Detection of Human Apolipoprotein E Genotypes by DNA Electrochemical Biosensor Coupled with PCR

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Background: Apolipoprotein E (apoE) is an important constituent of several plasma lipoproteins, mainly VLDL, HDL, and chylomicrons. It is involved in the redistribution of lipids in the liver and is implicated in growth and repair of injured neurons in the nervous system. apoE has also been associated with the risk of developing cardiovascular diseases and in familial type III hyperlipoproteinemia.

Methods: We developed a new procedure for detecting genetic polymorphisms of apoE in human blood samples. The procedure is based on coupling of DNA electrochemical sensors with PCR-amplified DNA extracted from human blood. The DNA electrochemical sensor incorporated single-stranded oligonucleotides immobilized on graphite screen-printed electrodes (SPEs) by adsorption at controlled potential. The hybridization reaction on the electrode surface was monitored by chronopotentiometric stripping analysis (PSA), using daunomycin as indicator.

Results: With use of two different probes, it was possible to investigate both DNA positions in which the apoE polymorphism takes place and thus to distinguish different genotypes. Real samples containing only complementary sequences gave a good increase in the area of the daunomycin peak (~600 ms) compared with the peak observed with the buffer. Samples containing 50% complementary sequences gave a much lower increase, and samples containing only mismatch sequences gave a decrease in the daunomycin area. The procedure was validated by comparison with a method based on polycrylamide gel electrophoresis.

Conclusion: The coupling of DNA electrochemical sensors with PCR allowed quick discrimination between the different genotypes of apoE.

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In recent years, there has been considerable interest in developing a DNA electrochemical biosensor for rapid applications in genetic analysis. DNA biosensors can detect the presence of genes or mutant genes associated with inherited human diseases. A DNA electrochemical biosensor generally is an electrode with an oligonucleotide immobilized on the surface.

Recently, some reports have indicated that electrochemical techniques are well suited for measuring hybridization events (1–4). The hybridization can be detected by redox-active metal/polypyridine complexes that associate selectively and reversibly with double-stranded immobilized DNA (5). In some cases, the redox-active metal causes a catalytic oxidation of guanine (6,7), and enhancement of the charged exchange has been reported. Alternatively, an electroactive antibiotic such as daunomycin intercalates in the double strand; in this case an enhancement of the charge is also obtained (8–10).

Most of the recent reports have concentrated only on synthetic oligonucleotides, showing that the hybridization can be monitored by variation of current or potential values. Only a few authors (3) have reported the coupling of PCR-amplified DNA from real samples with electrochemical DNA biosensors to obtain reliable measurement of clinical interest.

This report describes the use of a disposable (using thick-film technology) electrochemical DNA sensor (carbon electrode with an immobilized oligonucleotide on the surface and daunomycin as electrochemical indicator) coupled with PCR amplification of DNA extracted from human blood for the genetic detection of apolipoprotein E (apoE)3 polymorphism.

3 Nonstandard abbreviations: apoE, apolipoprotein E; SPE, screen-printed electrode; PSA, potentiometric stripping analysis; SCE, saturated calomel electrode; and SSC, saline sodium citrate.
apoE is an important constituent of several plasma lipoproteins, mainly VLDL, HDL, and chylomicrons. It is involved in the redistribution of lipids in the liver and is implicated in growth and repair of injured neurons in the nervous system. apoE has been associated with the risk of developing cardiovascular diseases and in familial type III hyperlipoproteinemia (11).

Human plasma apoE is a 299-amino acid protein composed of a single polypeptide chain. In humans, apoE presents a genetic polymorphism: it is present as three major isoforms, E2, E3, and E4, related to two polymorphic sites on codons 112 and 158 of the gene located on chromosome 19. These isoforms arise from three alleles, e2, e3, and e4, respectively, combined in six different genotypes.

The molecular bases of the apoE polymorphism are cysteine-arginine interchanges: apoE2 contains cysteine residues at positions 112 and 158; apoE3 contains a single cysteine at position 112 and an arginine at position 158; apoE4 contains arginine residues at both positions (12). This polymorphism leads to the presence of six different phenotypes in the human population: three homozygous (E3/E3, E2/E2, and E4/E4), and three heterozygous (E2/E3, E2/E4, and E3/E4). These protein substitutions correspond to nucleotide substitutions in the DNA regions encoding common apoE isoforms: allele e2 has a thymine in codons 112 and 158 of the sequence; e3 has a thymine in codon 112 and a cytosine in codon 158; e4 has a cytosine in both codons (13).

