Clin Chem Lab Med 2003; 41(4):475–481 © 2003 by Walter de Gruyter · Berlin · New York

Electrochemical DNA Sensor for Detection of Single Nucleotide Polymorphisms

Leonel P.J. Marques[1], Isa Cavaco[1,2], José P. Pinheiro[2], Vera Ribeiro [1,2] and Guilherme N.M. Ferreira [1,3]*

[1] Centre for Molecular and Structural Biomedicine, University of Algarve, Faro, Portugal
[2] CMQA-ADQ/FCT, University of Algarve, Faro, Portugal
[3] Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Lisbon, Portugal

In recent years there has been an increased interest in using biosensors for the recognition and monitoring of molecule interactions. DNA sensors and gene chips are particularly relevant for directly applying the information gathered from the genome projects. In this work electrochemical techniques are used to develop methodologies to detect DNA polymorphisms in human genes using cytochrome P450 3A4 (*CYP3A4*) as a model gene. *CYP3A4* 1B* oligonucleotides were immobilized on the surface of a gold electrode and hybridized with fully complementary oligonucleotide sequences as well as with mismatched sequences corresponding to the *CYP3A4* 1A* reference sequence. The methodology developed is based on double-stranded DNA's ability to transport charge along nucleotide stacking. The perturbation of the double helix pi-stack introduced by a mismatched nucleotide reduces electron flow and can be detected by measuring the attenuation of the charge transfer. The methodology developed could identify *CYP3A4* 1A* homozygotes by the 5 µC charge attenuation observed when compared with DNA samples containing at least one *CYP3A4* 1B* allele. Clin Chem Lab Med 2003; 41(4):475–481

Key words: Biosensors; DNA sensors; Genotyping; Single nucleotide polymorphism; Electrochemistry.

Abbreviations: CC, chronocoulometry; CV, cyclic voltammetry; *CYP*, cytochrome P450; ds, double-stranded; MB, methylene blue; ODN, oligonucleotide; PBS, phosphate buffered saline; RFLP, restriction fragment length polymorphism; SNP, single nucleotide polymorphism; ss, single-stranded.

Introduction

One of the greatest challenges of modern biomedicine is the integration of new genetic information into procedures that can be implemented in rapid, cost effective, and reliable methods to genotype and to identify gene function, in order to be used as diagnostic tools in disease settings.

DNA sensors are particularly relevant for directly applying the information gathered from genome projects and polymorphism databases. Such devices are expected to have a considerable impact on diagnostic and health care in the near future since analysis throughputs can be increased several-fold with complete data standardization (1–3). Polymorphism identification, sequence recognition, pathogen identification, expression profiling, and mutation detection are examples of current applications of DNA sensors (4–11).

Briefly, in DNA sensors a solid surface (the sensor surface) is programmed with specific single-stranded DNA (ssDNA) sequences designed to probe samples and recognize particular gene loci through complementary hybridization with target samples. The critical issue in DNA sensing is the generation of a sensitive and selective signal to measure the hybridization analysis result (12). In the majority of the current DNA sensor applications this is achieved indirectly by gathering a signal generated by specific reporter groups, usually a fluorescent molecule, used to label the target molecules. These approaches require labeling the samples with the reporter groups prior to the sensing reaction, which constitutes a common source of irreproducibility and contamination and is critical for the sensitivity of the overall analysis (12, 13). To avoid such bottlenecks, the use of methodologies to directly measure the hybridization analysis results without involving sample pre-treatment is desirable (12).

From such a perspective, interest in electrochemical detection of DNA hybridization has recently increased. The electrochemical activity of nucleotide residues in DNA and RNA (8, 14, 15), particularly adenine and guanine, as well as the long range ability of double-strand DNA (dsDNA) to transport charge through the double helix pi-stack (16–18), enable the direct and real-time transduction of hybridization reactions (9, 10).

Different electrochemical transducing methodologies are described in the literature. Cyclic voltammetry (CV), square wave voltammetry and chronocoulometry (CC) are the most common techniques in the detection of hybridization reactions in gold, mercury, and carbon paste electrodes (6–15).

