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DNA electrochemical biosensors

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Abstract Disposable electrochemical DNA-based biosensors are reviewed; they have been used for the determination of low-molecular weight compounds with affinity for nucleic acids and for the detection of the hybridisation reaction. The first application is related to the molecular interaction between surface-linked DNA and the target pollutants or drugs, in order to develop a simple device for rapid screening of toxic or similar compounds. The determination of such compounds was measured by their effect on the oxidation signal of the guanine peak of calf thymus DNA immobilised on the electrode surface and investigated by chronopotentiometric analysis. The DNA biosensor is able to detect known intercalating compounds, such as daunomycin, polychlorinated biphenyls (PCBs), aflatoxin B1, and aromatic amines. Applicability to river and waste water samples is also demonstrated. Disposable electrochemical sensors for the detection of a specific sequence of DNA were realised by immobilising synthetic single-stranded oligonucleotides onto a graphite screen-printed electrode. The probes became hybridised with different concentrations of complementary sequences present in the sample. The hybrids formed on the electrode surface were evaluated by chronopotentiometric analysis using daunomycin as indicator of the hybridisation reaction. The hybridisation was also performed using real samples. Application to apolipoprotein E (ApoE) is described, in this case samples have to be amplified by PCR and then analysed by DNA biosensor. The extension of such procedures to samples of environmental interest or to contamination of food is discussed.

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Introduction

In recent years there has been an increase in the use of nucleic acids as a tool in the recognition and monitoring of many compounds of analytical interest [1, 2]. Nucleic acid layers combined with electrochemical transducers produce a new kind of affinity biosensor. An interesting application of a DNA biosensor will be the testing of water, food, soil, and plant samples for the presence of analytes (carcinogens, drugs, mutagenic pollutants, etc.) with binding affinities for the structure of DNA. Binding of small molecules to DNA and, generally, DNA damage by ionising radiation, dimethyl sulfate, etc., has been described [3–8]. The DNA-trapped compounds can either be detected directly if they are electroactive molecules or via changes in electrochemical DNA signal [4–6, 10–13].

Moreover, DNA-based affinity biosensors can be used for the detection of selected oligonucleotide sequences. DNA biosensors can detect the presence of genes or mutant genes associated with inherited human diseases [9–12]. They can be employed to obtain early and precise diagnoses of infectious agents in various environments [7], or can be exploited for monitoring sequences for specific hybridisation events directly or by DNA intercalators [13], which form complexes with the nitrogenous bases of DNA.

This article reviews recent activity in the author's laboratory in developing DNA biosensors based on the coupling of thick-film disposable electrochemical transducers with DNA recognition layers. Single-use sensors have several advantages, such as avoidance of contamination among samples, constant sensitivity and reproducibility and ease of use because no pretreatment is needed [14, 15]. We developed a disposable biosensor based on the immobilisation of double-stranded (ds) or single-stranded (ss) DNA on the surface of carbon-based screen-printed electrodes (SPE) and the use of a fast chronopotentiometric method in order to measure guanine, analyte oxidation (when it is electroactive), and intercalating agent oxidation peaks accumulated on the electrode surface.

We report here some examples of the two main possible applications of DNA biosensors: immobilisation on the electrode surface of calf thymus DNA (ds or ss) in order to detect in water binding compounds with affinity on DNA structure investigating the electrode response to guanine base oxidable on carbon surface at about +1 V vs. Ag/Ag/Cl reference. The monitoring of such compounds in water or in waste water can alert on the possible presence of genotoxic compounds and can be useful as a screening device or continuous monitoring for such compounds.

Moreover, the other main application of DNA biosensors is the possibility to detect the hybridisation reaction between one synthetic single strand oligonucleotide (up to 23 bases) immobilised on the electrode surface and the complementary strand in solution. If the hybridisation reaction occurs, a double strand is formed on the electrode surface and can be detected through an intercalating agent electrochemically active at +0.4 V vs. Ag/Ag/Cl like daunomycin. Such compound is preferably trapped on the electrode surface when the double strand is present, therefore it behaves as an hybridisation monitor agent.

An "interrogation" of the electrode surface reveals if daunomycin has been trapped and then if the hybridisation has occurred. This event has been coupled with the amplification reaction (Polymerase Chain Reaction, PCR) necessary to amplify a specific piece of DNA in buffer solution.

