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Enzyme-based impedimetric detection of PCR products using oligonucleotide-modified screen-printed gold electrodes

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Abstract

This paper describes the optimisation and the analytical performances of an enzyme-based electrochemical genosensor, developed using disposable oligonucleotide-modified screen-printed gold electrodes.

The immobilisation of a thiol-tethered probe was qualitatively investigated by means of faradic impedance spectroscopy. Impedance spectra confirmed that the thiol moiety unambiguously drives the immobilisation of the oligonucleotide probe. Furthermore, both probe surface densities and hybridisation efficiencies were quantified through chronocoulometric measurements.

Electrochemical transduction of the hybridisation process was also performed by means of faradic impedance spectroscopy, after coupling of a streptavidin–alkaline phosphatase conjugate and bio-catalysed precipitation of an insoluble and insulating product onto the sensing interface. Chronocoulometric results allowed discussion of the magnitude of hybridisation signals in terms of probe surface densities and their corresponding hybridisation efficiency. The genosensor response varied linearly (r^2 = 0.9998) with the oligonucleotide target concentration over three orders of magnitude, between 12 pmol/L and 12 nmol/L. The estimated detection limit was 1.2 pmol/L (i.e., 7.2×10^6 target molecules in 10 μL of sample solution).

The analytical usefulness of the impedimetric genosensor was finally demonstrated analysing amplified samples obtained from the pBI121 plasmid and soy and maize powders containing 1 and 5% of genetically modified product. Sensing of such unmodified amplicons was achieved via sandwich hybridisation with a biotinylated signaling probe. The electrochemical enzyme-amplified assay allowed unambiguous identification of all genetically modified samples, while no significant non-specific signal was detected in the case of all negative controls.

Keywords: Screen-printed gold electrodes; Genosensor; DNA biosensor; Sandwich hybridisation; Alkaline phosphatase; Faradic impedance spectroscopy

1. Introduction

In the past few years, advances in robotics, microfluidics, electronics and high resolution optics have driven the impressive development of both DNA microarrays and real-time PCR systems. The majority of microarrays (pioneered by the *GeneChip*® from Affymetrix Inc. [http://www.affymetrix.com]) and all real-time PCR instruments (e.g., ABI Prism® 7900HT Sequence Detection System [http://www.appliedbiosystems.com]) rely upon the detection and quantitation of a fluorescent reporter, whose signal increases proportionally to the amount of hybridised target or amplified PCR product. Detection thus requires imaging equipment or fluorescence readers that generally are very expensive.

Besides these technologies that are already impacting the market, new research tools are expected to have a major influence in coming years. The recent appearance on the diagnostic market of electrochemical DNA microarrays (e.g., Motorola eSensor™ DNA Detection System [http://www.motorola.com/lifesciences/esensor/] and Xanthon Xpression Analysis System [http://www.xanthoninc.com]) is witnessing the enormous potential of electrode-based genosensors. To make DNA testing more convenient, more economically feasible and ultimately more widely used, the

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appealing promise of electrochemical detection technologies, is thus driving the intense research efforts of hundreds laboratories world-wide.

Electrochemical impedance spectroscopy (EIS) is the electrochemical technique where the electrode impedance is monitored as a function of the frequency of an applied alternative voltage (Bard and Faulkner, 2001). The use of both electrochemical and faradic impedance spectroscopy to probe the interfacial properties of surface-modified electrodes and thus for monitoring biorecognition processes was recently proposed by Willner and co-workers (Patolsky et al., 1998, 2001a,b) and a few other authors (Yan and Sadik, 2001; Nahir and Bowden, 2002; Marquette et al., 2002; Vagin et al., 2003).

We have recently described the use of oligonucleotide-modified screen-printed gold electrodes for enzyme-amplified sensing of nucleic acids (Carpini et al., 2004). A 1 nmol/L detection limit of a 195 bp biotinylated amplicon was achieved using α-naphthyl phosphate as the substrate of alkaline phosphatase and the differential pulse voltammetry as the electroanalytical method. In this paper, as an extension of our previous work, we report on the complete characterisation and further optimisation of our disposable genosensor. Immobilisation of the thiol-tethered probe that on a "classic" gold surface proceeds through the well-established process called chemisorption was qualitatively and quantitatively investigated by means of faradic impedance spectroscopy and chronocoulometry, respectively. Electrochemical transduction of the hybridisation process was also performed by means of faradic impedance spectroscopy, after coupling of a streptavidin–alkaline phosphatase conjugate and bio-catalysed precipitation of an insoluble and insulating product onto the sensing interface (Patolsky et al., 2001a). The magnitude of electroanalytical signals was quantified in terms of probe surface densities and the corresponding hybridisation efficiencies. Finally, sensing of unmodified amplicons was achieved via sandwich hybridisation with a biotinylated signaling probe. Performance advantages of the impedimetric genosensor are also discussed in this paper.

