captured by a magnetic field on the surface of tetrathiafulvalene (TTF) modified gold screen-printed electrodes (Au/SPEs). The amperometric response obtained at −0.15 V after the addition of hydrogen peroxide was used to detect the hybridization process. In order to improve the sensitivity of the determination and reduce the assay time, different variables of the assay protocol were optimized. A low detection limit (5.7 fmol) with good stability (RSD = 7.1%, n = 10) was obtained. The DNA nonspecific adsorption at the magnetic beads was negligible, the obtained results thus demonstrating the possibility to detect the hybridization event with great specificity and sensitivity. The developed method was used for the analysis of *Escherichia coli* DNA fragments (326 bases) in polymerase chain reaction (PCR) amplicons extracted from a cell culture. As low as 2.5 aM asymmetric PCR product could be detected with the developed methodology.

Detection of pathogenic bacteria is of great importance from the health and safety point of view. The most popular analytical methods used in pathogen detection still are those based on culture and colony counting methodologies because, although time-consuming, they offer good sensitivity, selectivity and reliability.[1] However, the need for 7−8 days to yield analytical results by these methods has resulted in the search for new rapid methods based on the use of novel technologies. Although biosensors are of great interest in this field, revealing equally reliable results in much shorter times, they still need to reach at least the same detection levels as traditional methodologies (10−100 cfu mL^{-1}). In particular, DNA biosensors, also called genosensors, based on the integration of a sequence-specific probe (usually a short synthetic oligonucleotide) and an electrochemical signal transducer are nowadays considered as the most attractive due to their simplicity, low instrumentation costs, and possibility for real-time accurate detection with very low detection limits.[2] Moreover, PCR amplification of the sample is still necessary due to the low abundance and extreme complexity of the nonamplified targets, and improved probe designs and sample pretreatments must be developed to allow efficient biorecognition events to occur at the transducer−solution interface.

Magnetic beads (MBs) offer a versatile tool for electrochemical DNA and protein biosensing. In fact, superparamagnetic particles provide a large surface area for nucleic acid attachment, that can thus be easily separated from the liquid phase with a small magnet and again dispersed immediately after the magnet is removed.[3,4] Furthermore, nonspecifically bound beads can be automatically removed via magnetically controlled washing.[5]

MBs modified with different recognition elements have been used for specific bioaffinity capture of different molecules. Thus, they have been applied in the fields of DNA hybridization detection,[6−10]

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immunoassay,[11−14] protein and enzyme immobilization,[15,16] cell separation,[17] and drug delivery.[18] As for its applications in DNA hybridization, apart from MBs-based electrochemical sensing methods,[6−8,10] recently gold nanoparticles-modified MBs[9,19,20] have been described. The earliest papers on the use of MBs in the electrochemical detection of DNA hybridization already emphasized the utility of these particles for discriminating against unwanted constituents of the samples, such as large excesses of noncomplementary chains and proteins.[21] Paleček et al.[22] proposed a new methodology consisting on performing DNA hybridization at one surface (MBs) and electrochemical detection at a different surface, the detection electrode, in order to minimize nonspecific adsorption of DNA, which causes serious difficulties especially in the case of the analysis of long target DNA chains. The application of MBs in the development of DNA hybridization sensors has recently been reviewed,[23] the major drawback of these methodologies consisting in the lack of simple, nonexpensive, and practical devices for MBs manipulation within these sensors.

The *Enterobacteriaceae* bacterial family, e.g. by *Escherichia*, *Enterobacter*, and *Klebsiella* genera, are facultative anaerobic, gram-negative, nonendospore forming, rod-shaped bacteria that are usually called enterics or coliforms which reflects their association with the intestinal tracts of humans and other animals.[24] Conventional coliform monitoring is based on the detection of β-galactosidase as the gene product of *lacZ* gene produced by coliform bacteria.[25] This is the basis for commercial colorimetric tests, such as the IDEXX Colilert, a U.S. EPA-approved test included in the Standard Methods for Examination of Water and Wastewater.[26] Detection of *E. coli* and other coliforms in food is highly relevant, and a number of recent food outbreaks in the USA, reported to Centres for Disease Control and Prevention (CDC)[27] involving the presence of *E. coli* in fresh spinach, lettuce, frozen ground beef patties, frozen pizza, and beef meat, have occurred in the past few years. Another route for infection is via swimming in contaminated water. The BEACH Act requires coastal and Great Lakes states to establish pathogen indication monitoring.[28] In this work, an enzyme-amplified strategy based on the coupling of the biotinylated *lacZ* gene synthetic target sequences to a streptavidin-peroxidase polymer has been developed. Thus, a biotinylated 25-mer capture synthetic probe was attached to streptavidin-modified magnetic beads, and hybridization with the biotinylated target was allowed to proceed. After binding the target to a streptavidin-peroxidase polymer, the resulting modified MBs were captured by a magnetic field on tetrathiafulvalene (TTF) modified gold screen-printed electrodes (Au/SPEs). The amperometric response obtained at −0.15 V after the addition of hydrogen peroxide was used to detect the hybridization process. The applicability of the developed method was tested by analyzing *E. coli* DNA fragments (326 bases) PCR amplicons from a cell culture.

