DNA-BASED BIOSENSOR FOR DETECTION OF GENETICALLY-MODIFIED ORGANISMS

A.G. Nica, M. Mascini* and A.A. Ciucu**

abstract: An electrochemical genosensor for the monitoring of hybridization was used in order to develop and characterize a DNA biosensor-based assay for the detection of genetically-modified organisms (GMOs). Screen-printed gold electrodes were modified with a suitable thiol-tethered DNA sequence (probe) related to the sequence of the 35S promoter (target) inserted in the genome of GMOs and responsible for regulating the transgene expression. An enzyme-amplified detection scheme was applied in order to quantify the electrochemical signal produced by reaction between probe sequence and target sequence (analyte). The assay was firstly characterized using synthetic oligonucleotides. Relevant parameters such as probe concentration, hybridization and enzymatic reaction time were investigated and optimized. Electrochemical techniques for DNA-modified electrodes control were used.

keywords*:* Biosensor; Screen-printed electrode, Genetically-modified organisms.

Introduction

In recent years there has been a considerable increase in the use of nucleic acids (DNA or RNA) as a tool in *recognition* and *monitoring* of many compounds of analytical interest (pollutants, toxic substances, antitumor drugs, pathogenic nucleic acid sequences etc.) due to the high *stability* and huge *variability* of nucleic acids sequences. Nucleic acids layers combined with electrochemical transducers produce a new kind of *affinity biosensors* for analytes of interest.

GMOs are referred to as living organisms whom genome has been modified by the introduction of an exogenous gene able to express an additional protein that confers new characteristics. The foreign DNA is usually inserted in a gene "cassette" consisting of an expression promoter, a structural gene (encoding region) and an expression terminator (Fig. 1).

Alternatively physical methods (e.g. particle gun) or chemical methods (e.g. polyethylene glycol or electroporation) may be used [1]. The promoter of the subunit 35S of ribosomal RNA of cauliflower mosaic virus (P35S) is widely used for the production of many transgenic vegetables, as soy Roundup Ready TM, maize Mais-Gard and the tomato Flavr Savr. Some genetically engineered plants are waiting for authorization, whereas others have already been approved by several countries: US, Canada, European Union, Switzerland, Australia, Argentina, Brazil and Japan. Concerning GM plants, new proteins usually confer

* Dipartimento di Chimica, Univerisita di Firenze, via della Lastruccia 3, Firenze, Italia
** Department of Analytical Chemistry, University of Bucharest, 90-92 road Panduri, Bucharest, ROMANIA

Analele Universităţii din Bucureşti – Chimie, Anul XIII (serie nouă), vol. I-II, pag. 85-94

herbicide tolerance [2], fertility/maturation modification or virus, fungi, parasite, drug or insect resistance [3]. Labelling of the genetically modified organism is not mandatory in US, but in Europe the novel food regulation will require the labelling.

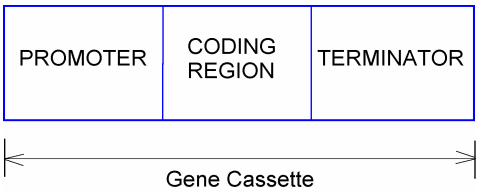

Fig. 1. *Schematic representation of a "gene cassette",*
consisting of a promoter, a structural gene (coding region) and a terminator

The ability of DNA and RNA complementary single strands to reform their double-helical structure was discovered more than 35 years ago [4]. Lately, new biosensor-based technologies have emerged toward the development of a fast low-cost practical diagnostic device. These technologies rely on the immobilization of a single-stranded DNA probe onto an electrochemical, optical, or piezoelectric transducer, which converts the recognition of the target sequence into an electrical signal (reviewed in [5]). In recent years, considerable efforts have been made to create an electrochemical sequence-specific DNA hybridization biosensor [5-l3]. Progresses in the development of electrochemical DNA-hybridization biosensors have been summarized in some excellent reviews [14-18].