The coupling of DNA biosensors with PCR amplification reaction is absolutely new and brings several advantages in the genetic identification of a selected species. Detection of specific base sequences in human, viral and bacterial nucleic acids is becoming increasingly important in several areas ranging from the detection of disease-causing and food contaminating organisms to forensic and environmental research.

We show here the first results obtained by electrochemical detection of the oligonucleotide target in solution after having immobilised an oligonucleotide complementary as a probe on the electrode surface. The novelty of the presentation is due to the fact that real samples are considered and that the target is amplified by the PCR reaction.

Experimental

Apparatus and chemicals. Electrochemical measurements were performed with an Autolab PGSTAT 10 electrochemical analysis system, with GPES4 software (Eco Chemie B.V., Utrecht, The Netherlands), in connection with a VA-Stand 663 (Metrohm, Milan, Italy). Screen-printed electrodes were printed with a Model 245 screen-printer, obtained from DEK (Weymouth, UK) using different inks obtained from Acheson Italiana (Milan, Italy). A graphite-based ink (Electrodag 421), a silver ink (Electrodag 477 RFU) and an insulating ink (Electrodag 6018 ss) were used. The substrate was a flexible polyester film obtained from Autotype Italia (Milan, Italy). A screen-printed three electrodes strip is realised with a graphite working electrode (surface area 3 mm^2), a graphite counter electrode and a silver reference electrode [14–15]. Calf Thymus DNA, daunomycin hydrochloride, NaCl, $C_3H_5Na_3O_7$ were from Sigma, Milan, Italy. Sodium acetate, acetic acid, KCl, methanol were from Merk Darmstadt, Germany. Oligonucleotide base sequences were obtained from Pharmacia Biotech (Milan, Italy).

Electrochemical measurements. All electrochemical measurements were carried out at room temperature. Chronopotentiometric analysis at a constant current (PSA) was performed with the following parameters; the potentials were sampled at a frequency of 33 KHz. The original signal (t vs. V) was elaborated and the derivative signal (dt/dE) versus the potential (E) was taken as the analytical signal. Using the GPES4 software this signal was smoothed and a baseline correction was generally performed.

DNA biosensor for binding compounds with affinity for DNA. The procedure is based on three steps: 1) calf thymus DNA immobilisation on the electrode surface, 2) dipping the electrode in the sample/blank solution, 3) electrochemical interrogation of the surface.

First the working electrode is cleaned by an electrochemical oxidation (+1.6 V for 2 min and +1.8 V vs. reference electrode for 1 min in 0.2 M acetate buffer pH 4.7), then the electrode is immersed in a stirred buffer solution containing 20 mg L^{-1} of single stranded or double stranded calf thymus DNA. This immobilisation step lasted for 2 min holding the electrode surface at a potential of +0.5 V vs. reference electrode. The electrode was washed with buffer solution. Then it was placed in the sample for 2 min for the interaction step. The electrode was washed with buffer solution and then a chronopotentiogram was carried out in 0.2 M acetate buffer pH 4.7, 0.01 M KCl, by using an initial potential of +0.2 V to +1.3 V and a constant current of +2 µA; by plotting the derivative signal dt/dE vs. V a peak, due to the oxidation of guanine [10–13], is obtained at around +1.0 V. The peak area is evaluated by the GPES4 software and the blank is subtracted.

In some cases the sample solvent was up to 10% of methanol to allow analyte dissolution; this concentration has no effect on the measurement value.

Analysis of river water samples and waste water samples. A preconcentration step for river water was found necessary to obtain clear signals with this technique. The water samples were generally concentrated according to [10]. The water samples were also analysed using a standard chromatographic procedure with an ion trap detector mass spectrometer (GC-ITDMS). Moreover, different industrial and civil waste water samples were analysed. The samples were collected during the BIOSET Technical Meeting [16, 17] and from the waste water treatment plant of the town of Florence. In this case the samples were only filtered through a 0.45 µm filter and buffered to obtain a final concentration of 0.2 M acetate buffer pH 4.7. The results obtained were compared with the Toxalert 100 (Merck, Darmstadt, Germany), a commercial device for toxicity testing based on luminescent bacteria; the procedure of sample analysis with Toxalert has been reported elsewhere [16–18]. Samples collected during the Bioset technical meeting in Berlin were compared also with the umu-assay test and the protein RTG2 test. The umu-assay [16] is a genotoxicity method based on the genetically engineered bacterium *Salmonella typhimurium* TA1535pSK1002 carried in the plasmid pSK1002 with the umuC-lacZ fusion gene. The values of the dilution steps by which an induction ratio lower than 1.5 was observed counts for genotoxicity. The factor pRTG-2 [16] is a test based on the protein RTG2 that is involved in inter-organelle communication between mitochondria, perixosomes and nucleus. RTG2p is required for expressions of several genes in respiratory deficient cells.