EXPERIMENTAL SECTION

Apparatus and Electrodes. Amperometric measurements were carried out with an ECO Chemie Autolab PSTAT 10 potentiostat (KM Utrecht, The Netherlands) using the software package GPES 4.9 (General Purpose Electrochemical System). A P-Selecta (Scharlab, Madrid, Spain) ultrasonic bath, an Optic Ivymen System constant temperature incubator shaker (Comecta S.A, distributed by Scharlab, Madrid, Spain), and a P-Selecta Agimatic magnetic stirrer were also used.

Gold screen printed electrodes (220AT, 4 mm diameter, Au/SPEs) purchased from Dropsens (Oviedo, Spain) were used. The format of these screen printed electrodes includes a silver pseudoreference electrode and a gold counter electrode.

A neodymium magnet (AIMAN GZ, Madrid, Spain) was used to control the attraction of the modified magnetic beads to the modified Au/SPEs surfaces. The magnetic separation during the washing steps was performed using a Dynal MPC-S (product no. 120.20, Dynal Biotech ASA, distributed by Oxoid, Madrid, Spain) magnetic separator.

Reagents and Solutions. Streptavidin-modified magnetic beads (10 mg mL^{-1}) (Dynabeads M-280 Streptavidin, product no. 112.05D) were purchased from Dynal Biotech ASA. Streptavidin-peroxidase polymer, ultrasensitive, (streptavidin-HRP), and hydrogen peroxide were purchased from Sigma (Spain) and used as received. Stock solutions of streptavidin-HRP (10 μg mL^{-1}) were prepared in a saline 0.01 M phosphate buffer solution containing 0.05% w/v Tween 20 (Aldrich, Spain), 0.138 M NaCl (Scharlau, Spain), and 0.0027 M KCl (Scharlau) (PBST, pH 7.4). Stock solutions of H_2O_2 (1 mM) were prepared in a saline 0.1 M phosphate buffer solution containing 0.138 M NaCl and 0.0027 M KCl (PBS, pH 7.4). More dilute solutions were prepared by suitable dilution with the same buffer solution.

The tested oligomers were synthesized by Sigma Genosys. Their base sequences appear in Table 1. These sequences were selected from the literature data,[25,29,30] where these oligonucleotides were used to detect coliform bacteria by PCR. The 25-mer one base mismatch strain is a mutant of the 25-mer target DNA,

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Table 1. Sequences of Oligonucleotides Used in the Sensor Development

oligonucleotide	sequence
biotinylated probe	5′-biotin-CAGGATATGTGGCGGATG AGCGGCA-3′
biotinylated target	5′-TGCCGCTCATCCGCCACATATCCTG-biotin-3′
biotinylated noncomplementary	5′-GGCCATCGTTGAAGATGCCTCTGCC-biotin-3′
biotinylated one base mismatch	5′-TGCCGCTCATCAGCCACATATCCTG-biotin-3′
lacZ forward primer	5′-ATGAAAGCTGGCTACAGGAAGGCC-3′
lacZ reverse primer	5′-biotin-GGTTTATGCAGCAACGAGACGTCA-3′

with one base changed (as underlined). Furthermore, Table 1 also includes the primers employed for the amplification of a 326-base region of the *E. coli lacZ* gene using asymmetric PCR.

DNA stock solutions (nominally 100 μM) were prepared in a buffer solution containing 10 mM Tris-HCl (Sigma), and 1 mM EDTA (Sigma) (TE, pH 8.0). More dilute solutions of the oligomers were prepared in a 50 mM Tris-HCl (Scharlau) and 20 mM NaCl buffer solution (Tris-HCl, pH 7.2). The final concentration of the biotinylated probe solutions was determined by UV−vis molecular absorption spectrometry using a Thermo Scientific NanoDrop 1000 Spectrophotometer (NanoDrop Technologies, Inc.). Thus, these concentrations (in μg mL^{-1}) were calculated from the absorbance measurement at 260 nm, taking into account the quality data sheet given by the supplier, and following the expression:

$$C = A_{260}\mathrm{OD}_{260}D_{\mathrm{f}} \quad (1)$$

where OD_{260} is the optical density (1 OD_{260} = 50 μg mL^{-1} for ds-DNA, 40 μg mL^{-1} for ss-DNA, and 20 μg mL^{-1} for ss-oligonucleotides), and D_{f} is the applied dilution factor. All oligonucleotide stock solutions were stored frozen at −20 °C.