Recent reports have concentrated only on synthetic oligonucleotides, showing that the hybridization can be monitored by variation of current or potential values [19-22]. Only a few authors [23-25] have reported the detection of hybridization event by using PCR-amplified DNA from real samples with electrochemical DNA biosensors to obtain reliable measurement of clinical interest.

Prof. Mascini's group developed piezoelectric [26] and optical [27] genosensors for GMOs detection, providing useful tools for screening analysis in food samples.

In this paper we describe a simple, sensitive and selective screening method, which can be used for the detection of GMOs. This method associates the hybridization of DNA with an electrochemical biosensor. The system relays on DNA sensing based on the hybridization of a nucleic acid probe immobilized on the screen-printed electrode transducer and the complementary oligonucleotides (target) in solution. The immobilized probe is specific for 35S promoter sequence, characteristic of GMOs. Electrochemical methods consist in cyclic voltammetry (CV) and constant curent chronopotentiometry (CCCP) were used in order to detect hybridization reaction between DNA-modified sensor surface and target sequence (analyte).

Experimental

Reagents and materials

Aldrich provided sulfuric acid 95-98% (Catalog no. 25,810-5) and potassium chloride (Catalog no. 420,800-0). Potassium ferrocyanide (Catalog no. 22,768-4) and potassium ferricyanide (Catalog no. 20,801-9) were obtained from Aldrich.

Sodium dihydrogen phosphate monohydrate, (p.a., Cat. No 106346), di-sodium hydrogen phosphate dihydrate (p.a., Cat. No 106580) from Merk (Darmstadt, Germany) were used for phosphate buffer (PBS) preparation.

Fluka provided 6-mercapto-1-hexanol (MCH) (Catalog no. 63762) and bovine serum albumine (BSA) (Catalog no. 05490) and Sigma provided 5-bromo-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Catalog no. B 1911) and streptavidin-alkaline phosphatase conjugate (Catalog no. S 5795).

Diethanolamine (DEA) was from Sigma (Catalog no. D 8885).

Synthetic oligonucleotide for DNA probe (having group C_6-SH at the 5' termination) was purchased from Sigma-Genosys (Cambridge, UK) and biotinylated synthetic oligonucleotides were purchased from Pharmacia Biotech (Uppsala, Sweden).

5'-mercaptohexyl-DNA probe sequences was 5'HS-$(CH)_2$-GCT CCT ACA AAT GCC ATC ATT GCG A-3', target DNA sequence (complementary strand) was 5'biotinyl-TCG CAA TGA TGG CAT TTG TAG GAG C-3' and non-complementary DNA strand was 5'-biotinyl-TGC CCA CAC CGA CGG CGC CCA CGG A-3'.

Apparatus

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

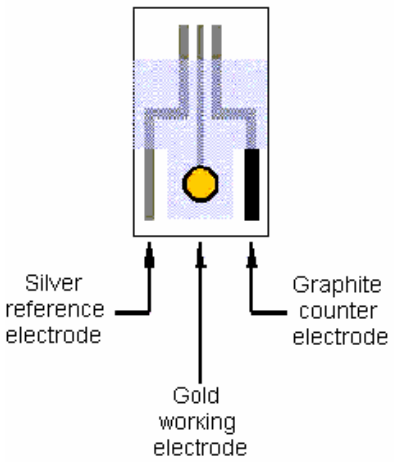

Fig. 2. *Screen-printed electrodes configuration*

Screen-printed electrodes were printed with a Model 245 screen printer, type DEK (Weimouth, UK) using inks obtained from Acheson Italiana (Milan, Italy). Graphite-based, silver, gold and insulating inks (Electrodag) were used. Working electrode was made from gold ink and geometrical area was 7 mm^2. Reference and auxiliary electrodes were made from silver ink and graphite ink, respectively (see Fig. 2).

All electrochemical measurements were carried out at room temperature in a 2 ml PTFE beaker.