DNA biosensor for hybridisation measurements. The procedure can be divided into three steps: 1) probe immobilisation, 2) interaction with the sample containing the target oligonucleotide for the hybridisation reaction, 3) indicator binding and electrochemical investigation of the surface. The probe immobilisation starts with an electrochemical pretreatment of the graphite electrode surface (see before); then it is immersed in a stirred citrate buffer solution (300 mM NaCl-30 mM $C_3H_5Na_3O_7$, pH 7.4, denoted as 2XSSC) containing 4 mg L^{-1} of synthetic oligonucleotide (probe) for 120 s at a potential of +0.5 V vs. reference electrode [12]. Daunomycin solution

(10 μM) is the hybridisation marker employed in this application. The electrode modified with oligonucleotide sequences (the DNA biosensor) is immersed for 6 min in the sample solution (containing the target) buffered by 2XSSC and in this period hybridisation on the surface of the electrode takes place. The probe, after rinsing, is immersed into the stirred daunomycin solution for 2 min at room temperature in the dark, and washed again. The electrochemical investigation of the surface was performed carrying out a chronopotentiogram in the range of +0.2 V–1.3 V with a constant current of +2 μA. A signal related to the oxidation of daunomycin is obtained at around +0.4 V. Only one measurement is allowed. If a second scan is performed no signal can be observed. We can conclude that under these conditions the daunomycin intercalated on the double strand is completely oxidised in one step.

Hybridisation detection on PCR amplified clinical samples. For the investigation of the hybridisation reaction with real samples, 20 μL of the DNA fragments obtained from the amplification by PCR were diluted with 0.8 mL of hybridisation buffer. The sample, before the dilution, was denatured by heating it at 95°C for 5 min and then cooling the sample in an ice bath for 2 min. Then 50 μL of the sample were directly added on the DNA biosensor. The reaction was allowed to proceed for 8 min and then the biosensor was washed and immersed into a daunomycin solution for 2 min and then washed again. An anodic daunomycin peak area increase was observed by chronopotentiometry. The reported results correspond to the difference between peak area of daunomycin for the hybridised oligonucleotide decreased by the blank value. The samples used for the ApoE polymorphism were previously characterised by PCR and polyacrylamide gel electrophoresis as described in [12].

Results

DNA sensor for binding compounds with affinity for DNA

Preliminary studies were performed to identify general assay conditions which affected the electrochemical signal of the guanine oxidation peak, like ionic strength, pH, buffer composition, DNA concentration and form (single stranded and double stranded) [11].

With the optimised conditions, we performed several preliminary experiments for evaluating the variation of the area of the guanine peak using single stranded or double stranded DNA immobilised and different compounds of environmental interest (Table 1) are analysed. The results are expressed as response % (R%).

Experiments carried out in buffer solution show that the ssDNA guanine peak area is higher than the dsDNA guanine peak area (more than 2 times); this can be explained considering that the guanine base in single-stranded DNA is reported to be more available for oxidation than in double-stranded DNA [11].

Some compounds such as aromatic amines decrease the peak area (even when low concentrations were present), some other increase it. This can be explained with lower or higher availability of guanine for oxidation at the electrode surface; with aromatic amines we can deduct a binding of the guanine with the structure of the DNA which prevents its oxidation, while with other chemicals we can suppose an interaction with the whole structure (especially with the dsDNA) giving an higher availability of the guanine for the oxidation. Similar results were also reported in [6].

From Table 1 it is noteworthy to point out that 2-anthramine and 2-naphthylamine gave similar decrease with ds and ssDNA while with the other two amines (1,2 diaminoantroquinase and acridine orange) the decrease of ssDNA is higher than with dsDNA. Therefore, the structure of the compounds should be also considered.

Moreover, compounds like daunomycin showed an increase in the guanine peak area when using dsDNA. In this case we observe a linear increase with the daunomycin concentration in the range 1–10 mg L^{-1}. We attribute such results to the intercalation of daunomycin between the base pairs in the double stranded DNA inducing an easy oxidation of guanine [11]. Any variation of the guanine oxidation peak was observed when ssDNA biosensor was used in that concentration range.