2. Experimental

2.1. Materials and reagents

Dithiothreitol (DTT), 6-mercapto-1-hexanol (MCH), streptavidin–alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), BCIP/nitro blue tetrazolium mixture [BCIP/NBT (cat. no. B-1911)], bovine serum albumin (BSA), Tris–HCl and diethanolamine were obtained from Sigma–Aldrich (Milan, Italy). Hexaammineruthenium(III) chloride was obtained from Aldrich. Di-sodium hydrogenphosphate, potassium hexacyanoferrate(III and II), sulphuric acid, magnesium and potassium chloride were purchased from Merck (Milan, Italy). NAP-10 columns of Sephadex G-25 and the *Taq* polymerase were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). MilliQ water was used throughout this work. Synthetic oligonucleotides were obtained from MWG Biotech AG (Germany):

- Immobilised probe (DNA-SH): 5′-HS-$(CH_2)_6$-GCT CCT ACA AAT GCC ATC ATT GCG A-3′
- Target oligo: 5′-biotin-TCG CAA TGA TGG CAT TTG TAG GAG C-3′
- Non-complementary oligo: 5′-biotin-GGC AGA GGC ATC TTC AAC GAT GGC C-3′
- Signaling probe: 5′-TTG ATG TGA TAT CTC CAC TGA CG-biotin TEG-3′

Prior to use, the thiol-modified probe was treated with DTT and then purified by elution through a NAP-10 column of Sephadex G-25. All oligonucleotide stock solutions were prepared in 0.5 mol/L phosphate buffer (pH 7) and stored frozen.

2.2. Amplification of 35S promoter sequences by polymerase chain reaction (PCR)

The pBI121 plasmid incorporating the 35S promoter sequence was extracted from *Escherichia coli* using a QIAprep Kit (Qiagen, Italy), according to the manufacturer protocol. A 195 bp region of the 35S promoter was then amplified using a DNA Thermal Cycler PTC-150 (MJ Research Inc., USA) according to the procedure described by Pietsch et al. (1997) and using the following primers:

- P35S top: 5′-GCT CCT ACA AAT GCC ATC A-3′
- P35S bottom: 5′-GAT AGT GGG ATT GTG CGT CA-3′

Successful amplification was confirmed by gel electrophoresis. As the negative control, a 217 bp fragment of the lectin gene was amplified from soybean genomic DNA (0% Roundup Ready™ CRM; Fluka, Italy) using the following primers:

- Lectin top: 5′-TGC CGA AGC AAC CCA AAC ATG ATC C-3′
- Lectin bottom: 5′-GCC CAT CTG CAA GCC TTT TTG TG-3′

Amplicon concentrations were determined by fluorescence measurements, using the Picogreen™ dye and a TD-700 fluorometer (Analytical Control, Milan, Italy).

PCR products obtained from 0, 1 and 5% genetically modified soy (GMO Soya Bean Powder: 0, 1 and 5% Roundup Ready™ Fluka) and 0, 1 and 5% genetically modified maize (GMO Maize Powder: 0, 1 and 5% MON-810) were kindly provided by the Laboratorio di Sicurezza Alimentare ed Ambientale, Dipartimento di Chimica e Chimica Industriale, Università di Genova, Italy.

2.3. Electrochemical pretreatment and biomodification of the sensor surface

Materials and procedures to screen-print the gold electrode transducers are described in our previously published paper (Carpini et al., 2004).

Prior to immobilisation of the thiol-tethered DNA probe, a multiple-pulse amperometric pretreatment of the gold surface was carried out in a stirred 0.5 mol/L H_2SO_4, 10 mmol/L KCl solution. The following triple-potential pulse sequence: −0.3 V for 0.30 s; 0.0 V for 0.30 s and +1.0 V for 0.15 s (150 cycles) was applied.

The gold working electrode surface of these planar sensors was then exposed to the thiolated oligonucleotide solution (10 μL, 1.3 μmol/L in 0.5 mol/L phosphate buffer, pH 7). Chemisorption was allowed to proceed overnight (≈16 h) with electrodes stored in Petri dishes to protect the solutions from evaporation. The immobilisation step was followed by treatment with a spacer thiol. A 10 μL drop of 1 mmol/L aqueous solution of MCH was placed onto the probe-modified surfaces for 30 min. Prior to the hybridisation reaction, the modified electrodes were washed twice with 15 μL of phosphate buffer.

2.4. Hybridisation

Hybridisation experiments were carried out using both synthetic biotinylated target sequences in a direct format and mixtures of unmodified PCR products and biotinylated signaling probe in a sandwich-like assay.

In the first case, probe-modified gold electrodes were exposed to a 10 μL drop of the biotinylated target sequence solution (in 0.5 mol/L phosphate buffer) for 20 min. A biotinylated non-complementary sequence was used as the negative control.

In the latter, DNA samples obtained by PCR amplification were first diluted with a 0.5 mol/L phosphate buffer solution containing the biotinylated signaling probe. The amplicon dilution was typically 1:5, while the signaling probe concentration was always kept constant at 1 μg/mL. Double-stranded DNAs were then thermally denatured by using a boiling water bath (5 min at 100 °C); amplicon strand re-annealing was retarded by cooling the sample in an ice-water–NaCl bath (≪0 °C) for 5 min. A 10 μL aliquot of this solution was finally placed directly onto the probe-modified electrodes for 1 h. Non-complementary PCR products were used as the negative control.