Other solutions employed, prepared in deionized water, were a B&W buffer solution (10 mM Tris-HCl, 1 mM EDTA (Panreac, Spain), and 2.0 M NaCl) to prepare the biotinylated probe to be immobilized at the MBs, and a 0.5 M tetrathiafulvalene (TTF) (Aldrich) solution prepared in acetone (Scharlau) for the Au/SPEs modification procedure. H_2SO_4 (Scharlau) was also used. All chemicals used were of analytical-reagent grade, and deionized water was obtained from a Millipore Milli-Q purification system.

Procedures. *Pretreatment of the Gold Screen Printed Electrodes.* The pretreatment of the Au/SPEs involved placing on their surface a 50 μL drop of a 0.5 M H_2SO_4 solution containing 10 mM KCl and cycling the potential 10 times between 0.0 and +1.2 V with a scan rate of 100 mV s^{-1}; then, the electrodes were rinsed with water and dried under a N_2 flow. Their behavior in acidic solution was analogous to that reported in literature for gold wire electrodes.[31] Thus, characteristic gold oxide formation and reduction peaks were observed at +1.08 and +0.57 V, respectively.

Magnetic Beads Modification. MBs modification was carried out using a procedure based on that recommended by Dynal Biotech ASA (Oslo, Norway). A volume of 15 μL of streptavidin-coated magnetic beads were transferred into a 2.0 mL centrifuge vial. The microspheres were washed twice with B&W buffer and resuspended in 40 μL of the same buffer containing 100 pmol of biotinylated probe. The probe was captured onto the beads during 30 min at 37 °C with gentle mixing (600 rpm).

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Subsequently, the probe-modified MBs were washed twice with 100 μL of 50 mM Tris-HCl buffer solution (pH 7.2) and resuspended in 90 μL of the same buffer solution containing the desired amount of biotinylated target. The reaction was left to proceed for 30 min at 37 °C under stirring conditions (600 rpm). The hybrid-attached beads were washed twice with 100 μL of Tris-HCl buffer solution. At this time, labeling of the target with the streptavidin-HRP polymer enzyme was done. Enzyme tag took place by adding 100 μL of 10 μg mL^{-1} streptavidin-HRP polymer solution prepared in PBST buffer solution. The labeling procedure was left to proceed for 30 min at 37 °C with gentle mixing (600 rpm). The enzyme-tagged DNA/beads assembly was then washed five times, for 5 min each, with 100 μL of PBST.

Deposition of Magnetic Beads onto the TTF-Au/SPEs. Activated Au/SPEs were modified by placing on their surface a 5 μL drop of 0.5 M TTF solution and let to dry at ambient temperature. Modified MBs obtained as described above were immobilized on the TTF-Au/SPEs surface by placing a neodymium magnet under the electrode surface.

Amperometric Measurements. Amperometric responses were obtained by pipetting a 45 μL drop of 0.1 M PBS (pH 7.4) on the modified-MBs/TTF-Au/SPEs surface. When a steady-state current was obtained, at an applied potential of −0.15 V vs Ag/AgCl, a 5 μL drop of 3.5 mM H_2O_2 was deposited on the bioelectrode surface and the change in the signal recorded.

Bacteria Culture and DNA Extraction and Isolation. The bacterial strain used was *E. coli* W ATCC11105 (American Type Culture Collection). The pure culture was grown in a model G25 incubator shaker (P.A.C.I.S.A., New Brunswick Scientific Co. Inc. Edison, NJ) at 30 °C for 24 h before the bacterial concentration was determined by the conventional surface plate counting method. Then it was centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Dupont Instruments) at 3500 rpm for 10 min to pellet the bacterial cells. The pellet was washed with 4−5 mL of TEG buffer (50 mM glucose (Merck), 10 mM EDTA (Merck), 25 mM Tris-HCl (MP Biomedicals, Inc.), pH 8.0), and the formed suspension was centrifuged again at 3500 rpm for 10 min. Then, 8.5 mL of TEG buffer and 0.2 mL of 0.5 M EDTA were added to the recovered pellet, and the bacterial cells were lysed with lysozyme (Sigma). Afterward, the proteinaceous debris was digested with 1 mg of Proteinase K (Roche) in 1 mL of a 1% SDS (Bio-Rad) solution in TEG buffer at 60 °C for 3−4 h. Sodium acetate (Merck) (final concentration 0.3 M) and 10 mL of phenol (Sigma) were then added, and the sample was centrifuged. The collected aqueous phase was subjected to the same phenolization process till it remained clear, and then remaining phenol was eliminated by treatment with Sevag (chloroform/isoamilic alcohol 24:1, Merck) followed by centrifugation. The supernatant containing *E. coli* W genomic DNA was mixed with 6 v of isopropanol (Merck). Isolated DNA was let to dry at room temperature, washed with 70% ethanol and then with absolute ethanol (Merck) and dissolved in 2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