Procedures

Preparation of DNA-modified gold electrodes

Preparation of DNA-modified electrodes was a multi-step procedure, as presented in Fig. 3. This procedure consists in surface pretreatment, immobilisation of DNA probe (25-mer), post-treatment with MCH, hybridisation with biotinylated target DNA sequence, coupling with the streptavidin-alkaline phosphatase conjugate and incubation with the enzymatic substrate (BCIP/NBT) of alkaline phosphatase.

a) Gold electrode surface pretreatment followed an electrochemical method (cyclic voltammetry). Electrochemical cell was a 2 ml PTFE beaker, with all electrodes (working, reference and auxiliary electrode) printed on the same substrate of polyester flexible film. CV parameters were: U_{min} = - 0.3 V, U_{max}= + 1.6 V, υ = 100 mV/s, n = 4, where is U_{max} first vertex potential, U_{min} is second vertex potential, where, υ is scan rate, n is number of scans (see Fig. 4). As electrolyte H_2SO_4 0.5 M (in KCl 0.1 M) was used. After pretreatment, electrodes were kept in PBS until next step.

b) Immobilization of DNA probe (25-mer) consists in placing 10 μL of 5'-mercaptohexyl-DNA solution (10 μg/mL in PBS) onto the gold working electrode surface overnight. Next day the electrode surface was carefully rinsed with purified water and electrodes were kept in PBS until used.

c) Post-treatment of gold electrodes with MCH: 10 μL of 1 mmol/L MCH aqueous solution were placed onto the probe-modified electrode for 30 min.

d) Hybridization of probe-modified surface with target sequence was accomplished by placing 10 μL of 5'-biotinylated target sequence (complemantary strand) in PBS for 20 min. After that, electrodes were washed with DEA buffer and kept wet until used.

e) Coupling with the stptavidin-alkaline phosphatase conjugate: 10 μL of the enzymatic conjugate (1 U/mL in DEA buffer, *p*H 9.6 + 8 mg/mL BSA) were reacted biotinylated hybrid for 20 min.

f) Incubation of enzyme-labeled DNA-modified electrodes with enzymatic substrate: 30 μL of BCIP/NBT mixture were incubated onto the biomodified sensor surface for 20 min. Incubation of enzymatic layer with substrate produces an insoluble product which precipitates in the mixed monolayer of MCH/enzyme-conjugate biotinylated hybrid. The precipitate acts as barrier which impedes the charge and mass transfer of redox probe in the electrochemical measurement (CV or CCCP).Electrodes were carefully washed with KCl 0.1 M and kept wet until measured.

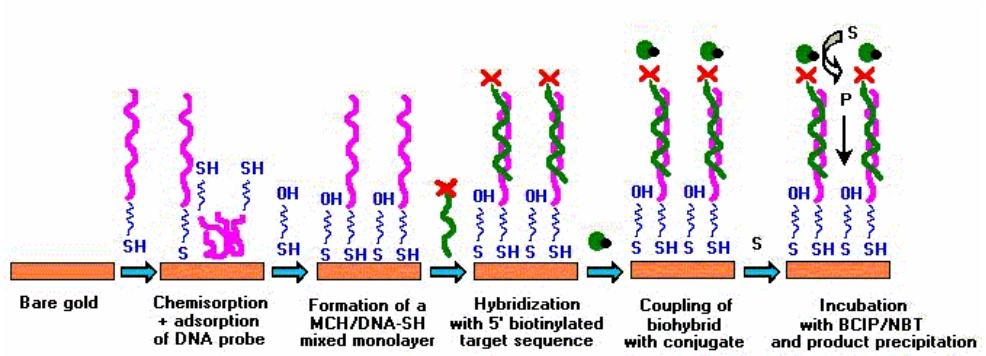

Fig. 3. *Preparation of electrodes using a multi-step procedure*

Electrochemical measurements

Electrochemical detection based on CV and CCCP techniques was used for assessment of hybridization of target DNA sequence with probe sequence immobilized onto sensor surface. A redox probe detection scheme based on $[Fe(CN)_6]^{3/4-}$ (1 mM in KCl 0.1 M) was applied in both CV and CCCP measurements.