Aflatoxins are metabolites produced by some strains of the mould *Aspergillus flavus*. They are among the most potent environment mutagens and are implicated as liver carcinogens. The binding of the aflatoxin B1 to both native and denatured DNA has been demonstrated [19]. We obtained a gradual decrease of the guanine peak in the presence of increasing levels of the aflatoxin B1 with a ssDNA in the range 10–30 mg L^{-1}. Any effect in this concentration range was observed when dsDNA was used. However, we have to point out that for such compounds the ppm range is not relevant for environmental interest being often present at ppb or lower level.

Moreover, since some compounds, such as aromatic amines (that constitute a very important class of environmental pollutants), are electroactive we compared the oxidation signal of these compounds with the effect of them on the oxidation peak of the guanine. In Fig. 1 the effect of 2-anthramine on dsDNA coated electrodes is reported: Fig. 1 A chronopotentiograms for some concentration val-

Table 1 Compounds tested with single stranded or double stranded calf thymus DNA immobilised on screen printed electrodes. Calf thymus DNA immobilisation: 20 mg L^{-1} of single stranded or double stranded calf thymus DNA in 0.2 M acetate buffer solution pH 4.7 for 2 min at +0.5 V vs. reference electrode. Chronopotentiometry conditions: in 0.2 M acetate buffer solution pH 4.7, KCl 0.01 M, with a stripping current of +2 μA and an initial potential of +0.2 V. The area is reported with each measurement repeated 4 times

Compounds tested	Calf thymus dsDNA R%	Calf thymus ssDNA R%
1,2-Diaminoanthraquinone (0.05 mg L^{-1})	–50 ± 7	–10 ± 4
2-Anthramine (0.05 mg L^{-1})	–38 ± 6	–26 ± 9
2-Naphthylamine (1.5 mg L^{-1})	–48 ± 13	–31 ± 10
Acridine orange (0.05 mg L^{-1})	–40 ± 4	–19 ± 5
Daunomycin (5 mg L^{-1})	+44 ± 16	+3 ± 1
Phthalates mixture (20 mg L^{-1})	+8 ± 6	+10 ± 7
PCB 105 (0.4 mg L^{-1})	+50 ± 20	+10 ± 8
Aflatoxin B1 (10 mg L^{-1})	–3 ± 1	–15 ± 3
Cisplatin (30 mg L^{-1})	+50 ± 8	–10 ± 2
Hydrazine (20 mg L^{-1})	+2 ± 0.5	–20 ± 6

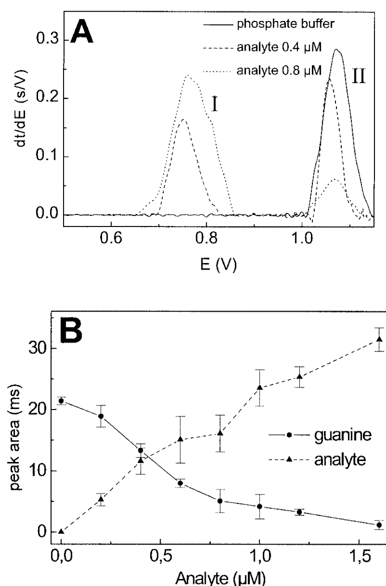

Fig. 1 Chronopotentiometric response of dsDNA biosensor for 2-anthramine; **A** chronopotentiograms for some concentrations, **B** calibration curves. dsDNA immobilisation: 120 s at +0.5 V in calf thymus dsDNA 20 μg mL^{-1} in acetate buffer 0.25 M (pH 4.7); accumulation: 120 s at open circuit in 0.1 M phosphate buffer solutions (pH 7.4); measure: chronopotentiometry at constant current (+2 μA) in acetate buffer 0.25 M (pH 4.7), KCl 0.01 M