PCR Amplification. Asymmetric PCR, a PCR procedure that predominantly produces ssDNA, was used to amplify a 326-bp DNA fragment, containing the target sequence (25-mer) for the direct hybridization detection. The 100 μL PCR reaction mixture contained 3 μL of *E. coli* W genomic DNA extract (130 ng/μL),

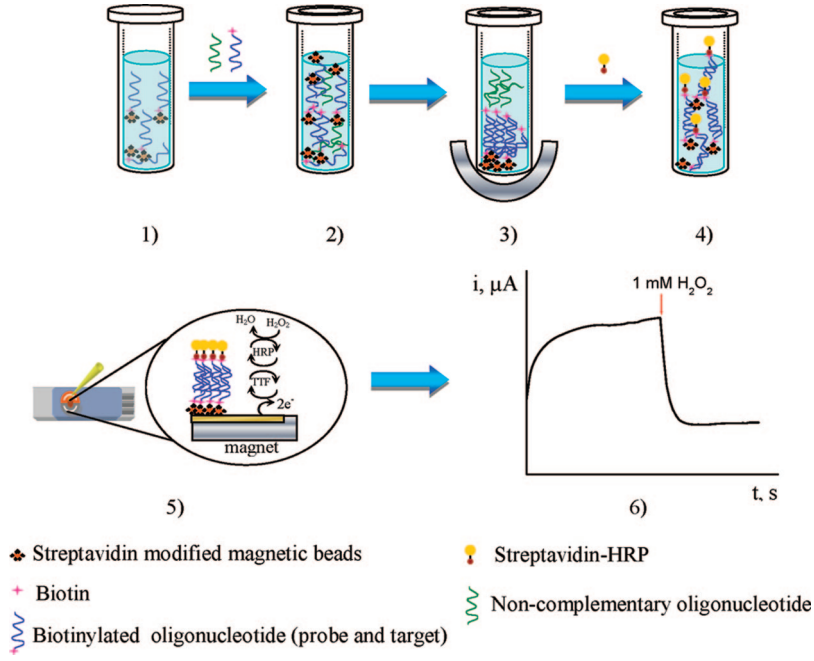

Figure 1. Schematic representation of the enzyme amplification protocol: (1) probe-modified magnetic beads washing step; (2) hybridization with the target *lacZ* gene probe; (3) hybrid-modified magnetic beads separation and noncomplementary oligonucleotide extraction; (4) enzymatic labeling with streptavidin-HRP; (5) hybrid-modified magnetic beads deposition on the TTF-Au/SPEs; (6) amperometric detection of the mediated reduction of H_2O_2 with TTF.

1.6 μL of a 5 μM forward primer solution, 16 μL of a 5 μM reverse biotinylated primer solution (biotinylated reverse primer to forward primer concentration ratio 10:1),[29] and other components following the protocol for PCR with *Taq* DNA polymerase. All PCR experiments were conducted in an Applied Biosystems 2720 thermal cycler. The PCR conditions were denaturation of *E. coli* W genomic DNA at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min (denaturation), 60 °C for 1 min (annealing), 72 °C for 1 min (extension), and 3 min final extension. During the process, the lower concentrated forward primer acted as a "limiting primer" and double-stranded target DNA fragments were produced when both primers were present in the PCR mixture. However, after the limiting primer was consumed, the remaining primer (biotinylated reverse primer) continued to amplify the target DNA, which resulted in the PCR products predominantly being biotinylated single-stranded *lacZ* gene fragments.

Following amplification, the PCR products were purified using a High Pure PCR Product Purification Kit (Roche), screened by gel electrophoresis in 1.5% agarose gel, and visualized by ethidium bromide (EtBr) staining.