CV parameters were: $U_{min} = -0.3$ V, $U_{max} = +0.6$ V, $\upsilon = 100$ mV/s, n = 1, where is U_{max} first vertex potential, U_{min} is second vertex potential, where, υ is scan rate, n is number of scans. For CV measurements, anodic peak current was considered when compared different electrodes.

CCCP parameters were I = 10 μA and t = 50 s where I is constant curent which was imposed to flow in the cell and t is the time of measurement. For CCCP measurements, the final equilibrium potential (taking into account the overpotential required for passing the 10 μA constant current) was considered in order to compare different electrodes.

For CV and CCCP were prepared sets of six different electrodes (1, 2, 3, 4, 5 and 6) following the preparation procedure. Thus, all six electrodes are prepared in the same mode until the formation of a mixed monolayer of MCH/DNA-SH (steps a, b and c in preparation procedure). After that, electrodes 1 and 2 were exposed to enzymatic conjugate solution and to the substrate solution (steps e and f in preparation procedure); electrodes 3 and 4 were exposed to non-specific biotinylated target sequence solution (1 μg/mL), to enzymatic conjugate solution and to the substrate solution (steps d, e and f in preparation procedure); electrodes 5 and 6 were exposed to specific biotinylated target sequence solution (10 μg/mL), to enzymatic conjugate solution and to the substrate solution (steps d, e and f in preparation procedure).

Results and discussion

Preparation of DNA-modified gold electrodes

Electrochemical cleaning of electrodes (pretreatment) of electrodes was used in order to remove the impurities from the electrodes surface. The increase in oxidation and reduction

peaks (not shown) in CV voltammograms underline cleaning process. The curents remained constant after four scans.

Immobilization of DNA probe was achieved due to chemisorption of SH group onto gold surface of electrode. Because chemisorption of 5'-mercaptohexyl-DNA is not the only phenomenon which takes place onto gold surface, we tried to remove the amount of wikly adsorbed DNA. For that reason we employed a post-treatment procedure in order to obtain a highly ordered MCH/DNA-SH monolayer. By comparing DNA-modified electrodes with and without post-treatment (results not shown) we decided to keep the post-treatment reaction of sensor surface with MCH for electrode preparation procedure.

The influence of DNA probe concentration on signal was investigated. We checked different DNA probe concentrations (1, 10, 20, 50, 100 µg/mL) and we decided that the most suitable probe concentration is 10 µg/mL in order to achieve high signals with lowest amount of DNA probe (see Fig. 4). dI represents difference in anodic peak currents of redox probe ($[Fe(CN)_6]^{3/4-}$) for a DNA probe-modified electrode and an electrode without immobilization step in the preparation procedure. The currents were recorded in CV experiments and ΔI is plotted versus probe concentration in Fig. 4.

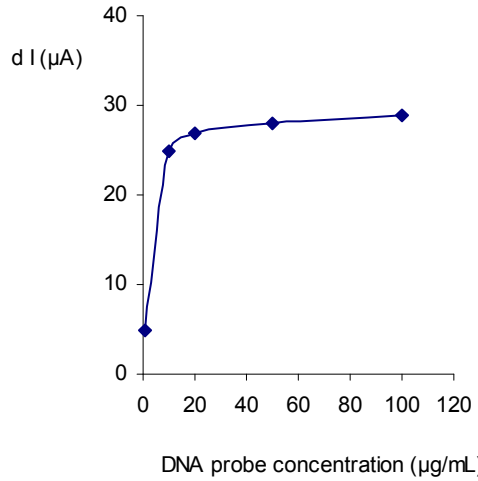

Fig. 4. *Influence of probe concentration on signal variation*

The detection scheme is based on hybrid formation between DNA probe and target sequence. In order to see if the sensor selectively recognizes the complementary DNA sequence we compared electrodes that were exposed to specific (complementary) biotinylated DNA and to non-specific (non-complementary) biotinylated sequence.