The apoE polymorphism is functional and influences a variety of physiological and pathological processes. Most patients with type III hyperlipidemia are homozygous for the E2 isoform, and the same isoform is associated with decreased cholesterol and β-lipoprotein; apoE3 is the most common isoform.

Moreover, several independent studies have shown that the inheritance of one or more e4 alleles increases an individual’s risk of developing atherosclerosis and Alzheimer disease. Conversely, the inheritance of one or more e2 alleles confers protection against Alzheimer disease and is associated with lower lipid concentrations. Thus, a rapid and simple genotyping test is needed for any laboratory that takes part in clinical research related to apoE.

Several methods have been developed for apoE typing at the DNA level, using genomic DNA for Southern blots with allele-specific oligonucleotide probes (14, 15). The sensitivity of these methods has been increased by the use of PCR to amplify APOE sequences for dot-blot without allele-specific oligonucleotide probes (16).

Here we report the development of a DNA biosensor capable of detecting apoE polymorphisms, using DNA fragments amplified by PCR. The procedure involves the use of an electroactive indicator, daunomycin, which intercalates the double-stranded DNA (8–10, 17). Synthetic oligonucleotide (23mer; the probe) is immobilized on the graphite electrode surface by adsorption at a controlled potential. The DNA sensor obtained is incubated with the target solution. The probe is then immersed in the daunomycin solution, which intercalates the hybrid formed on the electrode surface. The daunomycin anodic peak is used to detect the presence and amount of the complementary sequence by chronopotentiometric transduction (18).

The hybridization reaction has been performed on synthetic oligonucleotides (23mers) and on real samples of PCR-amplified DNA from human blood. The system was able to perform apoE typing of blood samples.

Materials and Methods

Daunomycin hydrochloride was purchased from Sigma Chemicals. The oligonucleotide base sequences were obtained from Pharmacia Biotech as sodium salts.

The probes and target nucleotides (based on point mutations that cause human diseases) were 23mer oligonucleotides:

 Probe 1 (5'-CAGGCGGCACACAGTCCTCCA-3') has a sequence that is complementary to a fragment of allele e3 and surrounds codon 112.
 Probe 2 (5'-CACTGGACTGCTTTGTAGG-3') has a sequence that is complementary to a fragment of allele e3 and surrounds codon 158.
 Oligo 2 (5'-ACCTGGACAAGTGCCAGG-3') has a sequence that is the same as a fragment of allele e2 and surrounds codon 158.
 Oligo 3 (5'-TGAGGGACGTGTCCCAGG-3') has a sequence that is the same as a fragment of allele e3 and surrounds codon 112.
 Oligo 3b (5'-ACCTGGGACCAGTGCCCAGG-3') has a sequence that is the same as a fragment of allele e3 and surrounds codon 158.
 Oligo 4 (5'-TGGAGGACGTGCCCAGG-3') has a sequence that is the same as a fragment of allele e4 and surrounds codon 112.

The mismatched bases are in bold.

Buffer solutions were prepared from AnalAR grade, DNase- and RNase-free reagents and deionized water.

Electrochemical experiments were performed with an AUTOLAB PGSTAT 10 electrochemical analysis system, with a GPES4 software package (Eco Chemie), in connection with a VA-Stand 663 (Metrohm).

SPs were printed with a Model 245 screen printer, obtained from DEK, using different inks obtained from Acheson Italiana. A graphite-based ink (Electrodag 421), a silver ink (Electrodag 477 SSFU), and an insulating ink (Electrodag 6018 SS) were used. The substrate was a polyester flexible film (Autostat HTS) obtained from Autotype Italia. The working electrode surface area was 20 mm². A saturated calomel electrode (SCE) and platinum wire (3 mm diameter) were used as reference and auxiliary electrodes respectively.
ELECTROCHEMICAL MEASUREMENTS
Potentiometric stripping analysis (PSA) at a constant current was performed as follows: the potentials were sampled at a frequency of 33 kHz, and the derivative signal (dt/dE) was recorded vs the potential using a range of 0.2–1.05 V for potential and a constant current of +1 μA in buffer solution [2× saline sodium citrate (SSC), containing 300 mmol/L NaCl-30 mmol/L C6H5Na3O7, pH 7.4].

The daunomycin peak area after baseline fitting was used as the analytical signal, and all electrochemical measurements were carried out at room temperature in a 5-mL polytetrafluoroethylene beaker.

HYBRIDIZATION DETECTION USING SYNTHETIC OLIGONUCLEOTIDES
The procedure consisted of the following steps performed on a new SPE: probe immobilization, hybridization with sample containing the target oligonucleotide, indicator binding, and electrochemical investigation of the surface.