After hybridisation, the sensors were washed twice with 15 μL of DEA buffer (diethanolamine 0.1 mol/L, $MgCl_2$ 1 mmol/L, KCl 100 mmol/L; pH 9.6).

2.5. Labeling with alkaline phosphatase and incubation with the enzymatic substrate

The biotinylated hybrid obtained at the electrode surface was reacted with a 10 μL drop solution containing 1 U/mL of the streptavidin–alkaline phosphatase conjugate and 8 mg/mL of BSA in DEA buffer. After 20 min, the genosensors were washed twice with 15 μL of DEA buffer.

The enzyme-modified surfaces were then incubated with 30 μL of the BCIP/NBT mixture for 20 min. After precipitation of the insoluble and insulating product and prior to impedimetric measurements, the sensors were washed with 0.1 mol/L KCl for 10 s.

2.6. Electrochemical measurements

All electrochemical measurements were performed with an AUTOLAB PGSTAT 10 digital potentiostat/galvanostat. The GPES 4.9004 software (Eco Chemie BV, Utrecht, The Netherlands) was used for multiple-pulse amperometry, cyclic voltammetry and chronocoulometry; the FRA2 module was used for faradic impedance experiments. All potentials were referred to the screen-printed silver pseudo-reference electrode; the experiments were carried out at room temperature (25 °C).

Faradic impedance measurements were carried out in the presence of 1 mmol/L $[Fe(CN)_6]^{3/4-}$ redox probe (equimolecular mixture in 0.1 mol/L KCl). An alternative voltage of 10 mV in amplitude (peak-to-peak), within the frequency range 100 kHz–50 mHz, was superimposed to the applied bias potential. The dc potential was set up at +0.13 V, the formal potential of the $[Fe(CN)_6]^{3/4-}$ redox probe. Experimental spectra, presented in the form of complex plane diagrams (i.e., Nyquist plots), were fitted with proper equivalent circuits using the facilities of the FRA2 software 4.9004 (EcoChemie). Both charge transfer resistance and Δ charge transfer resistance values were taken as analytical signals.

3. Results and discussion

Fig. 1 displays the schematic representation of the impedimetric genosensor. Unmodified PCR samples were recognised via sandwich hybridisation, subsequent coupling of a streptavidin–alkaline phosphatase conjugate and enzymatic conversion of a suitable substrate into an insoluble and insulating product.

In our previous paper (Carpini et al., 2004), we have shown that thiol-tethered oligonucleotides do not require any pretreatment of the screen-printed gold surface to be immobilised on it. Surface pretreatments were thus completely omitted, since naphthol electrochemistry was also not significantly affected by them. In contrast, specific pretreatment of the sensors was developed in the case of the impedimetric genosensor, mainly with the aim of reducing the background impedance value. A variety of electrochemical procedures were investigated, including the widely reported cyclic voltammetric scanning in 0.5 mol/L H_2SO_4 (Hashimoto et al., 1994). Comparing all investigated pretreatments, a multiple-pulse amperometric approach, similar to that proposed by van Bennekom and co-workers (Hoogvliet et al., 2000), was found to be the most favourable (data not shown). This procedure was carried out in a 0.5 mol/L H_2SO_4–10 mmol/L KCl solution and consisted of three pulses of 0.30, 0.30 and 0.15 s at −0.3, 0.0 and +1.0 V, respectively. One hundred and fifty cycles of such a triple-pulse regime were applied. This pretreatment was

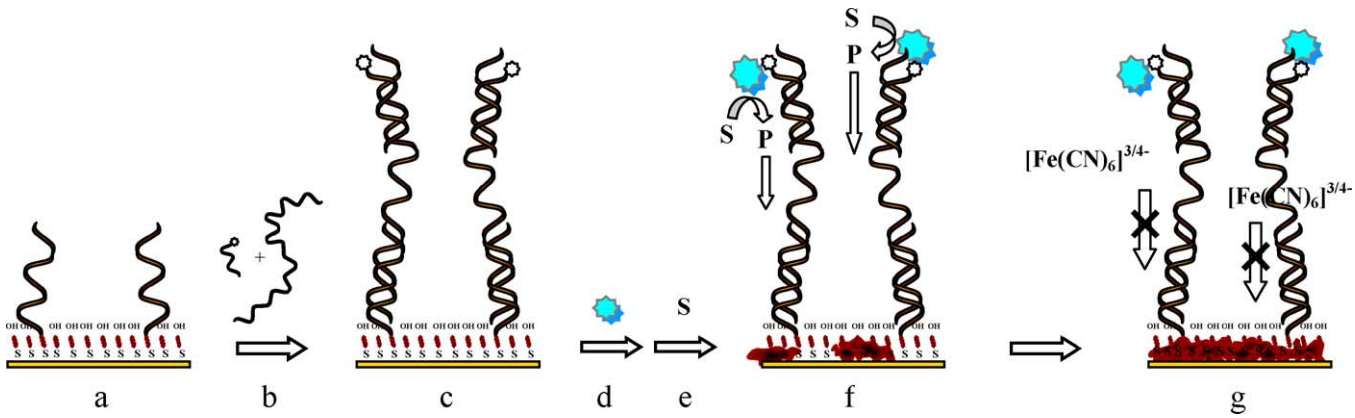

Fig. 1. Schematic representation of the impedimetric genosensor (sandwich hybridisation assay). Unmodified PCR products (b) were captured at the sensor interface (a) via sandwich hybridisation with the surface-tethered probe and a biotinylated signaling probe. The biotinylated hybrid (c) was then coupled with a streptavidin–alkaline phosphatase conjugate (d) and finally exposed to the substrate solution (e). The bio-catalysed precipitation of an insulating product (f) blocked the electrical communication between the gold surface and the $[Fe(CN)_6]^{3/4-}$ redox probe (g); faradic impedance spectroscopy was finally used to detected the enhanced electron transfer resistance.

the shorter and, more important, assured the lower and more reproducible background impedance.

