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ELECTROCHEMICAL BIOSENSORS FOR EVALUATION OF CONTAMINANTS IN FOOD

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This paper describes the application of electrochemical disposable biosensors in food analysis, which have recently been developed in our laboratory. The disposable biosensors, based on acetylcholinesterase inhibition potential, were exploited for testing the presence of organophosphorus and carbamatepesticides in water, fruit, and vegetable samples. The paper further describes preliminary tests for the detection of genetically modified organisms and hybridisation by coupling the DNA biosensors with the polymerase chain reaction.

Keywords:
acetylcholinesterase, biosensors, DNA, genetically modified organism, pesticides, screen-printed electrodes

For the past couple of years, food industry has been using electrochemical biosensors to control food (1, 2). The biosensors combine the inherent specificity of biological recognition processes and the sensitivity and speed of electrochemical measurements (3–5). This paper brings the recent results of their application in detection of pesticides (6–9). Organophosphorus and carbamate pesticides have gradually replaced the organochlorines and, although they are characterised by low environmental persistence, they generally show higher acute toxicity. The toxicity has a direct impact on human health because it causes an irreversible inhibition of acetylcholinesterase which is an enzyme involved in the nerve-impulse transmission. The measurement of the anticholinesterase activity of these pesticides can be used to as a screening test for the evaluation of the presence of these compounds in food matrices. The inhibition can be evaluated using screen-printed electrodes for amperometric or voltammetric measurements. Screen-printed electrodes are planar devices, based on different layers of ink printed on a plastic or ceramic substrate. The interest in these devices is due to the possibility of making them disposable. In electrochemistry a disposable sensor has the advantage of not suffering from the electrode fouling that can result in the

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loss of sensitivity and reproducibility. Moreover, these devices can be miniaturised and made suitable for on-site measurement. This paper brings some results using a disposable screen-printed carbon electrode. The carbon surface was modified by deposition of a mediator, tetracyanoquinodimethane (TCNQ). Acetylcholinesterase cleaves the acetylthiocholine added to the sample to thiocholine. The amount of thiocholine produced is directly related to the enzyme activity and can be evaluated by differential pulse voltammetry (DPV). DPV is a faster technique than the earlier used chronoamperometry (8), especially in combination with screen-printed electrodes.

In this approach, the enzyme inhibition is referred to as total anticholinesterase activity (TAA) and is expressed as »equivalent μg/L« of a reference pesticide. The TAA index does not measure the concentration of a specific compound, but is related to a class of compounds and to their toxicity. Therefore, the TAA index indicates whether a sample should undergo further analysis (10).

Moreover, this paper reports results obtained with a DNA-based biosensor for transgenic organisms. There is an increasing need of screening methods to identify genetically modified organisms (GMOs) in food (11). Currently the most cultivated GMOs are soya and maize, and the control and detection of these foodstuffs are important issues (12). Our strategy has been to immobilise sequences of 25-mer single-stranded oligonucleotides (probe) on carbon screen-printed electrodes. The immobilised oligonucleotides complement the sequence of the most common inserts in the GMOs: the Promoter 35S and the TNOS terminator (11). The food samples were previously amplified by polymerase chain reaction (PCR). The ability of the probe to recognise the target sequences was first tested using synthetic full complementary target oligonucleotides. Non-complementary synthetic oligonucleotides were used as negative control. An electrochemical marker (daunomycin) was used to signal the probe-target interaction (13, 14) that intercalates the double-stranded DNA. The procedure consisted of four steps:

1) the synthetic oligonucleotide was immobilised onto the electrode surface by adsorption at controlled potential;
2) the obtained DNA biosensor was incubated with the amplified sample or with the solution containing the target;
3) the probe was then immersed in the daunomycin solution, which intercalates the hybrid formed on the electrode surface; and
4) finally, the daunomycin anodic peak was used to detect whether hybridisation took place.