The probe immobilization consisted of an electrochemical pretreatment of the graphite electrode surface (+1.8 V for 120 s using 0.25 mol/L acetate buffer, pH 5.0). The pretreated electrodes were then immersed in a stirred 2× SSC buffer solution containing 4 mg/L synthetic 23mer oligonucleotide (probe) for 120 s at a potential of +0.5 V vs SCE. This is the so-called DNA biosensor.

The electrode was then washed with buffer solution for 30 s. Occasionally, the oligonucleotide immobilization process was estimated by measuring the peak area of guanine residues at approximately +1.0 V in 0.25 mol/L acetate buffer, pH 5.0, by chronopotentiometric stripping analysis.

Daunomycin solution (10 μmol/L) was the hybridization marker and was prepared daily in 2× SSC.

The electrodes modified with oligonucleotide sequences were immersed for 6 min in the sample solution (containing the target) buffered by 2× SSC, where hybridization takes place. The probe was then rinsed in the 2× SSC buffer solution, immersed into the stirred daunomycin solution for 2 min at room temperature in the dark, and then washed with the buffer solution. The electrochemical investigation of the surface consisted of performing chronopotentiometric analysis in 2× SSC, using an initial potential of 0.2 V and a constant current of +1 μA. Under these conditions, a peak related to the oxidation of daunomycin was obtained at approximately +0.4 V. Only one measurement was allowed. If a second chronopotentiometric analysis was performed, no peak was observed. We concluded that in these conditions, the daunomycin adsorbed is completely oxidized in one step. Cyclic voltammetry scans showed oxidation and reduction peaks of daunomycin, but in chronopotentiometry, the reduction do not occur. It is possible that the oxidized form of daunomycin is not available for electrochemical reduction after the chronopotentiometric scan. It is not clear whether the oxidized form is more soluble in the buffer and diffuses from the electrode or whether the oxidation to 1.05 V irreversibly oxidizes the daunomycin.

The reported results corresponded to the increase in the peak area of daunomycin obtained related to the peak area value when the target concentration is zero.

PCR AMPLIFICATION OF DNA FROM BLOOD SAMPLES
DNA was extracted from peripheral blood by salting out (19, 20). PCR amplification was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus) using oligonucleotide primers F4 (5’-ACAGAATTGCCCCGCCGCTTGTA-CAC-3’) and F6 (5’-TAAGCTTGCCAGGGCTGAAGGAGA-3’), as described in Hixon and Vernier (13). Each amplification reaction was carried out in 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2 mmol/L MgCl2, 200 mmol/L of each deoxynucleoside triphosphate, 0.4 mmol/L of each primer, 100 mL/L dimethyl sulfoxide, 0.025 kU/L Taq polymerase, and 1 μg of genomic DNA in a final volume of 100 μL.

After a first step of denaturation at 95 °C for 5 min, each of the 30 cycles of amplification consisted of 1 min of primer annealing at 60 °C, 2 min of extension at 70 °C, and 1 min of denaturation at 95 °C.

The amplification products were fragments of 244 bp containing the two polymorphic codons, 112 and 158.

HYBRIDIZATION DETECTION IN PCR-AMPLIFIED CLINICAL SAMPLES
For investigation of the hybridization reaction with real samples, 20 μL of the DNA fragments obtained from the PCR amplification were diluted with 0.8 mL of hybridization buffer. Before dilution, the sample was denatured by heating at 95 °C for 5 min and then cooling in an ice bath for 2 min. A 50-μL aliquot of the sample was added directly onto the DNA biosensor. The reaction was allowed to proceed for 8 min, and then the biosensor was washed with hybridization buffer. The biosensor was immersed in a daunomycin solution for 2 min and then washed with buffer. An anodic daunomycin peak area increase was observed by PSA. The reported results corresponded to the difference between the peak area of daunomycin for the oligonucleotide hybridized minus the area obtained for the single-stranded oligonucleotide.

The samples used for the measurements had been characterized previously by restriction isotyping with HhaI and polyacrylamide gel electrophoresis, as described by Hixon and Vernier (13).