RESULTS AND DISCUSSION

The disposable amperometric DNA hybridization sensor developed in this work is based on a sandwich configuration where a magnetic particle-labeled probe hybridizes to a biotinylated-DNA target that captures a streptavidin-HRP polymer. Figure 1 shows a schematic representation of the enzyme amplification protocol used. A total of 25-base single-stranded probes, specific to *Enterobacteriaceae lacZ* gene, were bound to streptavidin-modified magnetic beads. After hybridization and capturing of streptavidin-HRP, the DNA-modified magnetic beads were immobilized on top of TTF-modified Au/SPEs by means of a neodymium magnet placed under the electrode surface. The hybridization process was

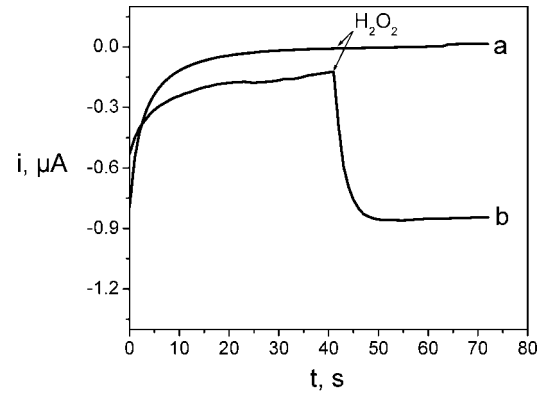

Figure 2. Amperometric signals obtained at the TTF-Au/SPEs electrode modified with the magnetic beads before (a) and after (b) the hybridization process. Supporting electrolyte, 0.1 M PBS buffer (pH 7.4) solution. [H_2O_2] = 1 mM, TTF loading, 2.5 μmol. Magnetic beads mass, 100 μg. Incubation conditions: biotinylated probe loading, 100 pmol, t_{inc} = 30 min. Hybridization conditions: biotinylated target loading, 10 pmol, t_{hyb} = 30 min. Enzymatic labeling: enzyme polymer loading, 1 μg, t_{inc} = 30 min. E_{app}= −0.10 V vs Ag/AgCl.

monitored following the addition of 0.35 mM H_2O_2. TTF incorporated on the electrode enhanced the electron transfer between HRP and the electrode surface according to the sequence:

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow HRP_{ox} + 2H_2O \quad (2)$$

$$HRP_{ox} + 2TTF \rightarrow HRP_{red} + 2TTF^+ \quad (3)$$

The generated TTF^+ was electrochemically reduced at the appropriate potential.

As it can be seen in Figure 2, curve a, no amperometric response was observed at an applied potential of −0.10 V (vs Ag/

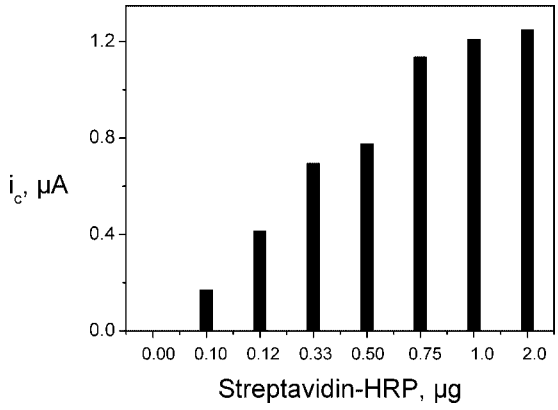

Figure 3. Effect of the streptavidin-HRP polymer loading on the amperometric signal at the HRP-dsDNA(MB)-TTF-Au/SPEs. Other conditions as in Figure 2.

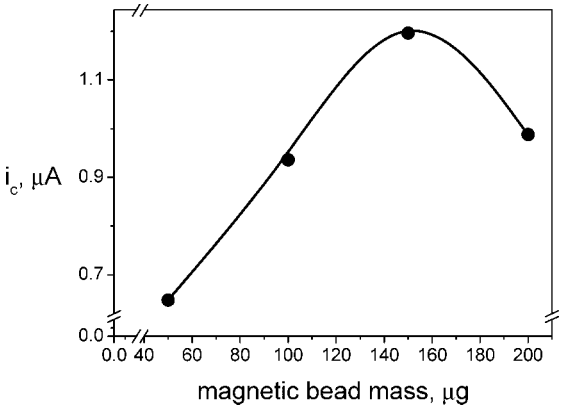

Figure 4. Effect of the modified-magnetic beads amount immobilized on the TTF-Au/SPEs on the resulting amperometric signal. Other conditions as in Figure 2.