The electrochemically active surface value of the screen-printed gold electrodes was also confirmed by cyclic voltammetric evaluation of the gold oxide reduction peak obtained in 0.5 mol/L H_2SO_4 solution. The value $7.05 \pm 0.35\,mm^2$ [calculated by assuming 482 $\mu C\,cm^{-2}$ to be the charge required for the reduction of a monolayer of gold oxide (Hoogvliet et al., 2000)] was in good agreement with that previously evaluated (Carpini et al., 2004).

Self-assembly of thiol-derivatised oligonucleotides onto gold surfaces has been investigated using a number of analytical approaches, including XPS and ^{32}P-radiolabeling (Herne and Tarlov, 1997), neutron reflectivity (Levicky et al., 1998), surface plasmon resonance (Peterlinz et al., 1997), quartz crystal microbalance (Bardea et al., 1999) and electrochemical methods (Hashimoto et al., 1994; Steel et al., 1998). In this work, the features of the DNA probe immobilisation process and the formation of DNA-SH/MCH mixed monolayers were qualitatively investigated by faradic impedance spectroscopy, using the $[Fe(CN)_6]^{3/4-}$ mixture as the redox probe. A combination of physical coverage by the oligonucleotides and electrostatic repulsion between the negatively charged redox ions and the DNA polyanionic backbone (Yang et al., 1998) were expected to account for an increase of the electron transfer resistance between the electrode and the redox probe in solution. Pretreated screen-printed gold sensors were first modified with a 10 μL drop of the thiolated oligonucleotides solution (1.3 μmol/L in 0.5 mol/L phosphate buffer) and then exposed to the 1 mmol/L solution of MCH. Both unmodified oligonucleotides (denoted as "DNA") and the phosphate buffer solution were used as negative controls. Impedance spectra, obtained for DNA-SH-modified, DNA-modified and bare electrodes before the MCH pretreatment, are reported in Fig. 2A. While an electron transfer resistance of about 10 MΩ characterised DNA-SH-modified surfaces, values of 488 ± 10 and 23 ± 4 kΩ were detected for DNA-modified and bare electrodes, respectively. The comparison between the last two values clearly indicates some non-specific adsorption (physisorption) of unmodified sequences. Analogous spectra, recorded after MCH post-treatment, highlighted impressive changes at the electrode interfaces (Fig. 2B). An electron transfer resistance of 256 ± 42 kΩ was measured at DNA-SH/MCH surfaces, indicating a massive displacement of non-specifically adsorbed thiol-tethered oligonucleotides. More interestingly, DNA/MCH-modified sensors exhibited nearly the same electron transfer resistance measured for bare/MCH electrodes, 35 kΩ versus 39 kΩ, thus confirming the quantitative displacement of unmodified oligonucleotides by the spacer thiol.

Qualitative information extracted from impedance spectra was integrated with the quantitative measurement of probe surface densities, according to the method developed by Steel et al. (1998). Briefly, this method relies on the chronocoulometric measurement of a highly charged cationic redox marker, $[Ru(NH_3)_6]^{3+}$. In low ionic strength buffers, this metal complex exchanges with cations naturally compensating the polyanionic backbone of the oligonucleotides. Therefore, at equilibrium conditions, the amount of $[Ru(NH_3)_6]^{3+}$ electrostatically associated to the phosphate groups is directly proportional to the number of immobilised probe molecules. A series of sensors was exposed to thiolated oligonucleotide solutions (1.3×10^{-3}, 1.3×10^{-2}, 0.13, 1.3 μmol/L in 0.5 mol/L phosphate buffer) with chemisorption allowed to proceed overnight. After treatment with MCH, each subset of modified sensors was exposed to the biotinylated complementary sequence (60 nmol/L), while the phosphate buffer was used as the negative control. The results of chronocoulometric measurements are displayed in Table 1.