In this work electrochemical techniques are used to develop methodologies for detecting DNA polymorphisms in human genes potentially involved in the modulation of individual cancer susceptibility as well as therapeutic efficacy. The electrochemical activity of a DNA intercalator is used to measure the variations on the charge transport through the DNA pi-stack occurring when mismatched nucleotides are present as in

*E-mail of the corresponding author: gferrei@ualg.pt

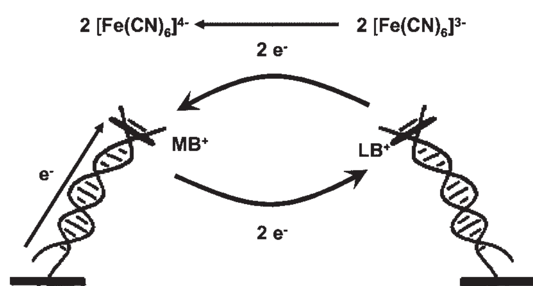

Figure 1 Schematic representation of the electrocatalytic cycle for a DNA intercalator (methylene blue; MB) and an electrochemically active compound (ferricyanide-$[Fe(CN)_6]^{3-}$) through DNA double helix. LB: leukomethylene blue.

the case of polymorphic locations (9, 10, 19). This DNA intercalator is reduced by the electron flow through DNA and releases the received electrons to an electrochemically active compound in the solution. An electrochemical catalytic cycle is therefore generated that is fed by electron flow from the electrode (9, 10, 19) (Figure 1). To detect this electron flow the most convenient technique is CC (9, 10). In this technique a fixed reduction potential is applied to the electrode and the evolution of current with time is followed generating a signal. The integration of this signal gives the amount of charge exchanged in the process, which is significantly larger for the perfectly hybridized sequences when compared with sequences including one mismatched nucleotide (9, 10).

A cytochrome P450 3A4 (*CYP3A4*) electrochemical DNA sensor was developed to identify the presence of variant **1B* alleles. *CYP3A4* is the main drug-metabolizing enzyme in human liver, being responsible for the biotransformation of at least 50% of the currently available therapeutic drugs, such as antiarrhythmics, cytostatics, and antimalarials (20, 21). *CYP3A4*1B* was the first variant described that has been proposed to affect the enzyme concentration, to be associated with advanced stage prostate cancer (22), and to be protective for secondary cancer caused by leukemia chemotherapy (23).

Materials and Methods

All chemicals used were pro-analysis or equivalent purity grade. Oligonucleotides (ODNs) were purchased from Invitrogen Corp. (Carlsbad, USA) or Hybaid (Interactiva Division, Ulm, Germany).

Preparation of DNA-modified gold electrodes

Single-stranded modified gold electrodes were prepared by immobilizing thiol-activated single-stranded oligonucleotides (ssODN, designated by probe) designed on the basis of the *CYP3A4* gene sequence (Genbank accession number AF280107). The *CYP3A4*1B* 17-mer probe sequence was designed in order to locate the polymorphic locus (underlined nucleotide) in the probe center and to have a melting temperature close to 50 °C. An additional requirement was the attachment of a mercaptohexyl group (-$SH(CH_2)_6$-) at the 5′ end to enable immobilization. The *CYP3A4*1B* 17-mer probe sequence used for immobilization is (5′-$SH(CH_2)_6$-CCTCTCTCCT-GCCCTTG-3′)

A gold electrode (2 mm diameter, Metrohm, Herisau, Switzerland) was cleaned by polishing the surface with an electrode polishing kit (Metrohm, Herisau, Switzerland) containing alumina. After polishing, the electrode was sonicated for 5 min sequentially in a 65% (v/v) solution of nitric acid (Riedel-de-Haen, Seelze, Germany), acetone (Riedel-de-Haen, Seelze, Germany), and Milli-Q water. This cleaning procedure was performed prior to each immobilization.

Cleaned electrodes were immersed overnight in 100 μl of a 5 μM probe solution in 100 mM phosphate buffer containing 100 mM NaCl, pH 7 (PBS buffer). After immobilization, the electrode was rinsed with PBS buffer and then immersed in a 0.1 mM 2-mercapto-1-ethanol solution (Fluka, Buchs, Switzerland) – mercaptoethanol – for 90 min and rinsed again with PBS buffer.

