

M. Minunni · S. Tombelli · E. Mariotti · M. Mascini
M. Mascini

Biosensors as new analytical tool for detection of Genetically Modified Organisms (GMOs)

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Abstract Three different biosensors for detection of Genetically Modified Organisms (GMOs) are presented. The sensing principle is based on the affinity interaction between nucleic acids: the probe is immobilised on the sensor surface and the target analyte is free in solution. The immobilised probes are specific for most inserted sequences in GMOs: the promoter P35S and the terminator TNOS. Electrochemical methods with screen-printed electrodes, piezoelectric and optical (SPR) transduction principles were applied.

1 Introduction

Biosensors for their characteristics (i.e. fast time response, low costs) are very attractive for new applications in different emerging fields like genetically modified organisms (GMOs) detection. The transducers applied here to GMOs sensing are based on electrochemical, piezoelectric and optical (Surface Plasmon Resonance) devices and the nucleic acid hybridisation reaction has been used as affinity reaction for analyte detection.

Piezoelectric and optical transduction principles are direct, label free methods, while the electrochemical transduction, in our case chronopotentiometric analysis at constant current performed on screen-printed electrodes, requires an additional step in which an electrochemical marker for the hybridisation reaction is added. We used daunomycin which intercalates in the DNA double helix and which is oxidised at the electrode surface once bound to DNA. The piezoelectric sensing was based on 10 MHz

AT-cut quartz crystals and the optical sensing on the commercially available instrument based on Surface Plasmon Resonance (SPR), Biacore XTM.

To develop the DNA sensors two different 25-mer oligonucleotides probes were immobilised on the different sensing surfaces: screen-printed electrodes, piezoelectric crystals and Biacore XTM sensor chips. These sequences are complementary to sequences of the most common inserts in the GMOs: the promoter 35S and the TNOS terminator. These probes recognise the complementary target sequence present in samples containing amplified genetic material for the region P35S and TNOS via PCR, using appropriate primers.

The sensors with the immobilised probe were first tested using synthetic complementary oligonucleotides and then exposed to PCR amplified DNA samples. The amplified samples are obtained from template DNA extracted from soy-bean flour Certified Reference Material (CRM) (Fluka).

2 Materials and methods

2.1. Chemicals

11-Mercaptoundecanol was synthesised in the laboratory [1]. Dextran and oligonucleotides were purchased from Pharmacia Biotech (Uppsala, Sweden), epichlorohydrin and N-hydroxysuccinimide (NHS) were from Fluka (Milan, Italy). Immobilisation buffer: NaCl 300 mM, Na₂HPO₄ 20 mM, EDTA 0.1 mM, pH 7.4. Hybridisation buffer: NaCl 150 mM, Na₂HPO₄ 20 mM, EDTA 0.1 mM, pH 7.4.

The sequences of the 5'-biotinylated probes (25-mer), of the complementary oligonucleotides (25-mer) and of the non-complementary oligonucleotide (23-mer) are given below. The piezoelectric and SPR sensors required biotinylation of the probe, while the electrochemical sensor requires no biotinylation.

Probe (35S): 5' biotin- GGCCATCGTTGAAGATGCCTCTGCC 3'

Probe (TNOS): 5' biotin- AATGATTAATTGCGGGACTCTAATC 3'

Target (35S): 5' GGCAGAGGCATCTTCAACGATGGCC 3'

Target (TNOS): 5' GATTAGAGTCCCGCAATTAATCATT 3'

Non-complementary strand: 5' TGCCACACCGACGGCGCCACC 3'

PCR reagents: The procedure for P35S and TNOS amplification is reported in Lipp et al.[2].