Chronocoulometric signals at 1.3×10^{-3} and 1.3×10^{-2} μmol/L DNA-SH/MCH-modified electrodes were not distinguishable from background values. This finding reflected the relatively low sensitivity of the chrono-

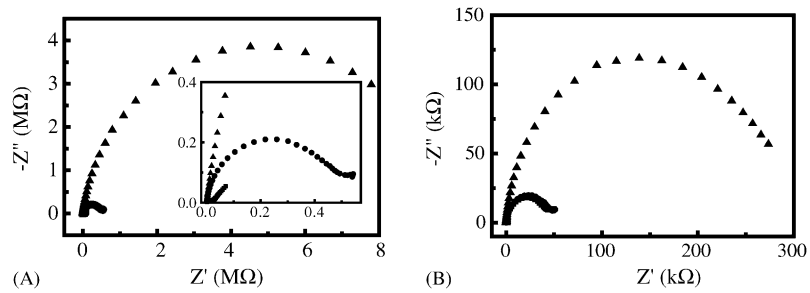

Fig. 2. (A) Nyquist plots for DNA-SH-modified (up triangles), DNA-modified (circles) and bare (squares) electrodes (inset is the magnification of the spectra for DNA-modified and bare electrodes). (B) Spectra recorded at DNA-SH-modified (up triangles), DNA-modified (circles) and bare (squares) electrodes after exposure to the MCH solution. Impedance spectra were recorded in the presence of the 1 mmol/L $[Fe(CN)_6]^{3/4-}$ redox probe (1:1 mixture in 0.1 mol/L KCl). An ac voltage of 10 mV in amplitude (peak-to-peak), within the frequency range 100 kHz–50 mHz, was superimposed to the applied bias potential (+0.13 V). Each measurement was repeated at least three times.

Table 1
Chronocoulometric and impedimetric results as a function of the DNA-SH probe concentration

Probe concentration (μmol/L)	Probe coverage (molecules/cm^2)	Hybridised (molecules/cm^2)	Hybridisation efficiency (%)	Charge transfer resistance (kΩ)
1.3×10^{-3}	–	*a* (–)	–	8 ± 3
		b (–)	–	57 ± 16
1.3×10^{-2}	–	*a* (–)	–	9 ± 4
		b (–)	–	164 ± 67
0.13	$(2.9 \pm 0.4) \times 10^{12}$	*a* (–)	–	36 ± 4
		b ($(2.1 \pm 0.1) \times 10^{12}$)	72	663 ± 164
1.3	$(1.46 \pm 0.00) \times 10^{13}$	*a* (–)	–	76 ± 2
		b ($(1.8 \pm 0.1) \times 10^{12}$)	12	663 ± 25

Letters *a* and *b* denote probe-modified sensors exposed to pure phosphate buffer and biotinylated target, respectively. Chronocoulometric measurements were carried out in the presence of 50 μmol/L $[Ru(NH_3)_6]^{3+}$ in 10 mmol/L Tris buffer (pH 7.4). Initial and final potentials were +0.1 and −0.4 V, respectively; a pulse duration of 2 s was applied. Faradic impedance spectra were recorded according to the conditions described in Fig. 2. Each measurement was repeated at least three times.

coulometric assay that was reported to be of about 1×10^{11} molecules/cm^2 (Steel et al., 1998). The number of probe molecules immobilised in the above-mentioned conditions was probably too low to be detected. On the contrary, clear signals were obtained at 0.13 and 1.3 μmol/L DNA-SH/MCH-modified electrodes. Calculated probe surface densities were found to be surprisingly similar to those reported for analogous immobilisation accomplished onto evaporated gold films (Steel et al., 1998). Hybridisation of the surface-tethered probes with the corresponding target sequence increases the number of $[Ru(NH_3)_6]^{3+}$ binding sites (nucleotide phosphates) at the electrode interface. The number of hybridised target molecules can be thus conveniently evaluated from the excess of $[Ru(NH_3)_6]^{3+}$ found comparing hybrid- and probe-modified electrodes. According to the literature, the hybridisation efficiency (i.e., the percentage of surface-bound probes undergoing hybridisation) increased as the probe density was diminished. Such a behaviour reflected the lower steric hindrance and electrostatic repulsion experienced by the target molecules at less tightly packed probe monolayers. It can be easily assumed that, although both probe coverage and hybridisation efficiency are not measurable, the latter approaches 100% at 1.3×10^{-3} and 1.3×10^{-2} μmol/L DNA-SH/MCH-modified electrodes.

Because of the non-destructive characteristics of chronocoulometric measurements, the same batch of electrodes was further processed. Oligonucleotide-modified sensors were exposed to the streptavidin–alkaline phosphatase conjugate and then incubated with the enzymatic substrate solution. The precipitation of an insoluble product, obtained through the bio-catalysed oxidative hydrolysis of the BCIP/NBT mixture, generated an insulating layer which inhibited the interfacial electron transfer of the $[Fe(CN)_6]^{3/4-}$ redox probe. The resulting charge transfer resistance values, detected by means of faradic impedance spectroscopy, were taken as the analytical signals. Depending on the extent of surface modification by the insoluble product, different equivalent circuits were required to fit experimental data (Fig. 3).

The constant phase element, *Q*, was always used instead of pure capacitive elements to take into account the rough-

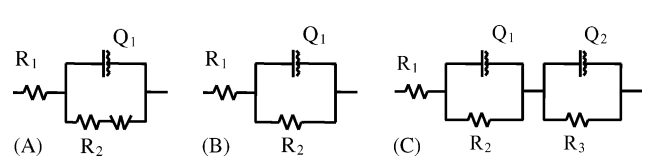

Fig. 3. Equivalent circuits used to fit experimental spectra (non-linear least squares method).

ness of the modified sensor surfaces (Macdonald, 1987). The equivalent circuit A [the "Randles equivalent circuit" (Bard and Faulkner, 2001)] was successfully applied to fit data acquired at weakly modified sensors. As the precipitate layer became thicker and the reaction kinetically slower, the frequency region where mass transfer is a significant factor disappeared. The Warburg impedance (W) was thus removed from the equivalent circuit (case B). At the most relevantly modified sensors, a new (QR) element had to be introduced (case C). It was postulated that the (Q_2R_3) factor served to model processes involving some redox probe "entrapped" below the insulating layer; however, its real meaning is still under investigation.