Double-stranded oligonucleotide (dsODN)-modified electrodes were prepared by hybridizing the ssODN-modified gold electrode with fully complementary (5′-CAAGGGCA-GGAGAGAGG–3′) or single nucleotide mismatch sequence (5′-CAAGGGCAAGAGAGAGG-3′), corresponding to the *CYP3A4*1B* and **1A* alleles, respectively. Hybridization reactions were performed in a 100 μl solution of 50 nM target ODN in PBS buffer for 270 min at room temperature.

Electrochemical measurements

CV and CC were performed using an Ecochemie Autolab PGSTAT 12 potentiostat, and the results were analyzed using the General Purpose Electrochemical System (GPES) software, version 4.8 (Ecochemie B.V., Utrecht, The Netherlands). A normal three-electrode configuration consisting of a modified, gold disk, working electrode, a homemade Ag/AgCl reference electrode, and a graphite counter electrode was used. The supporting electrolyte (PBS buffer) was deoxygenated *via* purging with nitrogen gas for 20 min, and the cell was blanketed with nitrogen during the experiments. CV was performed between −0.5 and 1.25 V at a 0.1 V/s scan rate, in 0 to 8 μM methylene blue (MB; Fluka, Buchs, Switzerland), and 0 to 2.5 mM potassium hexacianoferrate (III) (Merck, Darmstadt, Germany), hereafter designated ferrycianide. CC was performed with a −0.4 V applied potential for 5 s, in 2 mM hexacianoferrate (III) and 8 μM MB.

Three independent electrochemical experiments, either CV or CC, were performed and average values, standard deviations, and 95% confidence intervals for the mean values were calculated.

*Electrochemical detection of CYP3A4*1B in DNA samples*

Genomic DNA was extracted from whole peripheral blood samples (24), obtained during regular medical check-ups upon informed consent and approval by the Ethical Boards of the involved clinical institutions. The samples were tested for the identification of the *CYP3A4*1B* single nucleotide polymorphism (SNP), which corresponds to an A→G transition at −392 bp upstream of the transcription initiation site (22). A 124 bp fragment, in which the polymorphic site is located in a central position, was amplified using the following primers (Invitrogen Corp. Carlsbad, USA): forward, 5′-TGT GGC TTG TTG GGA TGA ATT TC-3′ and reverse, 5′-GGT TCT TAT CAG AAA CTC AAG TGG-3′. Amplification was carried out in a reaction mixture containing 2 μl of DNA (200–700 ng DNA), 1.5 mM $MgCl_2$, thermophilic DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), 0.2 mM of the four deoxynucleotide triphosphates (Stratagene, La Jolla, USA), 2.5 units of Taq polymerase (Promega Corp., Madison, USA)

and 0.6 μM of each primer. Touchdown PCR amplification was performed (Mastercycler 5332, Eppendorf, Germany) as follows: denaturation at 94 °C for 2 min, followed by 7 cycles of 30 s at 56 °C, 15 cycles of 45 s at 53°C, 5 cycles of 45 s decreasing 1 °C/cycle from 52 °C to 48 °C, and 15 cycles 45 s at 48 °C, and a final extension step for 2 min at 72 °C. An aliquot was analyzed by polyacrylamide gel (8%) electrophoresis for 2 h at 200 V, to check for amplification efficiency and specificity.

The PCR product was diluted to a concentration of approximately 50 nM in PBS buffer and denatured at 90 °C for 2 min. After rapid cooling, the ssODN-modified electrode was immersed in the solution (100 μl) for 270 min, so that the amplicon could hybridize with the immobilized probe. Electrochemical measurements, CV, and CC were carried out with the same parameters described above.