M. Minunni (✉) · S. Tombelli · E. Mariotti · M. Mascini
Università degli Studi di Firenze, Dipartimento di Chimica,
Via G. Capponi n. 9, Firenze, Italy
e-mail: minunni@unifi.it

M. Mascini
Università degli Studi di Teramo, Facoltà di Agraria,
Mosciano Stazione (TE), Italy

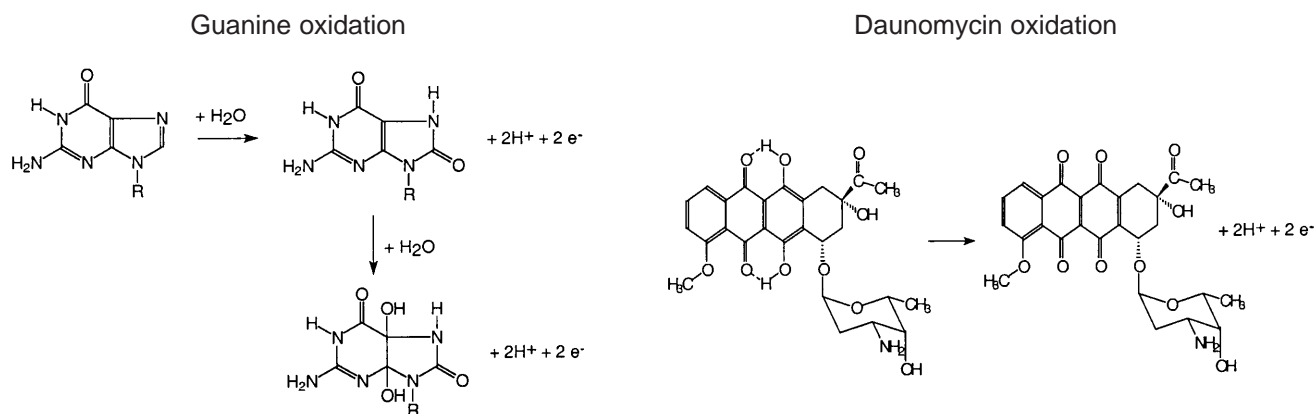
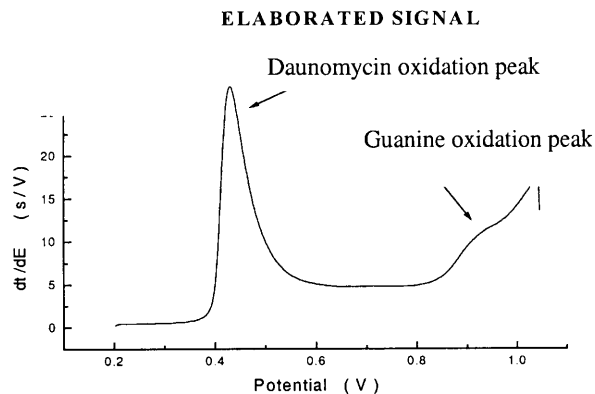
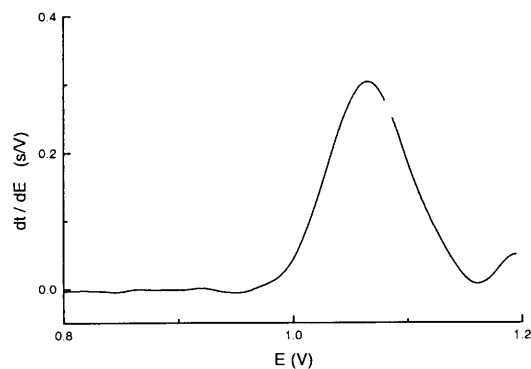


Fig. 1 Chronopotentiometric signal from the DNA sensor: peak of guanine oxidation (left) and peak of Daunomycin oxidation (right)

Buffers: Saline-sodium citrate Buffer (2XSSC) from Sigma (Milan, Italy) Acetate buffer (0.25 M pH 4.7).

2% Genetically Modified Flour Certified Reference Material (CRM) from Fluka, (Milan, Italy).