The charge transfer resistance values for probe- and hybrid-modified sensors as a function of the DNA-SH concentration are reported in the last column of Table 1. The impedimetric results confirmed the chronocoulometric experiments. Because of the higher sensitivity of the enzyme-linked assay, clear hybridisation signals were obtained even at 1.3×10^{-3} and 1.3×10^{-2} μmol/L DNA-SH/MCH-modified electrodes. Furthermore, comparable responses were observed for the 0.13 and 1.3 μmol/L DNA-SH/MCH-modified sensors. Despite the difference in the hybridisation efficiencies (72 and 12%, respectively), the actual number of hybridised target molecules at these surfaces was, in fact, rather similar (2.1×10^{12} molecules/cm^2 versus 1.8×10^{12} molecules/cm^2).

The composition of the enzymatic substrate solution had a crucial influence on the impedimetric results. The use of BCIP was widely reported by Willner and co-workers (Patolsky et al., 2001a; Willner et al., 2001); however, when BCIP solutions (up to 4 mg/mL) were used, no hybridisation signals were detected ($\Delta R = -13 \pm 13$ kΩ). The resulting precipitate (blue coloured) did not form a stable, compact layer on the screen-printed surface and, thus, it was easily washed away while immersing the sensor in the rinsing solution. A compact, dark brown insoluble layer was, on the contrary, obtained upon using the BCIP/NBT mixture. A dramatic increase of the impedance was thus observed at hybrid-modified sensors as a consequence of product precipitation ($\Delta R = 1230 \pm 151$ kΩ). The signals measured at sensors exposed to analogous concentrations of non-complementary sequence and pure hybridisation buffer were nearly the same ($\Delta R = -5 \pm 26$ and 4 ± 7 kΩ, respectively). Therefore, non-specific binding of the non-complementary sequence was assumed to be negligible. On the other hand, the affinity coupling of the streptavidin–alkaline phosphatase conjugate with the biotinylated hybrid and subsequent precipitation of the insoluble product effectively provided a means to detect such an interfacial hybridisation event.

Following a step-like behaviour, the analytical signals rapidly increased with the substrate incubation time up to 20 min and then reached a plateau. When probe-modified sensors were exposed to the non-complementary sequence and thus no biotinylated hybrid could be formed, the non-specific adsorption of the enzymatic conjugate was negligible. No significant increase of the electron transfer resistance was detected even after 120 min of incubation with the substrate solution (data not shown).

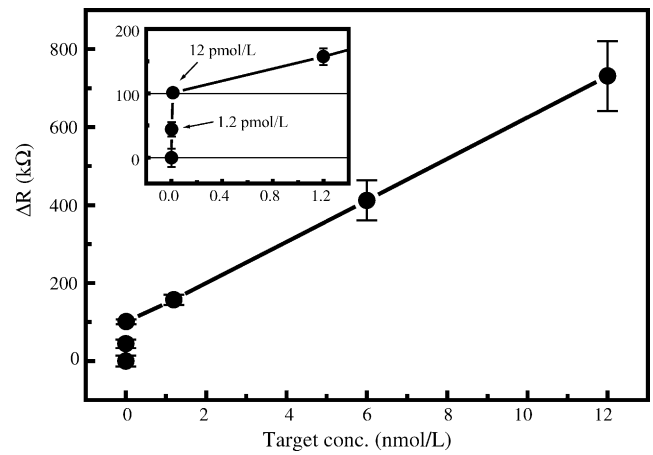

Fig. 4. Calibration plot for synthetic oligonucleotides. Probe-modified sensors were exposed to a 10 μL drop of the biotinylated target sequence solution (0, 1.2×10^{-3}, 1.2×10^{-2}, 1.2, 6, 12 nmol/L) for 20 min. Further details are available in Section 2.

To demonstrate the analytical performances of the impedimetric assay with synthetic oligonucleotides, a calibration experiment was designed. The genosensor response varied linearly ($r^2 = 0.9998$) with the target concentration over three orders of magnitude, between 12 pmol/L and 12 nmol/L (Fig. 4). Signals leveled off for higher concentrations, indicating that saturation of all available probes was achieved; moreover, the non-specific signal of biotinylated non-complementary oligomers was negligible up to 240 nmol/L (data not shown). Within the linear analytical range, the sensitivity was 52 kΩ/(nmol/L), with an estimated detection limit of 1.2 pmol/L (i.e., 7.2×10^6 target molecules in 10 μL of sample solution). Repetitive determinations of the 6 nmol/L target solution yielded reproducible signals with relative standard deviation of 10% ($n = 4$).