AgCl) when H_2O_2 was added to the TTF-Au/SPEs modified with the magnetic beads-labeled ss-DNA before the hybridization process. On the contrary, when the hybridization process took place, a noticeable reduction current was observed (Figure 2, curve b).

Optimization of Working Variables. Working variables affecting the whole sensing procedure were optimized. First, the influence on the amperometric signal of the enzyme polymer used to label the hybridized DNA was tested. As expected (Figure 3), the amperometric signal increased with the enzymatic polymer loading, leveling off for amounts higher than 1.0 μg, the value which was chosen for further studies. It should be noted that no response was observed with the electrode without HRP, indicating that direct reduction of H_2O_2 at the modified Au/SPEs was negligible at the applied potential.

The effect on the amperometric signal of the modified-magnetic beads amount immobilized on top of the electrode surface was also investigated. Obtained results are shown in Figure 4. As it can be seen, an increase in the amperometric signal was observed up to 150 μg, the signal decreasing for higher loadings, which is probably due to an increase in the electron transfer resistance for large modified-magnetic beads loadings.

The effect of other experimental variables affecting the electrode reaction was also tested. The nonconducting nature of the redox mediator employed (TTF) resulted in a decrease of the amperometric signal for TTF loadings higher than 2.5 μmol, as a

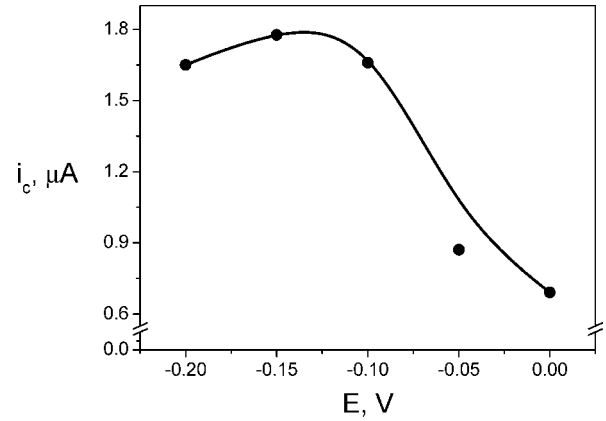

Figure 5. Effect of the applied potential on the amperometric response of HRP-dsDNA(MBs)-TTF-Au/SPEs. Magnetic beads mass, 150 μg; $[H_2O_2] = 0.35$ mM. Other conditions are as in Figure 2.

consequence of the electron transfer hindering in the presence of large amounts of this mediator on the electrode surface.[32,33] The influence of the enzyme substrate (hydrogen peroxide) concentration on the amperometric signal was studied within the 0.1−1.0 mM range (not shown). Taking into account that the amperometric signal increased sharply with the H_2O_2 concentration up to 0.35 mM leveling off for higher concentration values, also that high concentrations of H_2O_2 can result in the electrode surface poisoning, as well as that of irreversible denaturation of HRP to its higher oxidized inactive form at H_2O_2 concentrations higher than approximately 0.6 mM has been previously described,[34] a value of 0.35 mM was selected for further studies.

Finally, the influence of the applied potential on the amperometric analytical signal obtained after the hybridization process of the *lacZ* gene specific sequences was tested. Figure 5 shows that maximum responses were obtained at approximately −0.15 V (vs Ag/AgCl), which is in agreement with the behavior reported for electron transfers involving TTF, thus indicating that the electron flow through the dsDNA-modified magnetic beads is possible and that the TTF-mediated enzymatic reaction occurs. Accordingly, a −0.15 V potential value was selected to monitor the affinity reaction.

Reproducibility and Analytical Characteristics. Measurements with 10 different genosensors constructed in the same manner, for 5.0 nmol of target DNA, resulted in reproducible signals with a relative standard deviation (RSD) value of 7.1%. This result demonstrated the reliability in the sensor construction procedure, which is of great importance when working with single-use screen-printed modified electrodes, as well as the effectiveness of the binding between the biotinylated probe and the modified magnetic beads attracted to the TTF-modified screen-printed electrodes.