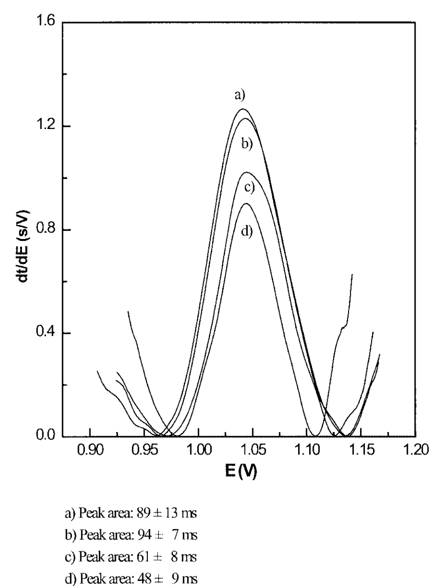

Fig. 2 Chronopotentiograms obtained using calf thymus ssDNA modified sreen-printed electrode with *a*) blank solution, *b*), *c*) and *d*) river water (100 times concentrated). Calf thymus ssDNA immobilisation: 20 mg L^{-1} calf thymus ssDNA for 2 min at +0.5 V vs. reference electrode. Chronopotentiometry conditions: in 0.2 acetate buffer pH 4.7, 0.01 M KCl with a stripping constant current of +2 μA and an initial potential of +0.2 V

ues and Fig. 1 B the two calibration curves. Increasing the concentration we obtain an increase in the amount of aromatic amines collected and oxidised to its cation radical (I) [6], and a decrease in the dsDNA guanine oxidation peak (II).

After such preliminary results we tried some application with real samples of river water. The results reported in Table 1 suggested to concentrate such samples in order to obtain some clear responses. One of the first results is depicted in Fig. 2 where some samples (b, c, d) are compared with a ssDNA sensor giving different and clear results. The area of sample b is similar to the buffer while c and d samples gave measurable smaller areas. Analysis by HPLC of some pesticides in the same samples gave the results reported in Table 2, where surprisingly we can notice that samples c and d are heavily polluted in comparison with sample b.

Some of these compounds, revealed by HPLC analysis, did not give any effect on the DNA sensor at such low level of concentration and we concluded that the DNA sensor revealed a cumulative effect.

Therefore, the sensor is not able to distinguish specific compounds of environmental concern but it could be conveniently used as a screening tool of toxicity (or genotoxicity) due to the short time involved in the measuring step.

Samples of water collected during the '2nd European Technical Meeting – Biosensors for Environmental Monitoring' (Berlin 1998) and waste water samples collected during the Bioset Technical Meeting for the Evaluation of the Performance of Waste Water Treatment Works (Barcelona 2000) were tested using the DNA biosensor.

Table 2 Results of the water samples obtained using a standard method with a Varian 3400 gas chromatograph coupled to a Finningan Mat 800 ion trap detector mass spectrometer (GC-IT-DMS)

Samples	b (ng L^{-1})	c (ng L^{-1})	d (ng L^{-1})
Desethyl-terbuthylazine	0	12	21
Carbofuran	0	210	101
Simazine	0	0	23
Terbuthylazine	79	28	81
Ethofumesate	6	184	83
Alachlor	0	0	27
Metolachlor	7	14	225

These technical meetings (supported from the EC) provide valuable opportunities to evaluate biosensors performance in field conditions with a portable apparatus; we participated by comparing the DNA biosensor outputs with other toxicity tests on several water samples. The results expressed as response % (R%) are reported in Table 3 and 4A [13]. Table 3 reports the correlation between different tests: the samples from the C series (industrial waste waters) gave low R% with the DNA-biosensor and also shows elevate inhibition for Toxalert® and high dilution values for the *umuC* assay. The samples collected in Barcellona Technical Meeting (Table 4A) show a good correlation between other toxicity tests such as the Toxalert® and the DNA biosensor. Toxalert® is a commercial device for toxicity based on luminescent bacteria. The system is quite compact and practical and most suitable for in field analysis with clear samples. Results obtained with such

Table 3 Comparison of dsDNA and ssDNA biosensor results and other genotoxicity tests for samples of water collected for the '2nd European Technical Meeting – Biosensors for Environmental Monitoring' (Berlin 1998)

Sample[a]	Genotoxicity umu-assay without S9	Genotoxicity Factor p RTG-2	ToxAlert®		dsDNA R%	ssDNA R%
			Dilution	inhib. %		
C0199A	1[c]	1.27[c]	1:4	3	70	70
C0566A	–	1.16[b]	1:80	41	63	67
C0992A	1[c]	1.4[d]	1:4	9	50	60
C1227A	1[c]	1.31[d]	1:4	39	12	63
C1702A	12[d]	1.32[d]	1:80	25	24	70
C1747A	384[d]	1.87[d]	1:80	56	10	5
C1794A	1[c]	1.14[b]	1:4	8	38	23
C2010A	192[d]	2.27[d]	1:80	40	40	40
C2596A	48[d]	1.5[d]	1:80	26	50	49
C2894A	12[d]	1.38[d]	1:80	51	53	33
K1	–	–	1:2	22	100	79
K3	–	–	1:2	5	100	87
R1	–	–	n.d.	n.d.	69	74
R3	–	–	n.d.	n.d.	77	83
R4	–	–	n.d.	n.d.	82	82
R7	–	–	n.d.	n.d.	100	76
S1	–	–	–	6*	87	76