PCR amplified targets were 195 bp fragments of the 35S promoter. Amplified fragments of the lectin gene (217 bp) and the PCR mix (i.e., the solution containing primers, deoxynucleosides triphosphate, *Taq* polymerase, etc., but lacking the P35S template) were used as negative controls. The immobilised probe (25-mer) was designed to hybridise with one of the termini of the amplicon (i.e., it largely corresponded to the top primer used for amplification). Therefore, the assay selectivity strongly depended on the use of a biotinylated signaling probe which specifically recognised another region of the PCR product. The specificity of such a recognition was verified by exposing the probe-modified sensors to the pure phosphate buffer, 132 nmol/L of the signaling probe (alone) and, finally, 8.6 nmol/L of the 35S amplified target and the lectin negative control in the absence and presence (132 nmol/L) of the signaling probe.

The results, summarised in Table 2, show the excellent selectivity of the biosensing interface. The electron transfer

Table 2
Impedimetric assessment of the sandwich hybridisation assay specificity

Samples	Δ Charge transfer resistance (kΩ)
Buffer	0 ± 35
Signaling probe	1 ± 7
Lectin	−3 ± 14
Lectin + signaling probe	−4 ± 8
35S target	33 ± 20
35S target + signaling probe	743 ± 184

Probe-modified sensors were exposed for 1 h to the pure phosphate buffer, the signaling probe (alone) and thermally denatured 35S and lectin amplicons in the absence and presence of the biotinylated signaling probe. Further details are described in the text.

resistance remained unchanged in the case of all negative controls, thus demonstrating the absence of any non-specific interaction of the signaling probe with both the surface-tethered oligonucleotide and the lectin amplicon. On the contrary, significant enhancement of the signal was observed when the 35S amplicon hybridised at the sensor surface, even in the absence of the signaling probe. Such an increase of the electron transfer resistance was attributed to the higher electrostatic repulsion experienced by the $[Fe(CN)_6]^{3/4-}$ redox probe because of the increased number of interfacial negative charges (phosphate groups). Strong signal amplification was clearly observed for the biotinylated sandwich hybrid, which allowed binding of the enzymatic conjugate and subsequent bio-catalysed precipitation of the insulating product.

The influence of the amplified target concentration on impedimetric signals is illustrated in Fig. 5. Although non-linearly, the measured electron transfer resistance rapidly increased with the sample concentration within the 86 pmol/L–86 nmol/L range. Linear relationship between signal and concentration could be probably obtained within a narrower concentration range. Analytical signals levelled off for concentrations higher than 8.6 nmol/L, again indicating the saturation of all available probes. The estimated detection limit was 86 pmol/L (i.e., 5.2×10^8 target molecules in 10 μL of sample solution).

The analytical performance of the impedimetric genosensor was also demonstrated in connection with the electrochemical monitoring of the progress of the PCR amplification. The Δ charge transfer resistance values

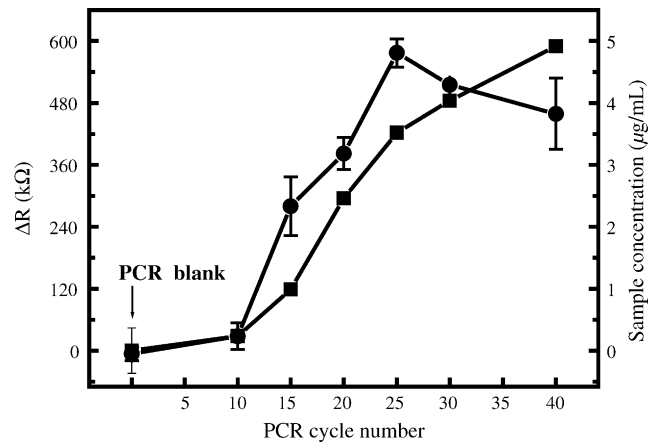

Fig. 6. Electrochemical monitoring of the progress of the PCR amplification. Δ Charge transfer resistance values (circles) were measured for probe-modified sensors exposed to a 10 μL drop of the amplified target solution (1:5 dilution in 0.5 mol/L phosphate buffer containing 132 nmol/L of signaling probe) for 1 h. Further details are available in Section 2. Samples concentrations (squares) were determined, after analogous dilution, by fluorometric measurement using the Picogreen™ dye.

obtained analysing samples individually subjected to 10, 15, 20, 25, 30 and 40 PCR cycles are displayed in Fig. 6. Also shown in Fig. 6 is the corresponding sample concentration profile determined by fluorometric measurement using the Picogreen™ dye. An almost linear increase of the electron transfer resistance was observed between the 10th and 25th PCR cycle, followed by a little decrease. Clearly, no variations of the charge transfer resistance were found for the amplicon-free PCR blank, which was used as the negative control. Besides the good agreement between impedimetric and fluorometric results, Fig. 6 also highlights the high sensitivity of the enzyme-amplified approach. The amplified target sequences were in fact detected starting from the 10th cycle, i.e., after just a $2^{10} = 1024$ amplification factor.