MATERIALS AND METHODS

Pesticide detection

Acetylcholinesterase (AchE, EC 3.1.1.7) from the electric eel (530 U/mg protein), acetylthiocholine (ATCh chloride salt), and the mediator 7,7,8,8, tetracyanoquinodimethane (TCNQ) were purchased from Sigma (USA). Carbofuran was obtained from Poly-

science Corporation (USA). Standard solutions were prepared daily by dissolving the pesticide in the acetonitrile purchased from Sigma (USA). All other reagents were of analytical grade and were obtained from Merck (Germany). The water was from a reagent grade ion-exchange system from Millipore Inc. (USA).

All electrochemical measurements were performed using a computer controlled polarographic analyser model 433 A (Amel, Italy). The reagents and the procedure for electrode screen-printing were reported elsewhere (6–8). The electrochemical cell consisted of a screen-printed carbon working electrode and silver reference and counter screen-printed electrodes.

In DPV, the potential was scanned in a defined range and the pulses were superimposed on the potential ramp. The current was measured immediately before pulse application and at the end of the pulse, and the difference between the two currents was registered. Since DPV is a differential technique, the response is similar to the first derivative of a conventional voltammogram, that is, a peak.

To perform the measurement, the carbon surface of the working electrode was modified with 2 μl of a suspension obtained by mixing 5 μl of $1x10^{-3}$ mol/L TCNQ solution in acetonitrile and 50 μl Nafion®.

In order to optimise the determination of the acetylcholinesterase activity we developed the following procedure; 20 μl of a stock solution of enzyme (50 U/μl) and 20 μl of a $5x10^{-2}$ mol/L acetylthiocholine were mixed in 1 ml of buffer phosphate 0.1 mol/L at pH 7.5; the reaction was allowed to proceed for 5 min at room temperature. Then 100 μl of the solution were deposited onto the planar surface of the TCNQ-Nafionâ SPE and the DPV measurement was performed from +0.35 to +0.7 V vs. Ag pseudo-reference electrode; with the pulse amplitude of 50 mV, scan rate 25 mV/s and pulse width of 60 ms. The current peak obtained at + 0.5 V which was set as the analytical signal. To measure the total anticholinesterase activity using the acetylthiocholine sensor, 50 μl of pesticide standard solutions or river water samples were added to 1 ml of 0.1 mol/L phosphate buffer solution pH 7.5 with acetylcholinesterase (1 U/ml) and incubated for 10 min. Then we added 20 μl of the $5x10^{-2}$ mol/L acetylthiocholine solution. After 5 min, 100 μl of this solution were directly dropped on the surface of the sensor. The oxidation current peak obtained by DPV was measured (I_2) and compared with the oxidation current value obtained without pesticide (I_1).

The percent of inhibition (I%) was obtained according to the following formula:

$$I\% = 100\{(I_1 - I_2)/I_1\}$$

The total anticholinesterase activity can be expressed as the amount of compounds which inhibits cholinesterase in a percentage equival to inhibition by a known amount of a pesticide (such as carbofuran, a carbamate pesticide) taken as the reference compound. Carbofuran was chosen as the reference pesticide because it is widely use for crop protection and is one of the most frequently found pesticides in water samples taken from the River Arno. The procedure for standard gas-chromatography of the river water samples was described earlier (9).

GMO detection

Synthetic oligonucleotides were from Pharmacia Biotech (Milan, Italy). The PCR reagents and the procedure were reported elsewhere (14). Saline-sodium citrate buffer

(2xSSC, containing 300 mmol/L NaCl and 30 mmol/L $C_3H_5Na_3O_7$, pH 7.4), acetic acid, sodium acetate, daunomycin hydrochloride were from Sigma (Milan, Italy). Certified reference material (2% soya flour) was from Fluka, (Milan, Italy). The reagents and the procedure for electrode screen-printing were reported elsewhere (6, 7). The electrochemical cell consisted of a screen-printed carbon working electrode, a platinum counter electrode, and a saturated calomel reference electrode (SCE). The electrochemical experiments were performed with an Autolab PGSTA 10 electrochemical analysis system and a GPES4 software package (Ecochemie, the Netherlands). Chronopotentiometry records the variation of the potential (E) with time resulting from the application of a constant current to the electrode. In general, the derivative signal (dE/dt) is plotted vs. the potential, producing the analytical signal as a peak.