Results and Discussion
PROBE IMMOBILIZATION ON CARBON
The daunomycin peak area obtained as a function of the probe 1 concentration is shown in Fig. 1. The area increases linearly with concentration up to 2 mg/L, and then plateaus. A oligonucleotide concentration of 4 mg/L was generally used.
DNA BIOSENSOR FOR HYBRIDIZATION DETECTION OF SYNTHETIC OLIGONUCLEOTIDES

The increasing area of the daunomycin peak as a function of the complementary oligonucleotide concentration with probes 1 and 2, respectively, is shown in Fig. 2 (line a) and Fig. 3 (line c). When hybridization occurs, the area is higher and one can assume that more daunomycin is trapped on the electrode surface (intercalation in the double strand). However, the area of the daunomycin peak did not increase if the target sequence contained only one base mismatch [Fig. 2 (line b) and Fig. 3 (line d)]. Each measurement was repeated four times.

The results illustrated in Figs. 2 and 3 show that 1.0 mg/L of target is necessary to confirm the hybridization event.

With our conditions, the background area was ~2000 ms (Fig. 1), which is a relatively high value in comparison with the variation of area attributable to the presence of an excess of complementary strand. The high background is caused by the adsorption of daunomycin on the porous carbon surface and to nonspecific adsorption on the electrode surface after hybridization reactions with oligo 3, the complementary strand (line a), and oligo 4, which contains one mismatch (line b).

The immobilization time for probe 1 was 120 s at +0.5 V vs SCE in 2× SSC buffer (pH 7.4). Hybridization reactions took place for 6 min in solutions containing different concentrations of oligo 3 or oligo 4 in 2× SSC buffer (pH 7.4). PSA transduction in 2× SSC buffer (pH 7.4) with a constant current of 1 μA and an initial potential of 0.2 V. Δarea, difference in the daunomycin area between samples and the 2× SSC buffer; ms, milliseconds. Bars, SD.

The immobilization time for probe 2 was 120 s at +0.5 V vs SCE in 2× SSC buffer (pH 7.4). Hybridization reactions took place for 6 min in solutions containing different concentrations of oligo 3b or oligo 2 in 2× SSC buffer (pH 7.4). PSA transduction in 2× SSC buffer (pH 7.4) with a constant current of 1 μA and an initial potential of 0.2 V. Δarea, difference in the daunomycin area between samples and the 2× SSC buffer; ms, milliseconds. Bars, SD.

The immobilization time for probe 1 was 120 s at +0.5 V vs SCE in 2× SSC buffer (pH 7.4). Hybridization reactions took place for different lengths of time in solutions containing oligo 3 (2 mg/L) in 2× SSC buffer. PSA transduction in 2× SSC buffer (pH 7.4) with a constant current of 1 μA and an initial potential of 0.2 V. Δarea, difference in the daunomycin area between samples and the 2× SSC buffer; ms, milliseconds. Bars, SD.
samples and the 2× SSC buffer (pH 7.4). Hybridization reactions took place for 8 min with solutions of real samples diluted 40-fold with 2× SSC buffer (pH 7.4). PSA transduction in 2× SSC buffer (pH 7.4) with a constant current of 1 μA and an initial potential of 0.2 V. dt/dE, inverse of the derivative of the signal (E) with time (t).

The immobilization time for probe 1 was 120 s at 0.5 V vs SCE in 2× SSC buffer. This nonspecific adsorption limits the sensitivity of the biosensor, but to date we have not succeeded in reducing this effect. However, the biosensor is able to distinguish complementary and mismatch strands when the concentration is >1 mg/L.

All of the target oligonucleotides used in the experiments had a sequence of 23 bases; the mismatch had a base substitution in the middle of the sequence. The sequences of oligonucleotides oligo 3 and oligo 4 were identical to the one around codon 112 of the APOE gene; oligonucleotides oligo 3b and oligo 2 are the same as the sequence around codon 158 of the APOE gene. These are the positions where the APOE polymorphisms occur.

The influence of hybridization time is shown in Fig. 4. The response for a 2 mg/L target oligonucleotide increases with hybridization time up to 6 min; therefore, 6 min was generally used for all experiments with oligonucleotides.

These results confirm similar experiments reported previously (8) and show the possibility of detecting specific hybridizations in a short time.

### Table 1. Real samples tested with probe 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Genotype</th>
<th>Daunomycin ∆area, a ms</th>
<th>SD, b ms</th>
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<td>180</td>
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a Difference in the daunomycin area between the samples and the 2× SSC buffer.

b SD for the daunomycin area of the sample (background value ~2000 ms).

### Table 2. Real samples tested with probe 2.

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<th>SD, b ms</th>
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a Difference in the daunomycin area between the samples and the 2× SSC buffer.

b SD for the daunomycin area of the sample (background value ~2000 ms).
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References


2. Singhal P, Kuhr WG. Ultrasensitive voltammetric detection of