Table 3 summarises the analytical results obtained by analysing samples amplified from soy and maize containing 1 and 5% of genetically modified product. Negative control samples were both PCR blanks and products obtained using soy and maize wild-type DNAs during the amplification process. Further negative controls were 217 bp fragments of the

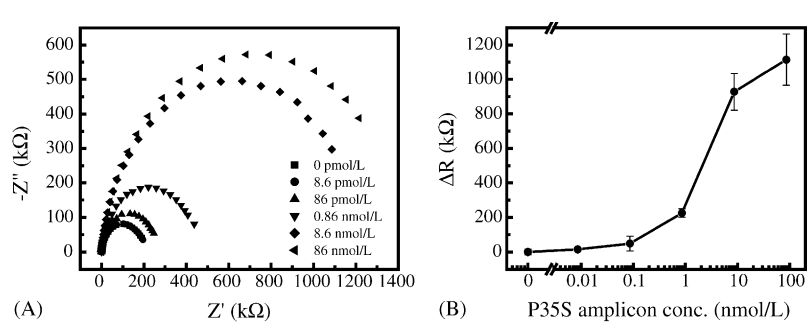

Fig. 5. Influence of the amplified target concentration on impedimetric signals. (A) Impedance spectra (Nyquist plots); (B) calculated Δ charge transfer resistance values. Probe-modified sensors were exposed to a 10 μL drop of the amplified target solution (0, 8.6×10^{-3}, 8.6×10^{-2}, 0.86, 8.6 and 86 nmol/L, in the presence of 132 nmol/L of signaling probe) for 1 h. Further details are available in Section 2.

Table 3
Electrochemical detection of the 35S promoter sequence in amplified samples obtained from GM Soy and Maize powders (GMO Soya: 0, 1 and 5% Roundup Ready™; GMO Maize: 0, 1 and 5% MON-810; negative controls: PCR blanks and lectin amplicons)

Samples	ΔR (kΩ)
PCR blank	12 ± 10
	−6 ± 6
	41 ± 20
Lectin	9 ± 3
	−4 ± 9
	−22 ± 16
GMO Soya 0% RR	−11 ± 7
	47 ± 23
	10 ± 6
GMO Soya 1% RR	586 ± 67
GMO Soya 5% RR	658 ± 163
	459 ± 78
	322 ± 66
GMO Maize 0% MON-810	−9 ± 3
	−13 ± 21
GMO Maize 1% MON-810	434 ± 66
	437 ± 31
GMO Maize 5% MON-810	417 ± 84

Probe-modified sensors were exposed to a 10 μL drop of the amplified target solution (1:5 dilution in 0.5 mol/L phosphate buffer containing 132 nmol/L of signaling probe) for 1 h. Further details are available in Section 2. Each sample was analysed at least in triplicate.

lectin gene. The electrochemical enzyme-amplified assay allowed unambiguous identification of all genetically modified samples, while no significant non-specific signal was detected in the case of all negative controls. Despite the original different content of genetically modified product, the 1 and 5% GM samples produced substantially similar electrochemical results, since that the final concentration of the amplicons was leveled off by the polymerase chain reaction itself. Therefore, the presence of GMOs could be properly quantified only for samples taken in the exponential phase of their amplification (as in the case of real-time PCR systems).

4. Conclusions

In this paper, as an extension of our previous work, we have reported the complete characterisation and further optimisation of our electrochemical genosensor, based on the use of disposable oligonucleotide-modified screen-printed gold electrodes.

The immobilisation of the thiol-tethered probe was qualitatively investigated by means of faradic impedance spectroscopy. Impedance spectra confirmed that the thiol moiety unambiguously drives the immobilisation of the oligonucleotide probe. Furthermore, both probe surface densities and hybridisation efficiencies were quantified through chronocoulometric measurements. Calculated probe surface densities were found to be surprisingly similar to those reported for analogous immobilisation, accomplished onto evaporated gold films.

Electrochemical transduction of the hybridisation process was also performed by means of faradic impedance spectroscopy, after bio-catalysed precipitation of an insoluble and insulating product onto the sensing interface. Chronocoulometric results allowed discussion of the magnitude of hybridisation signals in terms of probe surface densities and their corresponding hybridisation efficiency. Low hybridisation signals were clearly obtained when only a limited number of probe molecules was available for hybridisation at the sensor surface; significantly lower hybridisation efficiency was, on the contrary, observed when the probe molecules were too densely packed.

Compared to our previous work, the bio-catalysed precipitation of the insulating product and the use of faradic impedance spectroscopy as the electroanalytical method allowed a remarkable improvement of the sensitivity. The genosensor response varied linearly (r^2 = 0.9998) with the oligonucleotide target concentration over three orders of magnitude, between 12 pmol/L and 12 nmol/L. The estimated detection limit was 1.2 pmol/L (i.e., 7.2×10^6 target molecules in 10 μL of sample solution).

The analytical usefulness of the impedimetric genosensor was finally demonstrated analysing amplified samples obtained from soy and maize powders containing 1 and 5% of genetically modified product. Sensing of such 195 bp amplicons was achieved via sandwich hybridisation with a biotinylated signaling probe, in a flexible assay format that eliminates the need for using biotinylated primers during PCR amplifications. The electrochemical enzyme-amplified assay allowed unambiguous identification of all genetically modified samples, while no significant non-specific signal was detected in the case of all negative controls. While the genosensor presently acts as a *yes* or *no* system, work is currently in progress to improve its sensitivity and adapt it for the quantitative detection of GMO.

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