For the immobilisation of the probe onto the electrode surface, the specific oligonucleotides were diluted in an appropriate buffer solution (2xSSC) pH 7.4, reaching the final concentration of 4 μg/ml. The deposition was carried out applying the positive potential +0.5V vs. SCE, for 120 s. To check whether the immobilisation was successful, a chronopotentiogram was used to observe the guanine oxidation peak as confirmation of the deposition after the immobilisation.

The probe base sequences were:

GGCCATCGTTGAAGATGCCTCTGCC (P35S)
AATGATTAATTGCGGGACTCTAATC (TNOS)

The hybridisation reaction was first tested using synthetic oligonucleotides at different concentrations. The diluted oligonucleotide solution was prepared in 2xSSC buffer; 50 μl of the diluted solution was applied onto the working electrode surface for 4 min and then the sensor surface was washed with 2xSSC buffer; then the working electrode was immersed in a 10 μM daunomycin solution in 2xSSC buffer for 120 s. After the washing with a 2xSSC buffer, chronopotentiometry is carried out in 2xSSC buffer, applying a constant current of +1 μA in a potential range from +0.2V to +1.1V vs. SCE. The analytical signal is the daunomycin oxidation peak area around +0.45 V vs. SCE.

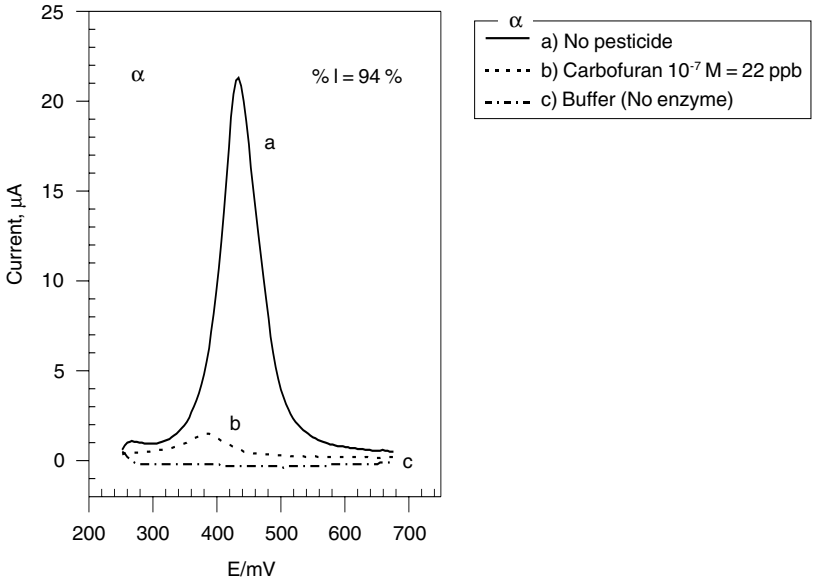
When the amplified samples from the certified material were analysed, the procedure continued with denaturation of the diluted PCR-amplified double-stranded DNA by heating at 95 °C for 5 min. and rapid cooling with ice for 2 min. The denaturated amplified sample (50 μl) was deposited onto the electrode surface to react with the immobilised complementary probe for 4 min. The steps for the synthetic oligonucleotides are the same as described above.