[a] The water samples analysed were collected from the lake Stoßensee in front of the Fischereiamt (S1), at the sewage plant Ruhleben (R1,3,4,7), from the wastewater of a cement industry (K1,3), from industrial effluents from Bitterfeld (C samples); [b] low toxic effect, [c] moderate toxic effect, [d] high toxic effect

Table 4 Waste water samples analysis with the DNA biosensor: A) Sample collected during the Bioset Technical Meeting in Barcelona (march 2000); B) some samples collected in the waste water treatment plants of the town of Florence. All the experiments were realised with dsDNA

4A)

	ToxAlert % inhibition	DNA biosensor Guanine peak % decrease (PSA)		ToxAlert % inhibition	DNA biosensor Guanine peak % decrease (PSA)
Lagosta influent	46 ± 1	–60 ± 7	Igualada influet	62 ± 2	–73 ± 4
Lagosta treatment	63 ± 1	–76 ± 5	Igualada treatment	71 ± 1	–96 ± 2
Lagosta effluent	31 ± 1	–36 ± 7	Igualada effluent	32 ± 2	–39 ± 8

4B)

Sample	Raw waste water		Treated waste water	
	ToxAlert Inhibition %	DNA biosensor Guanine peak % decrease	ToxAlert Inhibition %	DNA biosensor Guanine peak % decrease
1	35 ± 1	30 ± 2	63 ± 1	62 ± 11
2	85 ± 2	76 ± 11	34 ± 1	32 ± 3
3	82 ± 1	94 ± 3	56 ± 2	41 ± 6
4	89 ± 1	91 ± 9	53 ± 2	47 ± 6
5	89 ± 1	78 ± 3	45 ± 1	31 ± 19
6	87 ± 1	100	57 ± 1	19 ± 2
7	74 ± 1	65 ± 2	70 ± 1	42 ± 9
8	62 ± 1	56 ± 12	58 ± 1	47 ± 9
9	92 ± 1	100	54 ± 2	10 ± 10
10	78 ± 2	71 ± 6	83 ± 3	32 ± 13
11	76 ± 1	86 ± 5	69 ± 1	24 ± 5
12	90 ± 1	100	60 ± 1	8 ± 17
13	66 ± 1	97 ± 3	52 ± 1	64 ± 17
14	68 ± 1	41 ± 2	64 ± 1	22 ± 7

instrument are ready in 30 min. The performances of it are the nearest to those obtained with DNA biosensor (results obtained in 2 min whatever the turbidity of the sample). Therefore, recently we analysed a certain number of waste water samples with the two procedures and all results obtained so far are reported in Table 4B. The samples were analysed before and after the treatment and the methods show the same trend; generally the raw waste water samples are more toxic than the treated ones; nevertheless, for some treated water samples the toxicity value obtained

with Toxalert® is higher than that obtained with the biosensor; this is probably due to the use of luminescent bacteria which involve metabolic reactions, not present in the DNA biosensor measurement.

As a general remark, on the basis of our experience we conclude that both ss and dsDNA biosensors are convenient for environmental screening; dsDNA can also monitor the presence of molecules intercalating in the double helix, with ssDNA we can monitor the presence of molecules binding the guanine base.

The application of this DNA biosensor to the evaluation of the toxicity of waste water is interesting because it is a very fast measurement and it is in agreement with the request of international legislation.

DNA biosensor as detector of the hybridisation reaction

In such experiments an oligonucleotide is immobilised on the surface of the carbon disposable electrode and we called it "probe" and the complementary is in solution and is called "target". If the target is present in a suitable concentration hybridisation will occur and the daunomycin will be trapped in the double strand, giving an oxidation signal. Generally, a sequence of 20 nucleotides is considered enough to characterise a specific gene portion and then monitor unequivocally the presence of a certain organism.