*CYP3A4*1B detection by PCR-restriction fragment length polymorphism (RFLP) analysis*

Amplification was performed in a reaction mixture containing 2 μl of template DNA, 0.6 μM of each primer (Invitrogen Corp., Carlsbad, USA), forward, 5′-AAT GAG GAC AGC CAT AGA GAC AAG GGC A-3′ and reverse, 5′-CAA TCA ATG TTA CTG GGG AGT CCA AGG G–3′, 200 μM of each dNTP (Stratagene, La Jolla, USA), 1.5 mM $MgCl_2$, thermophilic DNA polymerase buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100), and 2.5 U of *Taq* DNA polymerase (Promega Corp., Madison, USA). Thirty cycles of amplification were performed including 45 s denaturation at 94 °C, 45 s annealing at 59 °C, and 45 s extension at 72 °C. The PCR product (122 bp) was digested with 5 U *Mva*I (Fermentas, Vilnius, Lithuania) for 3 h, at 37 °C, and analyzed by electrophoresis as before.

Results and Discussion

Gold electrode modification

DNA electrochemical sensors were prepared by immersing the clean electrode overnight in 100 μl of a 5 μM solution of thiol-terminated ODNs. Chemi-adsorption of thiol onto the gold surface is a fast process, completed in 240 min (25), and leads to the formation of densely packed monolayers with immobilized ODNs oriented in an upright position with respect to the electrode surface (25–27). The modified electrode was then immersed in a mercaptoethanol solution in order to remove unbound ssODN as well as to promote correct ssODN-surface orientation (25–27), and the dsODN modified electrode was obtained by immersing the ssODN-modified electrode in the target solution allowing complementary hybridization to occur.

CV in PBS buffer was therefore performed as a control assay for the immobilization of ODNs and for the follow-up of electrode hybridization and denaturation. As shown in Figure 2, two peaks are present that correspond to the gold oxide formation at the electrode surface (approximately 1.1 V) and the respective reduction peak (approximately 0.4 V) (28). Their intensities and peak potentials change according to the modification due to the different electrode surface status (Figure 2). The higher intensity observed for the modified electrodes suggests that higher amounts of oxides are formed at the electrode surface, which could be due to the higher electrostatic attraction of metal ions by the

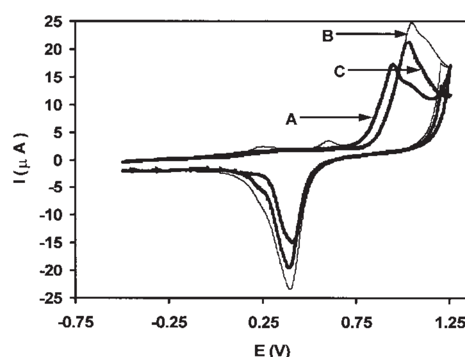

Figure 2 Cyclic voltammograms in buffer at bare gold electrodes (curve A), gold electrodes modified with ssODN (curve B), and dsODN (curve C).

negatively charged ODN layer (28). The intensity of these peaks, however, decreased for the double-stranded modified electrode. Although higher negative charges are present near the electrode surface, dsODN-modified electrodes are also more hindered and thus less surface space is available for the accumulation of metal ions (28). The different intensities of the oxide peaks, especially the reduction peak, thus enable a clear indication of electrode surface status, clean or modified with ODN, as well as the identification of the species immobilized (ss or dsODN).

Electrochemical genotyping – method development

In order to assess the potential identification of polymorphisms through charge transport along the dsODN film, a catalytic electrochemical cycle was generated using a DNA intercalator (MB) and a solution-borne electron acceptor (ferricyanide; Figure 1) MB is a DNA intercalator with pronounced electrochemical response (reduction potential: –0.33 V). It binds at the top of the immobilized dsODN film and is reduced by receiving the electrons flowing from the electrode surface through the DNA pi-stack (9, 10, 29). Leukomethylene blue (LB), which is the reduced form of MB, will then release the received electrons to reduce solution-borne ferricyanide, returning to the initial oxidized form (MB). The cycle continues as long MB can be reduced by electron flow, and is independent of the concentration of the involved species. Mismatched nucleotides lead to base stacking interruptions and are expected to have a pronounced effect on the observed catalytic cycle due to the diminished electron flow through the DNA film.