The influence of incubation time (with enzymatic substrate) on signal was investigated. We checked different incubation times (10, 20, 30 60, 120 min) and we decided that the best incubation time is 20 min for achieving high signals in shortest time (see Fig. 5). dI represents difference in anodic peak currents of redox probe ($[Fe(CN)_6]^{3/4-}$) for a modified electrode with a given incubation time and an electrode without incubation step in the preparation procedure. The currents were recorded in CV experiments and dI is plotted versus incubation time in Fig. 5.

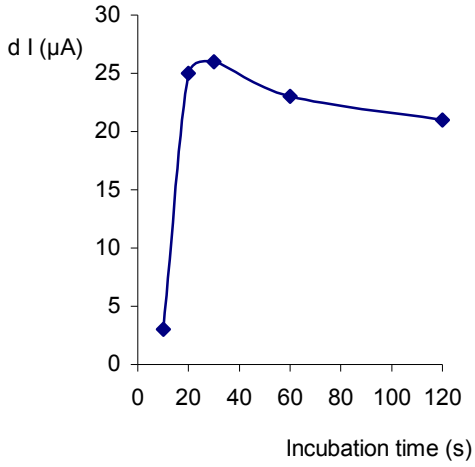

Fig. 5. *Influence of incubation time on signal variation*

Electrochemical detection

Sets of six electrodes (prepared as mentioned in "Procedures" section) were run in CV and CCCP experiments. For illustration, Figs. 6 and 7 are presented. Each curve corresponds to each electrode, thus curve 1 belongs to electrode 1, curve 2 belongs to electrode 2 and so on. Concentration of analyte (complementary biotinylated target) for electrodes 5 and 6 was 1 μg/mL and concentration of non-specific (non-complemantary) biotinylated target was ten times bigger in order to check the selectivity of the sensor. The responses in CV measurements for electrodes 5 and 6 were similar and represented a decrease in anodic (or cathodic) peak currents (a decrease of ~25 μA) comparing with electrodes 1, 2, 3 or 4 in Fig. 6.

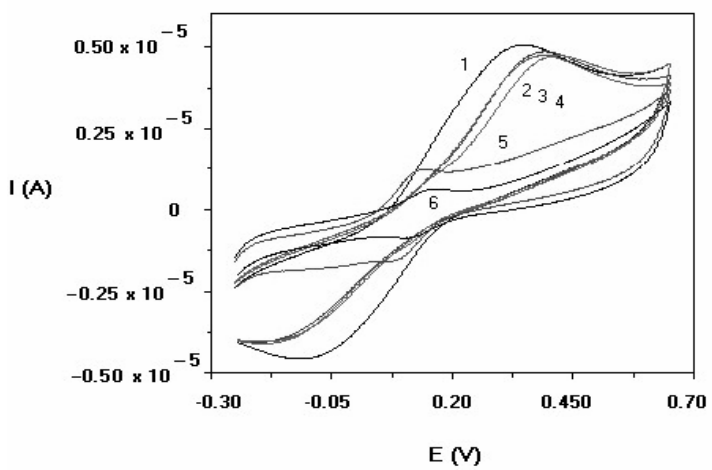

Fig. 6. *Cyclic voltammograms for six different electrodes*

A possible explanation for the decrease of the current for electrodes which performed a hybridization step could be insoluble product formation (which precipitates in the sensor biolayer) due to enzymatic reaction driven by alkaline phosphatase. This insoluble product could hinder the mass and charge transport which are responsible for reduction currents. The other effect of the precipitate is that of the shifting of the peak potentials for oxidation and reduction curves. The degree of the shift is in around 300 mV.

We expected that electrodes 1, 2, 3 and 4 to behave similarly because no precipitate could be formed in the layer and the corresponding currents to be higher than those of electrodes 5 and 6.