2.2 Instrumentation

A) *Electrochemical sensing*: Electrochemical experiments were performed with an Autolab PGSTAT 10 electrochemical analysis system, with a GPES4 software package (Ecochemie, Netherlands); B) *Piezoelectric sensing*: The frequency variations were continuously recorded using a quartz crystal analyser (Model QCA917, Seiko EG&G, Chiba, Japan): the data (the resonance frequency) are displayed on the main display screen and can be read directly by a computer connected to the QCA917 interface; C) *Optical sensing*: The SPR based instrument was the Biacore X™ commercially available from Biacore AB (Uppsala, Sweden).

2.3 Preparation of sensors

A) *Screen-printed electrodes*: the procedure is reported in Cagnini et al. [3]; B) *Piezoelectric crystals*: the procedure is reported in Tombelli et al. [4]; C) *Optical sensors*: We used gold sensor surfaces (Biosensor AB, Uppsala, Sweden) modified as reported by Tombelli et al. [4] for direct comparison between piezoelectric and SPR detection.

3 Sensor development

3.1 Electrochemical sensors

The chronopotentiometric screening at constant current was performed on screen-printed electrodes, used as disposable devices. Two different 25-mer probes, respectively for P35S and TNOS, were immobilised on screen-printed electrodes as reported by Marrazza et al. [5]. The specificity of the sensor was first studied by performing the hybridisation reaction adding, respectively 50 μ l of complementary and non-complementary oligonucleotides (target analyte). The daunomycin peak area was taken as analytical signal. Daunomycin, an intercalating agent, was used as hybridisation reaction marker. If hybridisation occurs, the marker will intercalate the double helix and its oxidation current will be observed when a potential scan is performed (Fig. 1).

For the Piezoelectric and Optical sensors biotinylated 25-mer probes were immobilised on streptavidin coated gold electrodes [4]. For the optical sensors the experimental conditions were: flow 5 μ l/min; temperature 25 °C.

After immobilisation, hybridisation reaction was performed as follows:

3.2 Piezoelectric sensor

200 μ l of the target sequence, in the hybridisation buffer were added to the cell well where the crystal was housed. The reaction was monitored for 20 minutes, then the crystal was washed with the hybridisation buffer in order to remove unbound oligonucleotides. The frequency was recorded until it reached a stable value (Fig. 2).

In all the experiments the single stranded probe was regenerated by a one minute treatment with 1 mM HCl allowing a successive hybridisation reaction to be monitored. Such treatment could be performed up to 10 times. All experiments were performed at room temperature (25 °C).

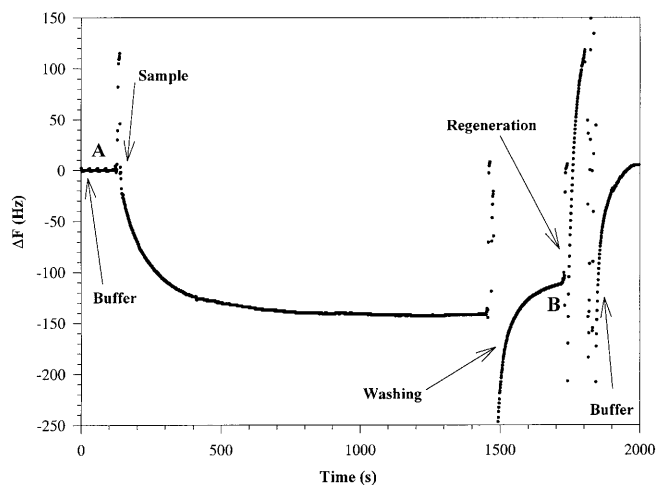


Fig. 2 Binding curve between the immobilised probe on the sensor surface and the target sequence in solution. The difference in frequency shift between points A and B is the analytical data

3.3 Optical sensor based on Surface Plasmon Resonance (SPR)

The commercially available instrument, Biacore XTM, was used for optical detection. 100 μ L at different concentrations of the target solution were added to the system (flow 33 μ L/min). The hybridisation reaction was monitored for 3 minutes, the sensor chip washed afterwards and regenerated as described above for the piezoelectric device. The signal was recorded after the washing step.

All experiments were performed three times.