Ferricyanide is a negatively charged, electrochemically active compound and therefore is expected to be repelled from the negatively charged dsODN film. Ferricyanide is expected to be kept away from the electrode surface and only residual electrochemical peaks should be observed in CV. However, the ferricyanide reduction/oxidation peaks at 0.20 V and 0.06 V, respectively, observed in CV at dsODN-modified electrodes (Figure 3A), are an indication that electrons are exchanged between solution-ferricyanide and the elec-

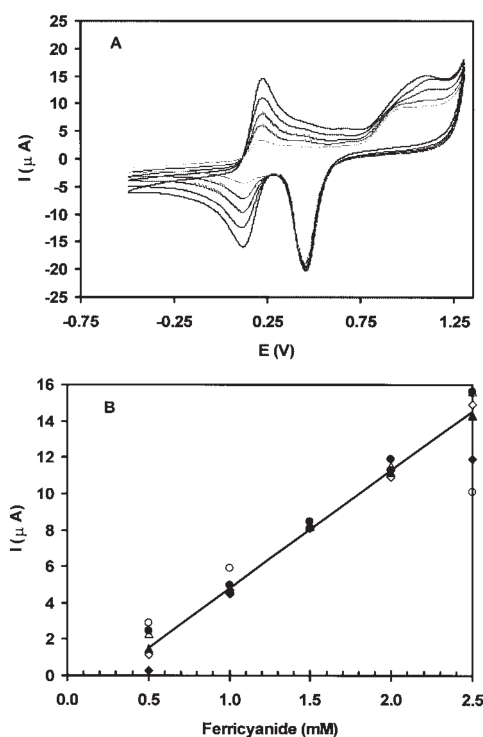

Figure 3 (A) Cyclic voltammograms for increasing ferricyanide concentration at dsODN-modified electrodes in PBS buffer. (B) Ferricyanide reduction peak intensity variation with ferricyanide concentration at dsODN-modified electrodes in PBS buffer containing 0 μM (open circles); 0.25 μM (dark diamonds); 0.5 μM (open diamonds); 1.0 μM (dark triangles); 2.0 μM (open triangles), and 4.0 μM (dark circles) methylene blue.

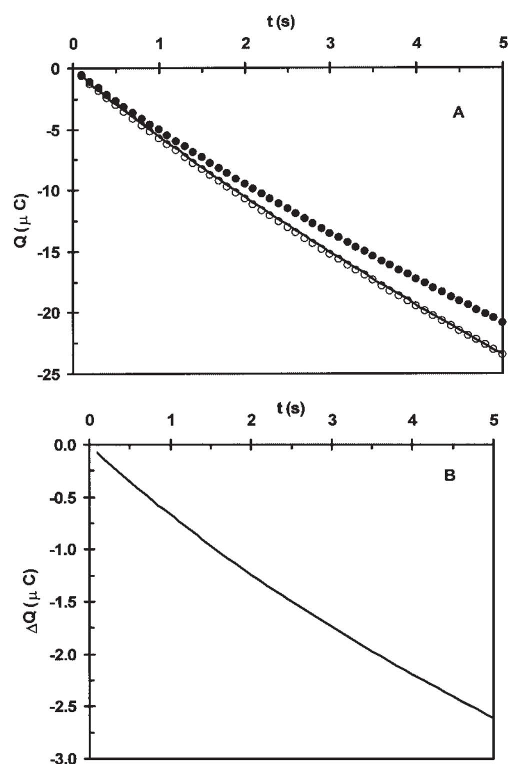

Figure 4 (A) Chronocoulometry at −0.4 V for 5 s in 2.0 mM of ferricyanide and 8 μM MB^+ for ssODN-modified electrode (solid line), ssODN-modified electrode hybridized with fully complementary oligonucleotide (open circles), and ssODN-modified electrode hybridized with mismatched oligonucleotide (closed circles). (B) Accumulated charge difference for fully complementary and mismatched samples.

trode surface. The peaks' intensity is linearly correlated with ferricyanide concentration and is independent of the MB concentration (Figure 3B).

CC can be used to monitor the electrochemical catalytic cycle described above if the applied potential is sufficient to guarantee the continuous reduction of the intercalated MB. In CC, a fixed reductive potential is applied to the electrode and current evolution within a certain time interval is integrated to obtain the charge involved in the electrochemical process.