A 21-mer oligonucleotide specific sequence for *Chlamydia trachomatis* was chosen as first model for our experiments; we immobilised it on the electrode surface and obtained the daunomycin signal (chronopotentiometry at constant current) for the different concentrations of the target sequence according to the procedure described. Figure 3 A shows the increasing area of the daunomycin peak as a function of the complementary oligonucleotide concentration. The electrical signal of daunomycin is much more evident when hybridisation occurs; note that the peak area did not increase when a 21-mer sequence non-complementary to the immobilised oligonucleotide was used in control experiments. The difference of the area obtained in the chronopotentiograms (increase compared to the single stranded immobilised oligonucleotide) is reported (Fig. 3 B) with each measurements repeated 4 times. Hybridisation lasted for 6 min and the signal was observed within the 0.2–3 mg L^{-1} of target range. With this approach 0.2 mg L^{-1} of target is necessary to confirm the hybridisation event.

Such results confirm similar experiments reported earlier [11] and show the possibility to detect specific hybridisation in a short time. However, the sensitivity of this assay is still insufficient to allow a direct assay without amplification step.

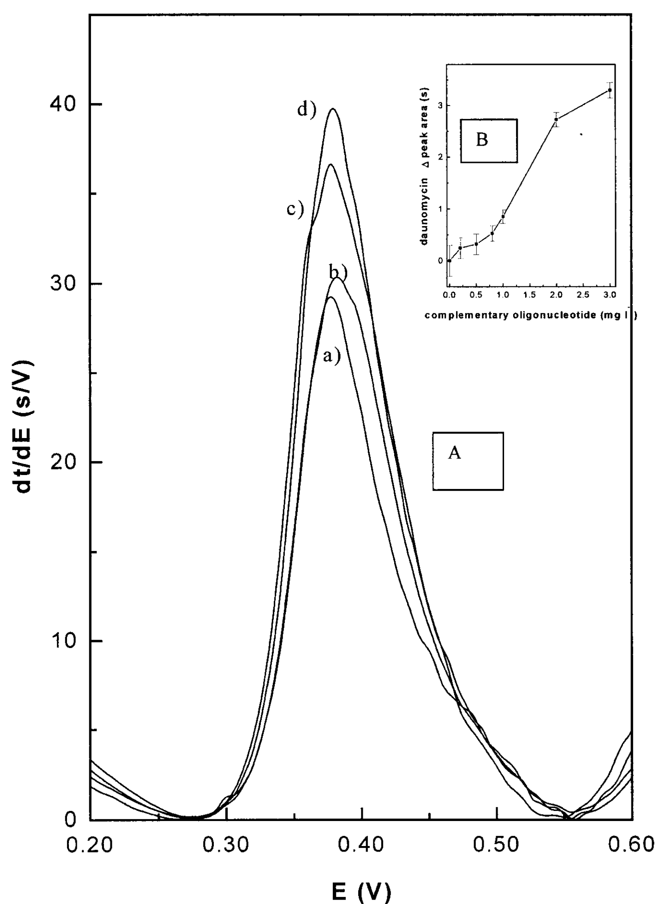

Fig. 3 (**A**) Chronopotentiograms for daunomycin *a*) at 5 mg L^{-1} 21-mer of *Chlamydia trachomatis* oligonucleotide modified screen-printed electrodes and followed increasing target concentrations *b*) 1 mg L^{-1} 21-mer non-complementary sequence, *c*) 0.5 mg L^{-1}, *d*) 1 mg L^{-1}. (**B**) Calibrations curve obtained with different amounts of target sequence. The results correspond to the difference between the daunomycin peak area for the hybrids minus that obtained for single 21-mer of *Chlamydia trachomatis* oligonucleotide. Probe immobilisation: 5 mg L^{-1} for 2 min at +0.5 V vs. reference electrode. Hybridisation: complementary oligonucleotide for 2 min at +0.5 V. Chronopotentiometry conditions: in 2×SSC buffer pH 7.4 with a stripping constant current of +2 μA and an initial potential of 0.2 V

DNA biosensor for hybridisation detection with clinical samples amplified by PCR

Apolipoprotein E (ApoE) is an important constituent of several plasma lipoproteins, it is associated with the risk of developing cardiovascular diseases [20]. The protein (299 amino acids) presents a genetic polymorphism with three major isoforms (E2, E3 and E4), related to cysteine-arginine interchanges at position 112 and 158. These amino acid substitutions correspond to nucleotide substitutions in the DNA regions. The corresponding allele ε2 has a thymine in codons 112 and 158 of the sequence, the ε3 has a thymine in codon 112 and a cytosine in codon 158, and the ε4 has a cytosine in both codons [13, 21].