4 Results and discussion

4.1 Experiment with synthetic oligonucleotides

The specificity of the sensor was studied with synthetic, fully complementary target oligonucleotides (see Material and Methods). Non-complementary, synthetic oligonucleotides were used as negative control.

4.1.1 Electrochemical sensors

In Fig. 3 a) and 3 b) the characteristic calibration curve between the immobilised probe and the DNA sample in solution is reported. A proportional relationship between the concentration of target and the recorded signal for both P35S and TNOS is observed. The specificity of the system is proven by unequivocally difference in the signals between complementary and non-complementary target sequences (data not shown).

4.1.2 Piezoelectric sensors

The results are reported in Fig. 4 a) and 4 b). The two curves show similar profiles with a linear region followed by a plateau. Measurements with the non-complementary oligonucleotides did not result in a measurable frequency shift.

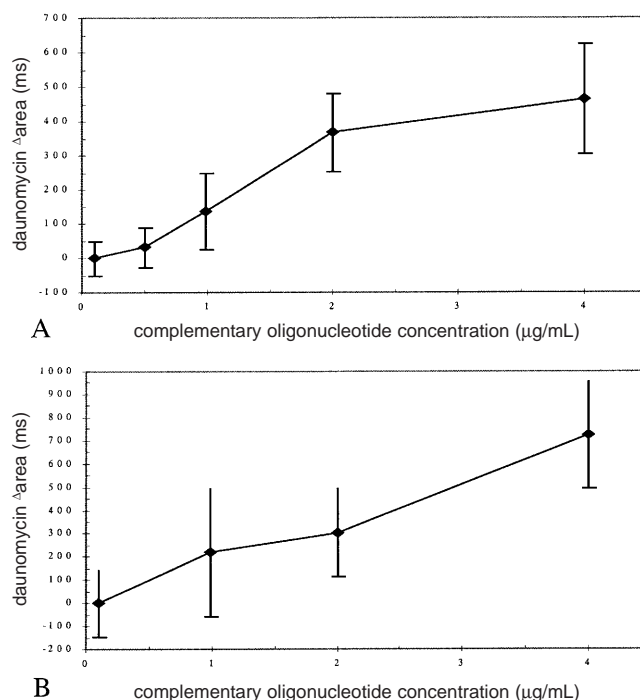


Fig. 3 Calibration curves for the electrochemical sensors with immobilised P35S and TNOS using the relative complementary oligonucleotides P35S (A) and TNOS (B)

4.1.3 Optical (SPR) sensors

The results are shown in Fig. 5 a) and 5 b). There is a linear dependence of the signal from the target analyte concentration in 0–100 nM range, both for TNOS and P35S target analyte. At higher analyte concentrations a surface saturation is observed. Measurements with the non-complementary oligonucleotides did not result in a significant signal (data not shown). The experimental detection limit was in the order of nanomoles, the CV was less than 5% for all tested concentrations.

4.2 Experiments with PCR-amplified real samples

The DNA fragments obtained from the amplification of 2% transgenic soya flour CRM by PCR were, diluted in the hybridisation buffer, denatured at 95 °C for 10 min and cooled in ice for 30 s. Then they were added to the sensors' surfaces. In order to test any possible interfering signal from the matrix (PCR amplified sample) a blank consisting in all PCR reagents exception for the amplified target was used. No interference of the matrix has been observed.

4.2.1 Electrochemical sensors

The results are shown in Fig. 6 where the analytical signal is reported vs. the sample dilution from 1 : 20 to 1 : 480. The