ODN-modified electrodes were hybridized with fully complementary and mismatched synthetic ODN targets, and CC at −0.40 V of 2 mM ferricyanide in the presence of 8 μM MB was performed (Figure 4A). As observed, the presence of a mismatched nucleotide leads to a significant 2.6 μC charge attenuation (Figure 4A) that increases linearly with time (Figure 4B). This is indicative of the diminished DNA-mediated electron transfer efficiency as a result of the local base stack perturbation (9, 10). Owing to the poor base stack, MB is not efficiently reduced to generate and to maintain the electrochemical catalytic cycle, which is reflected by reduced charge accumulation in CC (9, 10).

Charge accumulation (Q) at ssODN-modified electrodes was also examined. As shown in Figure 4A, this was the case with lower charge accumulation. This is an expected result since a monolayer of mercaptoethanol is formed at the electrode surface after ODN immobilization in order to saturate the unoccupied surface space. The CC activity at ssODN-modified electrodes demonstrates that the monolayer formed was not able to completely block the electrochemical activity of ferricyanide. Ferricyanide and MB can diffuse to the electrode surface, and direct catalysis, not DNA-mediated, occurs (9). The different charge transfer mechanisms are clearly demonstrated by the different slope and shape observed for the curves obtained when using single- and double-stranded ODN-modified electrodes (9) (Figure 4). In some cases, however, the curve obtained for the ssODN-modified electrode had a similar magnitude to the perfectly matched hybrid (data not shown). This was interpreted as evidence of the formation of an imperfect monolayer containing holes where the gold surface is exposed to the buffer.

The electrode surface status, *i.e.*, single- or double-stranded modification, was always assessed by the analysis of the gold oxide reduction peak (≈ 0.4 V) using CV (Figure 2), performed prior the CC experiments. This enabled the evaluation of the success of the hybridization and avoided misinterpretation due to false positives owing to possible hybridization failure.

In order to assess the method's ability to discriminate between homo- and heterozygotes, a solution of equal amounts of fully complementary and mismatch ODNs was prepared and hybridized to an ssODN-modified electrode. As expected, accumulated charges were sit-

uated between the two limits of fully complementary and mismatched ODNs (Figure 4A), suggesting that the method developed has the potential to discriminate heterozygotes.

Electrochemical genotyping – method validation and testing

In order to develop an alternative method for genotyping, the methodology must be validated with samples previously genotyped by standard methods (*e.g.*, PCR/RFLP). As such, ODN primers were designed to amplify a 124 bp fragment of the *CYP3A4* gene locating the targeted polymorphic loci in the amplicon center. Amplicons obtained from genomic DNA samples were genotyped by RFLP (Figure 5) and hybridized to ssODN-modified electrodes. After washing the electrode to remove unhybridized DNA, CV in buffer was performed to investigate the type of the monolayer film at the electrode surface (*i.e.*, single- or double-stranded DNA film), and electrochemical genotyping was performed by measuring the charge accumulation (Q) after 5 s in 2 mM ferricyanide and 8 μM MB.

Figure 6 shows the characteristic charge accumulated at the electrode surface after 5 s for homozygotes for *CYP3A4*1A* (genotype **1A/*1A*) and *CYP3A4*1B* (genotype **1B/*1B)* variants, as well as for heterozygotes for the mutation (genotype **1A/*1B*). Since the immobilized ODN (probe) was designed in order to be fully complementary for the **1B* variant, mismatched hybrids were obtained after hybridizing the electrode with **1A* samples. Therefore, samples presenting less charge accumulation correspond to **1A* samples. As previously mentioned, for heterozygotes the ratio of fully complementary/mismatch is equal. This is reflected in CC by the intermediate overall charge accumulation observed (Figure 6).