RESULTS AND DISCUSSION

Pesticide detection

The rate of thiocholine production in the presence of organophosphorus and carbamate pesticides, which bind to the enzyme acetylcholinesterase and inhibit it, drops with their increase. We tested different compounds using the described biosensors. Figure 1A

Figure 1 *Calibration curve of carbofuran A) DP voltammograms obtained in absence and in presence of two different concentrations of carbofuran and a scan obtained in the buffer without the enzyme; B) Inhibition curve of carbofuran obtained by plotting the inhibition value {i%=[(i$_1$-i$_2$)/i$_1$]x100} vs. concentration of inhibitor*



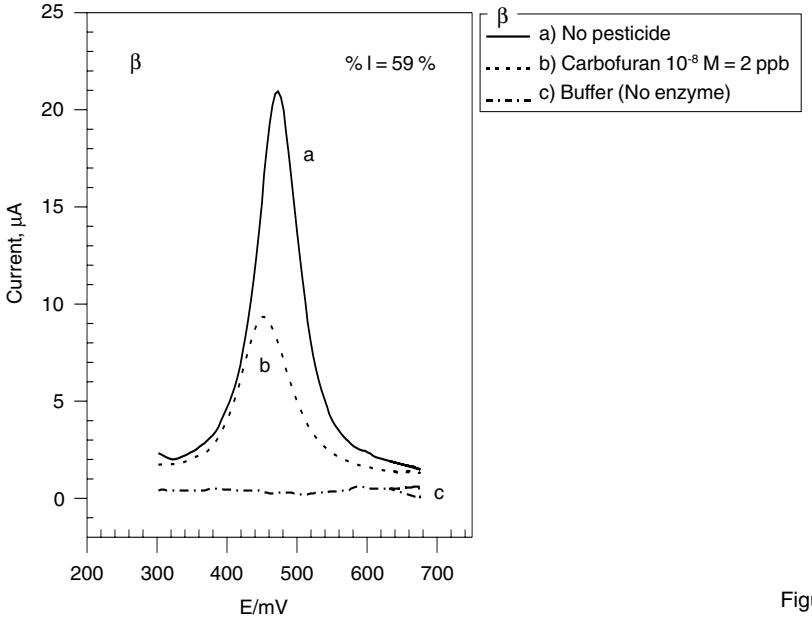


Figure 1a

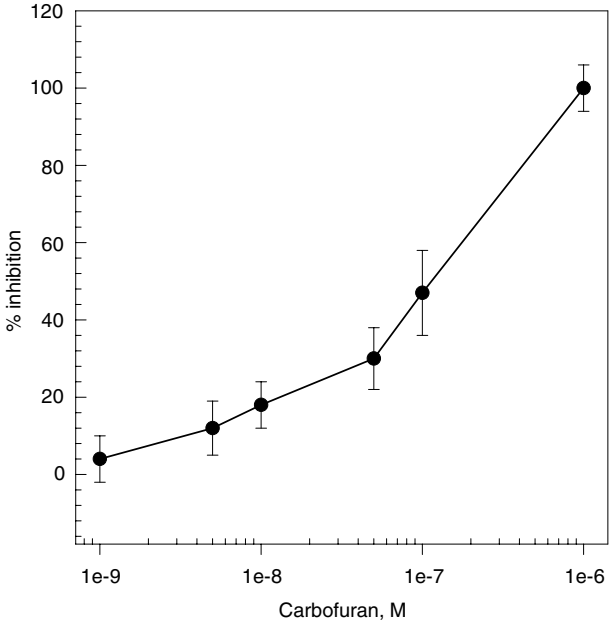


Figure 1b

shows the voltammograms obtained in the absence and in presence of two different concentrations of carbofuran, a carbamate pesticide considered as reference, since it is very common in agriculture. Figure 1B shows the calibration curve of this pesticide. Its limit of detection was $1x10^{-9}$ M.

Table 1 shows the results obtained with acetylthiocholine biosensor for the water analysis of the River Arno (9). Since the anticholinesterase pesticide concentration in the Arno water is very low, in many cases below 0.1 μg/L, we have pre-concentrated the samples using the solid-phase extraction (SPE) technique (9). The samples were pre-concentrated 100 times, shifting the detection limit to $9x10^{-11}$ M. Table 1 illustrates the results obtained using a standard method (gas chromatography) and the biosensor. There is a good correlation between the two sets of data. However, the results obtained by the biosensor were slightly greater than those obtained by the reference method, since the sensor determines a group of cholinesterase-inhibiting compounds. In fact, acetylcholinesterase activity may be inhibited by several compounds other than pesticides such as heavy metals and some toxins. The TAA is a general index combining these different contributions, although the effect of heavy metal on acetylcholinesterase activity is negligible compared to the effect of pesticides (8).