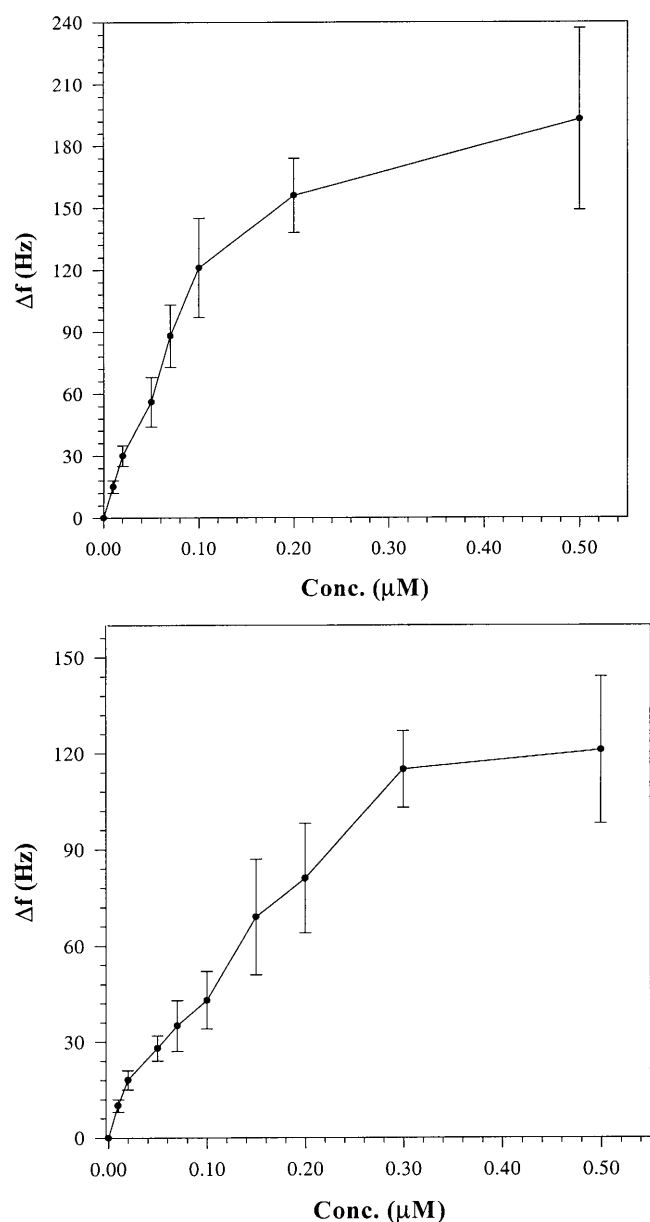


Fig. 4 Calibration curve for the piezoelectric sensors with immobilised P35S and TNOS using the relative complementary oligonucleotides P35S (A) and TNOS (B)

electrochemical signal reported as Δ area in ms is taken as the difference in recorded daunimycin peak area in the following conditions:

A) the electrode with the immobilised probe is hybridised with the complementary strand (sample) and exposed to daunimycin

B) the electrode with only the immobilised probes is exposed to daunimycin

In the case of P35S analysis the higher signal was observed at 1:480 dilution. In TNOS analysis only 3 sample dilutions were tested which gave almost the same electrochemical signal. The reproducibility for TNOS, is poor but this work is preliminar and need further investigations to optimise the experimental parameters.

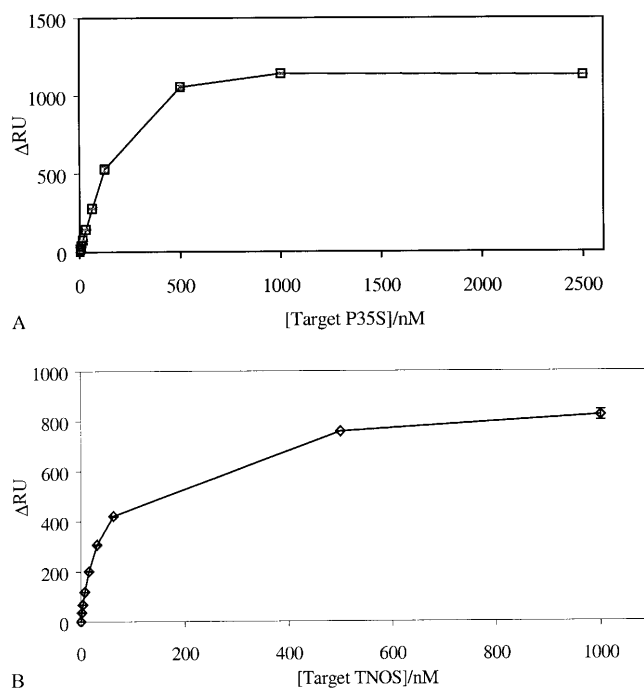


Fig. 5 Calibration curve for the optical (SPR) sensors with immobilised P35S and TNOS using the relative complementary oligonucleotides P35S (A) and TNOS (B)