With the use of the optimized experimental conditions, a linear calibration plot was constructed for target DNA in the $0-2.4 \times 10^{-7}$ M range, with a slope value of (5.3 ± 0.1) μA μM^{-1} and an intercept of (0.07 ± 0.01) μA ($r = 0.999$). Fairly low detection and determination limits were achieved without using PCR amplification. These values were 0.11 and 0.66 nM, respectively, as

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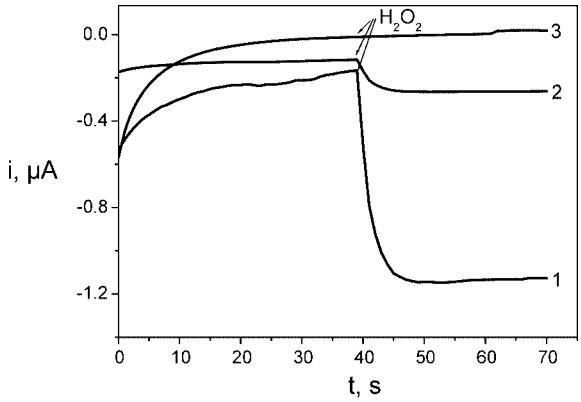

Figure 6. Amperometric signals obtained at the TTF-Au/SPEs modified with the magnetic beads after hybridization with the target sequence (10 pmol) (1), a single-base mismatch sequence (10 pmol) (2), and a noncomplementary sequence (10 pmol) (3). Other conditions as in Figure 5.

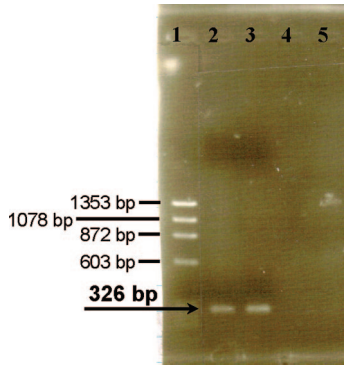

Figure 7. Gel electrophoresis detection of *E. coli lacZ* gene asymmetric PCR products. Lane 1, ϕX174 DNA-*Hae*III Digest ladder; lanes 2 and 3, asymmetric product using genomic DNA from *E. coli*; lanes 4 and 5, PCR blank control (purified water as PCR template).

calculated according to the 3 s/m and 10 s/m criteria, where m is the slope of the calibration plot and s was estimated as the standard deviation (n = 10) of the amperometric signals at the 50 fmol target DNA level. The detection limit achieved using the MBs-based HRP-amplified configuration described in this work is remarkably lower than the values obtained with the genosensors described previously in the literature for the same target DNA.[35,36]

The capture probes were also investigated for the response to noncomplementary and mismatch oligonucleotides. The obtained results are displayed in Figure 6. As it can be seen, no change in the amperometric response was observed when the capture probe-modified electrode was exposed to the noncomplementary oligonucleotide (curve 3). Concerning the single-base mismatch oligonucleotide (curve 2), the amperometric response was only a (17 ± 3) % of that obtained with the target DNA (curve 1). This indicated a much lower hybridization efficiency of single-base mismatch DNA compared with complementary target DNA, thus showing the ability to detect even a single-base mutation.

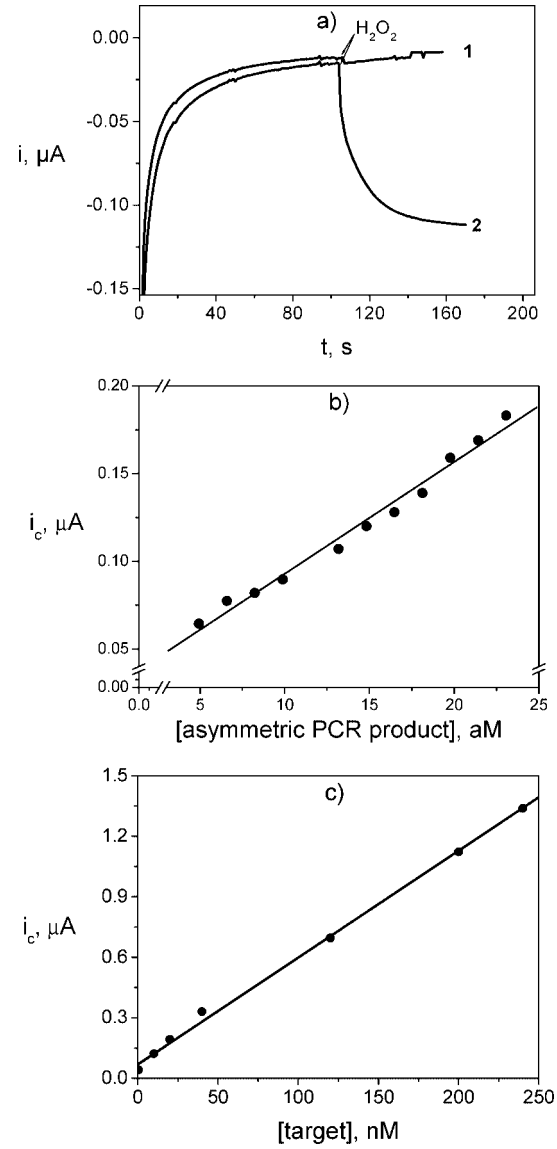

Figure 8. (a) Amperometric signals obtained for 13.2 aM PCR amplicons obtained using purified water (1) or *E. coli* genomic DNA (2) as PCR templates; (b) calibration plot obtained for the *E. coli* amplicon; and (c) calibration plot for model 25-mer target oligonucleotides. Other conditions as in Figure 6.