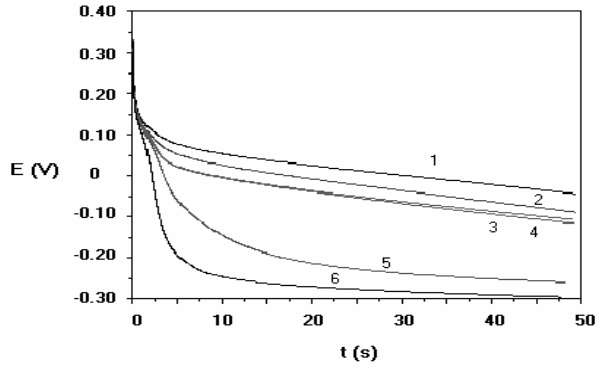

Fig. 7. *Chronopotentiograms for six different electrodes*

In good agreement with CV measurements, one can see that hybridization phenomenon could be revealed by chronopotentiograms in Fig. 7. Electrodes 5 and 6 which were exposed to hybridization with complementary DNA target sequence presented a much different final equilibrium potential at the end of the CCCP measurements. A reasonable explanation for this behaviour is that the overpotential required by electrodes exposed.

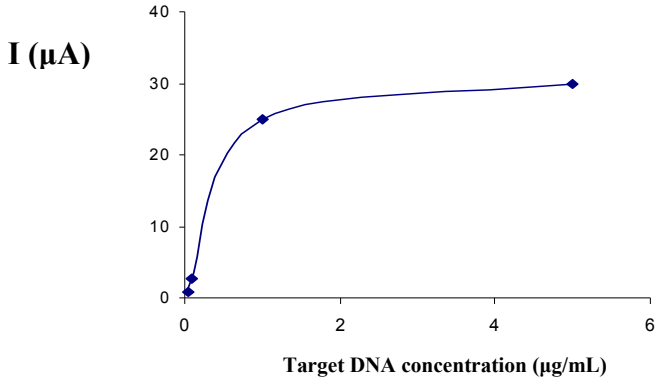

Fig. 8. *Calibration plot for detection of complementary DNA sequence*

CV measurements for different electrodes prepared in the same manner as electrodes 5 and 6 (step d, we used different concentrations of specific biotinylated target) allowed us to

obtain a calibration curve. As depicted in Fig. 8, limit of detection for the method was 0.1 μg/mL and the linear range of the response is 0.1-1 μg/mL.

Conclusion

With the progress of molecular biology, demand for gene analysis is increasing more than ever. Electrochemical gene analysis is one of the promising methods as far as the analytical speed and sensitivity is concerned. We described a novel electrochemical assay for nucleic acid sequence detection based on the DNA hybridization between an immobilized probe sequence and target sequence in sample solution.

A disposable, electrochemical DNA biosensor has been developed using screen-printed electrodes. The biosensor has been characterized using 25-mer oligonucleotides as model for the 35S promoter sequence and it was able to distinguish between full-matched (target), and non-complementary DNA sequences, with a detection limit of 0.1 μg/mL of target sequence. A 10 min hybridization time allowed a full characterization of each sample.

At the present stage of the technology, an amplification step is necessary to analyze real samples.

Further work includes the analysis of fragments of the 35S promoter sequence in DNA sample isolated from the biological sources amplified by polymerase chain reaction (PCR) and application of more sensitive electrochemical pulse techniques such as: Differential Pulse Voltammetry (DPV) and Square Wave Voltametry (SWV).

Acknowledgements

The authors are grateful to Prof. M. Mascini, University of Florence, for kindly providing the DNA sequences. Anton Ciucu is grateful to NATO fellowship awarded by the National Council of Research-Italy. The authors would like to thank also the National Institute of Biostructure and Biosystems, in particular their Research Grant in the frame of Project intitled "L'Impiego dei biosensori nella valutazione dell'esposizione occupazionale a inquinanti chimici e biologici".