Table 1 *Comparison of the results obtained by gas chromatography (GC-ITDMS) and the acetylthiocholinesterase biosensor. The river water samples were 100 times concentrated by solid-phase extraction. Carbofuran was the reference pesticide. Samples were collected in different days during spring-summer 1999*

Gas Chromatography		Biosensor	
Samples	**Carbofuran M**	**% Inhibition**	**Carbofuran *Equivalent Conc.* M**
1 (17/4/99)	2×10^{-13}	–	$<9 \times 10^{-11}$
2 (21/4/99)	2×10^{-13}	–	$<9 \times 10^{-11}$
3 (30/4/99)	2×10^{-13}	–	$<9 \times 10^{-11}$
4 (24/5/99)	1.2×10^{-10}	40 ± 9	1×10^{-9}
5 (25/5/99)	1.1×10^{-10}	30 ± 7	4×10^{-10}
6 (26/5/99)	1.0×10^{-10}	30 ± 7	4×10^{-10}
7 (27/5/99)	6.0×10^{-11}	15 ± 6	1×10^{-10}
8 (7/6/99)	1.5×10^{-10}	40 ± 9	1×10^{-9}
9 (21/6/99)	1.1×10^{-10}	40 ± 9	1×10^{-9}
10 (3/7/99)	4×10^{-13}	–	$<9 \times 10^{-11}$
11 (9/7/99)	5×10^{-13}	–	$<9 \times 10^{-11}$
12 (13/7/99)	2×10^{-13}	–	$<9 \times 10^{-11}$
13 (16/7/99)	8×10^{-13}	–	$<9 \times 10^{-11}$

Table 2 shows the results obtained analysing fruit and vegetable samples; 5 g of a sample were ground and treated with 15 ml of ethylacetate; 5 ml were then dried and dissolved in a 2 ml buffer. One millilitre was the sample volume used for the evaluation of pesticides.

Table 2 suggests that this simple procedure can be effective in analysing such samples, even *in situ*, that is, with a portable apparatus.

Table 2 *Inhibition values obtained by the analysis of fruit and vegetable samples*

Samples	Biosensor I%
Grape	93 ± 3
Grape	50 ± 2
Grape	0
Orange	40 ± 3
Pear	14 ± 1
Celery	30 ± 2
Melon	10 ± 1
Lettuce	31 ± 2

GMOs detection

Figure 2 shows calibration curves at different concentrations for complementary synthetic oligonucleotides using electrodes with P35S or TNOS probe immobilised on the surface of the working electrode. The daunomycin peak area is proportionally increasing with the concentration of complementary oligonucleotides either for P35S or TNOS probe. Figure 3 compares complementary with non-complementary signals. The difference is significant when the concentration is above 2 mg/kg.

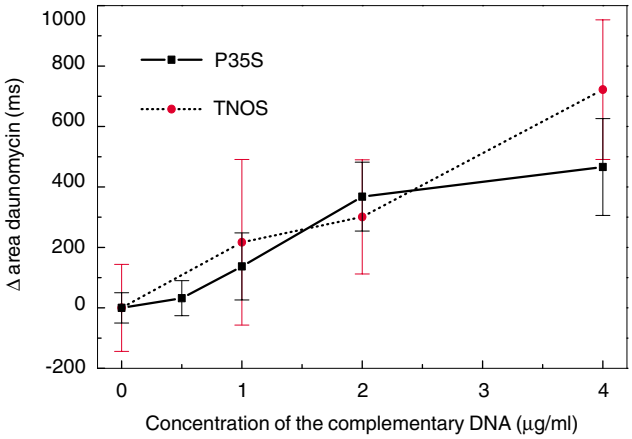


Figure 2 *Calibration curves for the sensors with immobilised P35S and TNOS using the relative complementary oligonucleotides*