We developed a new procedure for detecting genetic polymorphisms of ApoE in human blood samples, using two different probes (probe 1 and 2) to investigate both the positions where polymorphism takes place [13]. Probe 1 is characteristic of the alleles ε3 and ε2 (around the codon 112) and the 100% complementary sequence is rep-

Table 5 Real samples tested with probe 1. The 3rd column reports the difference in the daunomycin area between the samples and the 2XSSC buffer; the 4th column reports the standard deviation for the daunomycin area of the sample (the background value is 2000 ± 200 ms)

Sample	Genotypes	Daunomycin Δarea (ms)	Standard deviation (ms)
1	PCR blank	–346	171
2	PCR blank	–152	83
3	PCR blank	–343	179
4	PCR blank	–279	180
5	ε3/ε3	+468	135
6	ε3/ε3	+648	207
7	ε3/ε3	+564	64
8	ε3/ε3	+601	127
9	ε3/ε3	+12	144
10	ε3/ε4	+329	166
11	ε3/ε4	+162	176
12	ε3/ε4	+168	185
13	ε3/ε4	+128	81
14	ε3/ε4	+158	128
15	ε3/ε4	+18	192
16	ε4/ε4	–128	230
17	ε4/ε4	–189	88
18	ε4/ε4	–229	130
19	ε4/ε4	+84	201
20	ε4/ε4	–276	149

Table 6 Real samples tested with probe 2. The 3rd column reports the difference in the daunomycin area between the samples and the 2XSSC buffer; the 4th column reports the standard deviation for the daunomycin area of the sample (the background value is 2000 ± 200 ms)

Sample	Genotypes	Daunomycin Δarea (ms)	Standard deviation (ms)
1	PCR blank	–247	84
2	PCR blank	–97	65
3	ε3/ε3	+542	146
4	ε3/ε3	+719	218
5	ε2/ε3	+396	189
6	ε2/ε3	+225	182
7	ε2/ε3	+119	79
8	ε2/ε2	–289	185
9	ε2/ε2	+63	148
10	ε2/ε2	–226	256

resented by genotypes ε3/ε3, ε2/ε2 and ε2/ε3. The genotype ε4/ε4 presented the mismatch sequence; genotypes ε3/ε4 and ε2/ε4 had 50% of the two sequences together. Probe 2 is characteristic of the alleles ε3 and ε4 (around the codon 158) and the 100% complementary sequence is now represented by genotypes ε3/ε3, ε4/ε4 and ε3/ε4. The genotype ε2/ε2 presented the mismatch sequence; genotypes ε2/ε3 and ε2/ε4 had 50% of the two sequences together.

The DNA sensor gave a very clear response with complementary oligonucleotides and a very poor response with mismatched oligonucleotide. With real samples from human blood after PCR amplification the DNA sensor was able to give the results reported in Tables 5 and 6. Table 5 reports results obtained with probe 1. This probe was characterised by three kinds of samples: ε3/ε3 completely complementary (very high value of daunomycin signal), ε4/ε4 have the mismatch sequence and have low values (even negative), ε3/ε4 50% complementary (intermediate value of daunomycin signal).

In Table 6 the results are reported for probe 2. In this case the genotype ε3/ε3 is completely complementary, ε2/ε3 has 50% of complementarity and ε2/ε2 has the mismatch sequence. The results are easily divided into 3 groups with probe 1 and 3 groups with the probe 2. Only few samples, N.9, N.15 and N.19 of Table 5 and N.9 of Table 6 are of difficult interpretation and cannot be easily assigned to any group. This could be a new procedure to genotype blood samples.

Conclusions

The potential of DNA biosensors for DNA hybridisation and for detection of toxic compounds has been demonstrated. This procedure offers a sensitive, rapid and portable tool for several applications in clinical chemistry, in environmental and in food analysis.

The DNA biosensor for hybridisation detection is useful to detect specific sequences of oligonucleotides with high selectivity; this procedure requires an amplification step such as PCR in order to be readily applicable to real samples. Problems in clinical chemistry, such as blood genotyping for specific sequencing of environmental concern, or in food technology can be solved using electrochemical biosensors. They may be used in place of electrophoresis, which in several cases gives uncertain results because the amplified sequence is identified only from the molecular weight and not from the sequence itself. Moreover, it is demonstrated that calf thymus ssDNA biosensor interacts with low-molecular mass compounds of environmental concern and can thus be used as a general indicator of the presence of toxic compounds; it is a simple tool that can give reliable results directly at the site of interest.

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