REFERENCES

1. Motti, C., Dainese, E., Mascini, M., Minunni, M. and Cozzani, P. I. (2000) *Italy J. Biochem.* **49** 3.
2. Niederhauser, C., Gilgen, M. and Meyer, E. (1996) *Mitt. Gebeite Lebensm. Hyg*. **87** 307.
3. Minunni, M., Mascini, M., Mascini, M. and Cozzani, I. (2001) *Anal. Lett.* **33**(15), 3093.
4. Marmur, J., Rownd, R. and Schildkraut, C.L. (1963) **Progress in Nucleic Acids Research**, J.N. Davidson and W.E. Cohn, (Eds.), Academic Press, New York and London, 232.
5. Mikkelsen, S. R. (1996) *Electroanalysis* **8,** 15.
6. Hall, J.M., Moore-Smith, I., Bannister, J.V. and Higgins, I.J. (1994) *Biochem. and Molec. Biol. Int.* **32**, 21.
7. Hashimoto, K. and Ishimori, Y. (1994, *Anal. Chem*. **66**, 3830.
8. Johnston, D.H., Glasgow, K.C. and Thorp, H.H. (1995*) J. Am. Chem. Soc.* **117**, 8933.
9. Millan, K.M., Saraulo, A. and Mikkelsen, S.R. (1994) *Anal. Chem*. **66**, 2943.

10. Pang, D.W., Qi, Y.P., Wang, Z.L., Cheng, J.K. and Wang, J.W. (1995) *Electroanalysis* **7**, 774.

11. Wang, J., Palecek, E., Nielssen, P., Rivas, G., Cai, X., Shiraishi, H., Dontha, N., Luo, D. and Farias, P.A.M. (1996) *J. Am. Chem. Soc*. **118**, 7667.

12. Wang, J., Cai, X., Rivas, G. and Shiraishi, H. (1996) *Anal. Chim. Acta* **326**, 141.

13. Xu, X.H. and Bard, A.J. (1995) *J. Am. Chem. Soc*. **117**, 2927.

14. Pividori, M. I., Merkoci, A. and Alegret, S. (2000) *Biosens. Bioelectron*. **15**, 291.

15. Yang, M., McGovern, M.E. and Thompson, M. (1997) *Anal. Chim. Acta* **346**, 259.

16. Wang, J. (2000) *Nucleic Acids Res.* **28**(16), 3011.

17. Palecek, E. and Fojta, M. (2001) *Anal. Chem*. **73**, 74.

18. Palecek, E. (2002) *Talanta* **56**, 809.

19. Loewy, Z.G. and Pottathil, R. (1993) **Diagnostics in the Year 2000: Antibody, Biosensor and Nucleic Acid Technologies**, Singh, P., Sharma, B. P., Tyle, P. (Eds.), Van Nostrand Reinhold, New York, Chapter 22, 389–410.

20. McPherson, M.J., Quirke, P. and Taylor G.R. (Eds.) (1991) **PCR: A Practical Approach**, IRL Press (Oxford University Press), New York.

21. Erdem, A., Kerman, K., Meric, B., Akarca, U.S. and Ozsoz, M. (2000) *Anal. Chim. Acta* **422**, 139.

22. Yan, F., Erdem, A., Meric, B., Kerman, K., Ozsoz, M. and Sadik, O.A. (2001) *Electrochem. Commun.* **3**, 224.

23. Keller, G.H. and Manak, M.M. (1989) **DNA Probes**, Stockton Press, New York, Chapter 1, 18–22.

24. Napier, M.E. and Thorp, H.H. (1997) *Langmuir* **13**, 6342.

25. Minunni, M., Tombelli, S., Mariotti, E. and Mascini, M. (2001) *Fresen. J. Anal. Chem.* **369**, 589.

26. Mannelli, I., Minunni, M., Tombelli, S. and Mascini, M. (2003) *Biosens. and Bioelectron.* **18**, 129.

27. Mariotti, E., Minunni, M. and Mascini, M. (2002) *Anal. Chim. Acta* **453**, 165.