The 95% confidence intervals for the mean charge accumulated (Q) at the electrode surface after 5 s for triplicate chronocoulometric measurements of previously genotyped DNA samples are presented in Table 1. As shown (Table 1), the electrochemical genotyping methodology can distinguish *CYP3A4*1A* homozygotes, *CYP3A4*1A/CYP3A4*1B* heterozygotes, and *CYP3A4*1B* homozygotes. The 95% confidence intervals for the mean of the average charge accumulated at the electrode surface are $Q_m = -26.5 \pm 0.07$ μC, $Q_m = -30.8 \pm 0.06$ μC, and $Q_m = -33.59 \pm 0.02$ μC for **1A* homozygotes, **1A/*1B* heterozygotes, and **1B* homozygotes, respectively. These values correspond to

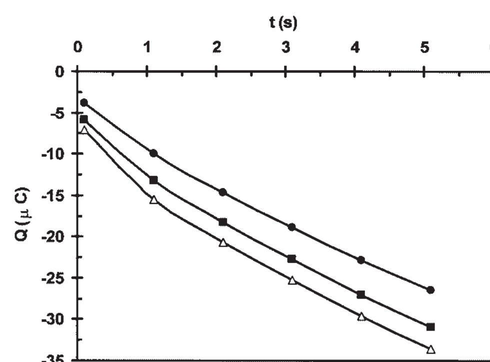

Figure 6 Chronocoulometry at −0.4 V for 5 s in 2.0 mM of ferrycianide and 8 μM MB^+ at ssODN-modified electrode hybridized with *CYP3A4*1B* homozygotes (open triangles), *CYP3A4*1B/*1A* heterozygotes (closed squares), and *CYP3A4*1A* homozygotes (closed circles).

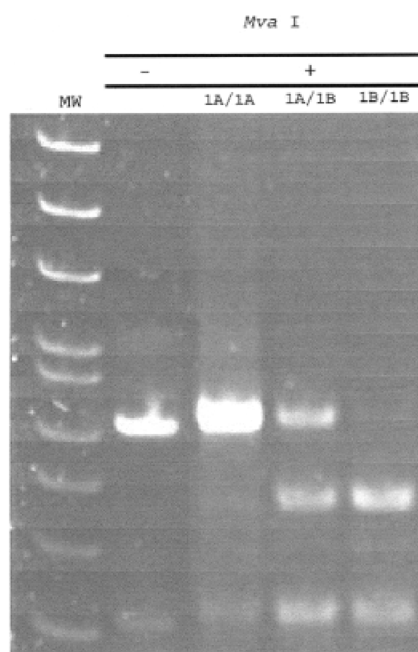

Figure 5 Polyacrylamide gel (8%) of a typical RFLP analysis. PCR products were analyzed before (–) (lane 2) and after (+) digestion with *Mva*I.Typical restriction patterns are shown for *CYP3A4*1A/*1A*, **1A/*1B*, and **1B/*1B* genotypes. *Mva*I digestion of the 122 bp PCR product gives rise to two fragments (97 bp and 25 bp) in the *CYP3A4 *1B* variant. MW: molecular weight marker: Φx174 digested with *Hinf*I.

Table 1 95% Confidence intervals for the mean charge accumulated ($Q \pm \sigma$) at the electrode surface for previously genotyped samples by RFLP and 95% confidence intervals for the mean of the average charge accumulated ($Q_m \pm \sigma_m$) for *CYP3A4*1A* homozygotes, *CYP3A4*1A/CYP3A4*1B* heterozygotes, and *CYP3A4*1B* homozygotes.

Sample	RFLP genotyping	$Q \pm \sigma$ (μC)	$Q_m \pm \sigma_m$ (μC)
# 1	**1A/*1A*	−27.89 ± 0.01	−26.5 ± 0.07
# 2	**1A/*1A*	−23.7 ± 0.06	
# 3	**1A/*1A*	−25.54 ± 0.04	
# 4	**1A/*1A*	−27.6 ± 0.06	
# 5	**1A/*1B*	−30.22 ± 0.03	−30.8 ± 0.06
# 6	**1A/*1B*	−30.18 ± 0.02	
# 7	**1A/*1B*	−29.13 ± 0.04	
# 8	**1A/*1B*	−33.91 ± 0.02	
# 9	**1A/*1B*	−31.30 ± 0.03	
# 10	**1B/*1B*	−33.59 ± 0.02	−33.59 ± 0.02