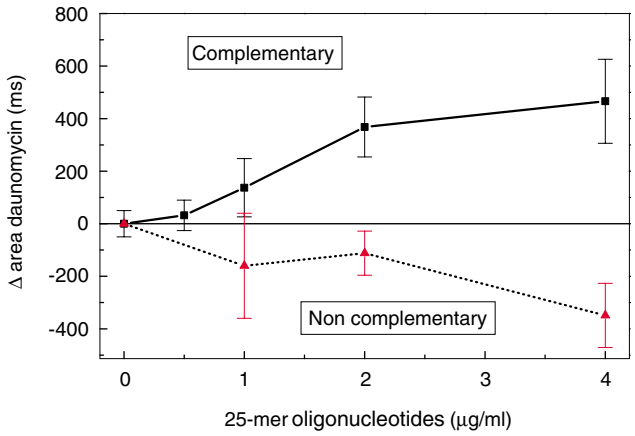


Figure 3 *P35S probe immobilised onto the electrode surface: response to the specific complementary and non-complementary 25-mer oligonucleotides*

The DNA probe was then applied to 2% transgenic soya flour (from certified reference material). In order to test a possible interfering signal in the matrix, a blank was tested with all PCR reagents, but without the target sequence and no interference was observed. Different dilutions of the amplified transgenic soya sample (2%) were assayed with both DNA biosensors with immobilised oligonucleotides (P35S or TNOS). Preliminary results are shown in Table 3 where the analytical signal is reported vs. the

Table 3 *Response of the electrode with P35S and TNOS probes in real samples amplified by PCR. Soya P35S-amplified samples were analysed with the P35S probe. Soya TNOS-amplified samples were analysed with the TNOS probe*

Dilutions	Soya TNOS	Soya P35S
1:20	0	+343 ± 380
1:40	+430 ± 240	-130 ± 130
1:80	+610 ± 350	-90 ± 180
1:160	+340 ± 250	0

sample dilution from 1:20 to 1:160. When the TNOS probe was immobilised on the sensor surface, the dilution of the relative amplified sample giving the higher response was 1:80. When the P35S probe was immobilised, an increase in the daunomycin peak area was observed only at the 1:20 dilution of the relative amplified sample. These preliminary experiments show the applicability of such devices in rapid evaluation of GMOs.

CONCLUSION

We have demonstrated that biosensors can offer fast and reliable results for food analysis. Acetylcholinesterase activity inhibition can be used to detect organophosphorus and carbamate pesticides in different food samples with minimal pretreatment. Such measurement can be used as a screening test before performing longer and expensive analysis.

Moreover, we have shown that the DNA biosensor is able to recognise oligonucleotides and, through PCR amplification, the presence of transgenic soya in certified reference material. The sensor is specific for the complementary sequence.

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Sažetak

ELEKTROKEMIJSKI BIOSENZORI ZA PROCJENU ONEČIŠĆIVAČA RADI POBOLJŠANJA KAKVOĆE HRANE

U radu su opisane neke primjene jednokratnih elektrokemijskih biosenzora za analizu hrane koji su nedavno razvijeni u našem laboratoriju. Biosenzori za jednokratnu upotrebu, bazirani na inhibiciji aktivnosti kolinesteraze primijenjeni su za ispitivanje prisutnosti organofosfornih i karbamatnih pesticida u uzorcima voda, voća i povrća. Razvijena je skupina DNK-biosenzora, a u ovom radu prikazani su rezultati preliminarnih ispitivanja detekcije genetički modificiranih organizama i hibridizacije vezanjem PCR i DNK-biosenzora.

Ključne riječi:
acetilkolinesteraza, biosenzori, DNK, genetički modificirani organizmi, pesticidi

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