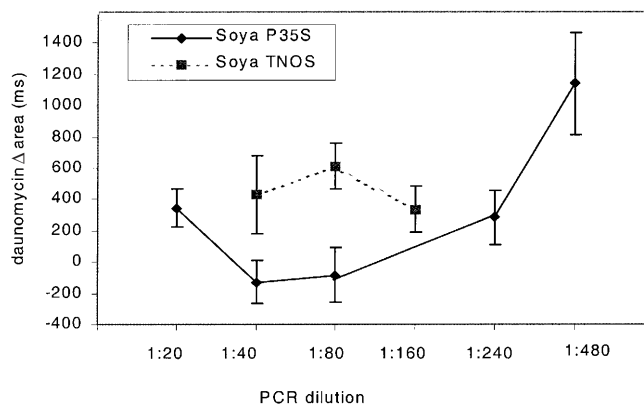


Fig. 6 Response of the electrodes with P35S and TNOS probes in real samples (PCR amplified)

4.2.2 Piezoelectric sensors

The results are reported in Tables 1 and 2 respectively for the P35S and TNOS probes. The P35S probe interaction with the transgenic soy samples gave a significant frequency shift for the samples diluted up to 1:20. For higher dilutions we recorded a negligible frequency shift. Also in TNOS case, the specific samples gave a measurable frequency shift if diluted 1:2 or 1:10. At higher dilutions negligible signals were observed.

No amplified sample was tested with the optical sensor.

Table 1 Results from PCR amplified transgenic material. Immobilised probe: P35S. Samples 1–3 are all amplified using primers for P35S. In the columns we report the origin of the template DNA and the dilution. The results are expressed as frequency shift (ΔF). Sample 5 is a non-complementary sequence of amplified DNA from T-NOS

Sample	Origin	Dilution	ΔF (Hz)
1	Soybean powder	1:20	20
2	Soybean powder	1:20	10
3	Soybean powder	1:40	0
4	Blank	1:10	0
5	Non-complementary DNA fragment	1:2	0

Table 2 Results from PCR amplified transgenic material. Immobilised probe: T-NOS. Samples 1–3 are all amplified using primers for T-NOS. In the columns we report the origin of the template DNA and the dilution. The results are expressed as frequency shift (ΔF). Sample 5 is a non-complementary sequence of amplified DNA from P35S

Sample	Origin	Dilution	ΔF (Hz)
1	Soybean powder	1:2	54
2	Soybean powder	1:20	6
3	Soybean powder	1:40	0
4	Blank	1:10	0
5	Non-complementary DNA fragment	1:2	0

5 Conclusions

We have demonstrated that biosensors could offer fast and reliable results for GMOs analysis. The proposed sensors are based on DNA hybridisation at the sensing surface be-

tween an immobilised probe, characteristic for GMOs (i.e. promoter P35S and terminator TNOS sequences present in the 98% of authorised GMOs) and the complementary, target sequence, in solution. These biosensors are able to detect the complementary synthetic oligonucleotides and the PCR amplified from 2% transgenic soy flour certified reference material, when tested. The sensors are specific for the complementary sequence as demonstrated by the results obtained with non-complementary samples. The DNA sensors are sequence specific, two of them (piezoelectric and SPR devices) are also label free and avoids the use of toxic chemicals, e.g. ethidium bromide used in electrophoresis, the reference method in GMOs analysis. Future work will be directed in optimising the experimental conditions in order to improve the reproducibility; sample from different GMOs sources will be also tested.

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