7.1±0.09 μC and 2.8±0.08 μC decreases for **1A* homozygotes and **1A/*1B* heterozygotes, respectively, when compared with the fully complementary hybrid formed at the electrode surface for **1B* homozygote samples. Although these are significant differences, only one previously genotyped **1B* homozygote sample was available for method validation (Table 1). Therefore, in order to ensure statistical significance of sensors' sample classification, electrochemical genotyping methodology was used to distinguish **1A* homozygotes from samples containing at least one **1B* allele (*i.e.*, heterozygotes or homozygotes for the polymorphism). Electrochemical sample classification was performed on the basis of normal distributions with the (average; standard deviation) parameters (26.5; 1.91) and (31.4; 1.95) for *CYP3A4*1A* homozygotes (validated with 4 samples and 12 independent measurements; Table 1) and for samples containing at least one **1B* allele (validated with 6 samples and 18 independent measurements; Table 1), respectively.

A blind assay was performed to test the methodology developed. Sixteen samples were genotyped independently by the electrochemical method and by RFLP (Table 2). Both electrochemical and RFLP genotyping gave similar results for *CYP3A4*1A* homozygotes considering two possible classifications: **1A* homozygotes and samples containing at least one **1B* allele. This discrimination failed only for one case (sample #17, Table 2), corresponding to a 6.25% failure frequency (Table 2).

In summary, an electrochemical genotyping methodology was developed with synthetic ODNs, validated with previously genotyped DNA samples and tested with DNA extracted from human subjects. The methodology developed was able to identify *CYP3A4*1A* homozygotes as well as samples containing at least one *CYP3A4*1B* allele. Furthermore, the method is fast (5 s) and reproducible. However, at this stage the method was not able to differentiate *CYP3A4*1B* homo- and heterozygotes. This apparent failure is due to poor discriminating power owing to the proximity of the accumulated charge curves for homozygotes **1A* and **1B*. Efforts are being undertaken to further optimize the methodology in order to enable full genotyping capacity by discriminating homozygotes and heterozygotes.

Table 2 Electrochemical and RFLP genotyping comparison.

Sample	$Q \pm \sigma_m$ (μC)	Electro-chemical genotyping	RFLP genotyping	Comparison
# 11	−28.95±0.03	?/*1B	*1B/*1B	+
# 12	−29.57±0.04	?/*1B	*1B/*1B	+
# 13	−28.5±0.02	?/*1B	*1A/*1B	+
# 14	−26.29±0.04	*1A/*1A	*1A/*1A	+
# 15	−27.96±0.01	*1A/*1A	*1A/*1A	+
# 16	−27.7±0.06	*1A/*1A	*1A/*1B	+
# 17	−28.93±0.03	?/*1B	*1A/*1A	−
# 18	−32.17±0.02	?/*1B	*1A/*1B	+
# 19	−26.39±0.03	*1A/*1A	*1A/*1A	+
# 20	−34.16±0.01	?/*1B	*1B/*1B	+
# 21	−29.71±0.01	?/*1B	*1B/*1B	+
# 22	−31.36±0.03	?/*1B	*1A/*1B	+
# 23	−29.274±0.003	?/*1B	*1A/*1B	+
# 24	−31.48±0.02	?/*1B	*1B/*1B	+
# 25	−27.60±0.02	*1A/*1A	*1A/*1A	+
# 26	−30.17±0.02	?/*1B	*1B/*1B	+

Genotypes are identified; (?) indicates failure in identifying the allele. Comparison is based upon identification of *CYP3A4*1A* homozygotes or samples containing at least one **1B* allele.

Acknowledgements

This work was partially supported by Fundação para a Ciência e a Tecnologia, Ministério da Ciência e Ensino Superior (Project POCTI/ESP/39589/01).

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Received 2 December 2002, revised 28 February 2003, accepted 6 March 2003

Corresponding author: Dr. Guilherme N.M. Ferreira, FERN, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
Phone: +351 289 800 900, Fax: +351 289 81 84 19,
E-mail: gferrei@ualg.pt