Detection of PCR Products. *Confirmation of Asymmetric PCR Amplification Using Gel Electrophoresis.* In order to improve the sensitivity of the developed method, asymmetric PCR was used to amplify a 326-bp DNA fragment containing the target sequence (25-mer) for the direct hybridization detection. The results obtained in the screening of the asymmetric PCR products by gel electrophoresis are shown in Figure 7. From left to right, the DNA size marker, asymmetric PCR product using genomic DNA from *E. coli*, for a concentration of 1.0×10^9 cfu/mL, and the PCR blank control (purified water as DNA template) can be seen. Electrophoresis confirmed the successful amplification of PCR products with the right size (326 bp) in spite of the lower EtBr staining efficiency for ssDNA.

Amperometric Detection of Amplicons Obtained by PCR Using Genomic DNA as Template. The PCR samples were diluted to the desired concentration using 50 mM Tris-HCl buffer solution (pH 7.2) and directly applied for the electrochemical detection. Thus,

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the desired amount of PCR product was used for each test following the same procedure used for synthetic oligonucleotides. As it can be seen in Figure 8a, curve 1, a PCR blank control, i.e., without addition of *E. coli* genomic DNA during amplification (using purified water as DNA template), yielded a nonsignificant amperometric signal. This indicated that nonspecific adsorption was negligible. Conversely, the presence of 13.2 aM PCR amplicons obtained using genomic DNA as PCR template gave rise to a remarkable amperometric response (Figure 8a, curve 2). The dependence of the amperometric signal on the concentration of the amplicon from *E. coli* is shown in Figure 8b. A linear range for the quantification of the PCR amplicon is observed from 5.0 to 23.0 aM giving a sensitivity of $(6.4 \pm 0.3) \times 10^{-9}$ A aM^{-1} and a correlation coefficient of 0.992. A decrease in sensitivity was observed for higher amplicon concentrations, which was probably due to the fact that folding of long DNA chains hindered the hybridization process, this effect being stronger in the presence of high concentrations of the large DNA chains containing the target sequence. For comparison purposes, Figure c shows the calibration curve for model 25-mer target nucleotides (see Reproducibility and Analytical Characteristics). The limits of detection and determination were calculated according to the criteria mentioned above taking *s* as the standard deviation (n = 10) of the amperometric signals from 4.9 aM asymmetric PCR product. These values were 2.5 and 8.2 aM, respectively, which obviously, means a significant improvement with respect to the results obtained when no PCR amplification was applied. The detection limit achieved is also significantly lower than those obtained for the determination of other bacteria such as *Salmonella* either using an electrochemical DNA-based PCR reusable sensor fabricated by the self-assembly of a MB-labeled DNA probe on a AuE surface[37] or using a configuration based on MBs and HRP and PCR amplification systems.[10]

An RSD value of 6.1% was obtained for 10 measurements at an asymmetric PCR product concentration level of 19.8 aM, which can be considered as quite good reproducibility for this kind of disposable genosensors and the low amplicon concentration assayed.

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CONCLUSIONS

Disposable magnetic hybridization DNA sensors, combining the use of magnetic beads for DNA isolation and hybridization and of an HRP-amplified detection strategy mediated by TTF, show a high sensitivity even without the use of PCR amplification. The use of magnetic beads allows the immobilization of the DNA hybrid on the Au/SPE transducer avoiding difficult and time-consuming immobilization procedures. In fact, the assay described in this work takes only hours (as opposed to days for culture-based techniques) to complete and uses inexpensive reagents for analysis. The developed sensors have also been demonstrated useful for rapid, specific, quantitative, and sensitive detection of PCR amplified products from *E. coli* bacterial cultures without complicated sample treatment procedures. Furthermore, the use of a hybridization sensor also avoids one of the major drawbacks of PCR analysis such as false-positive results. An extremely low detection limit of only 2.5 aM was achieved and, moreover, up to 30 sensors per day can be prepared and used. This design is supposed to be easily adapted for the detection of other relevant bacteria, since it requires only a single DNA probe for such purpose.

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