

# ELECTROCHEMICAL DNA BASED BIOSENSOR FOR THE DETECTION OF GENOTOXIC COMPOUNDS IN FISH BILE SAMPLES

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**abstract:** A disposable electrochemical DNA based biosensor for the detection of genotoxic compounds in fish bile samples as marker of PAHs (polycyclic aromatic hydrocarbons) exposed at contaminated sites has been reported. The DNA biosensor is assembled by immobilising double stranded *Calf Thymus* DNA onto the surface of a disposable carbon screen-printed electrode. The oxidation signal of the guanine base, obtained by a square wave voltammetric scan, is used as analytical signal to detect the DNA damage; the presence of low molecular weight compounds with affinity for nucleic acids is measured by their effect on the guanine oxidation peak. Preliminary analysis of some PAH metabolites standard solutions were performed, in order to establish their behaviour with DNA based biosensor. Also, in order to highlight the differences between the not exposed bile and the injected bile, a deconjugation (or hydrolysis) process of the bile has been performed. The applicability of such a biosensor for analysing bile samples of fish from sites with high ecological risk was evaluated.

**keywords:** Electrochemical DNA biosensor, Genotoxic compounds, Screen-printed electrode

## Introduction

Polycyclic Aromatic Hydrocarbons (PAHs) constitute a major environmental threat due to their carcinogenic properties. Increased prevalences of liver lesions and tumors have been observed in several fish populations in European and North American waters, and were linked to PAH pollution [1-4]. In order to quantitate PAH uptake by fish, biotransformation products (metabolites) can be determined in the bile fluid [5-9]. The Oslo & Paris Commission (OSPARCOM) has recently expressed its view that the determination of PAH metabolites in fish bile should be included in the new Joint Assessment and Monitoring Programme (JAMP) [10].

Analysis of PAH accumulation levels in fish tissue is usually not feasible due to rapid transformation (metabolism) into more polar and more easily excretable forms. The occurrence of biotransformation does, not, however, imply that PAHs are relatively

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harmless to metabolizing species. On contrary during metabolism some reactive intermediates can be formed that can bind to proteins and DNA [11,12].

Many studies have demonstrated that the presence of PAH metabolites in bile is well correlated with level of exposure [13-16], therefore bile PAHs metabolites can be determined as a biomarker of PAH exposure.

In recent years, the detection of DNA damage has been proposed as a useful tool for assessing biological effects of environmental pollutants. Consequently, a variety of DNA based assays are presently in use [17,18]. Nevertheless, the impact of PAH exposure at the level of individual genes and the subsequent utilization of these effects for biosensor development requires further attention.

The interactions between DNA and environmental pollutants can cause chemical or conformational modifications of nucleic acids and thus variation of the electrochemical properties of DNA. The presence of these compounds is measured by their effect on the guanine base: the changes in oxidation of the guanine peak, obtained by a square wave voltametric scan, is used as analytical signal [19].

In this paper, a rapid and low cost device, based on electrochemical DNA biosensor, is proposed as a screening tool for the detection of PAH exposure at contaminated sites. PAH exposure in marine organisms is often assessed by measuring the concentration of PAHs in their tissues. However fish caught at highly polluted sites often showed only trace levels of PAHs in the tissue, due to their ability to metabolise these compounds. Laboratory studies have demonstrated the presence of PAH metabolites in bile is well correlated with level of exposure [20 -23], as the gallbladder bile is major excretion route for PAH in fish. After biotransformation, PAH metabolites are excreted into the bile and concentrated. Thus, the bile can be used as an indicator of PAH exposure.

The developed DNA based biosensor is used as a rapid (8 minutes for each test) screening tool for the evaluation of an environmental sample.

## **Experimental**

### **Material and methods**

#### *a. Apparatus and reagents*

Electrochemical measurements were performed with a Palm Sens (Palm Instrument BV, Hoten Netherlands) which is controlled by a Pocket PC (fig. 1) and screen printed electrodes (SPE).

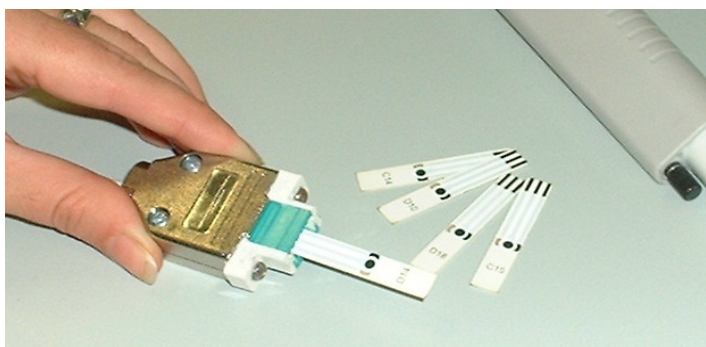


**Fig. 1** *Palm Sens connected with Pocket PC*

The Palm Sens Instrument is used for sensors or cells with two or three electrodes and the dynamic range allows applications as micro as well as macroelectrodes.

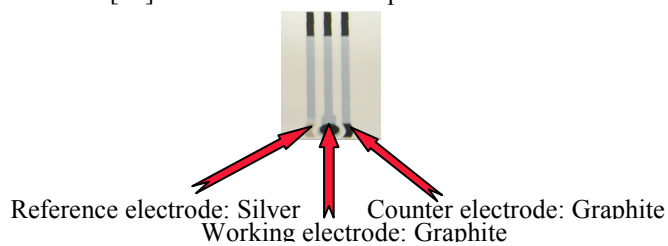
The Pocket PC software provides easy control of Palm Sens.

The electrochemical cells produced at the University of Florence is presented in fig 2.



**Fig. 2.** *Electrochemical screen printed cell*

These planar electrochemical cells can be used as “drop and sensors” and only 10  $\mu\text{L}$  of sample solution is required to perform the measurements. The cell consists of a circular graphite working electrode (diameter of 3 mm), a pseudo reference electrode and a graphite counter electrode (fig. 3). The procedure and the reagents to make screen printed electrodes were reported in literature [24]. Each electrode is disposable.



**Fig. 3.** *Screen printed electrode*

### *b. Samples preparation*

In order to minimize the matrix effect of bile on the electrochemical measurements, different dilutions of fish bile samples have been investigated (1:250, 1:1000, 1:3000, 1:5000, 1:10000). In this study, it was used the 1:5000 dilution being considered the best one. Crude bile sample (10 $\mu$ L) was first diluted in acetate buffer solution (0.25M, pH 4.74) containing 20% ethanol for a final dilution factor of 1:250. The second dilution step involved in the use of acetate buffer solution containing 1% ethanol for final dilution factor 1:5000. Crude bile samples were stored at +20°C. Glass vials were used for the preparation of all solutions.

### *c. DNA biosensor*

The electrode surface was pretreated by applying a potential of + 1.6 V for 2 min. and a potential of 1.8 V for 1 min.

The biosensor was assembled by immobilizing double stranded calf thymus DNA at fixed potential (+ 0.5 V vs. Ag screen printed pseudo-reference electrode, for 300 s) onto the screen printed electrode surface. During the immobilization step, the strip was immersed in acetate buffer solution containing 50 ppm of double stranded calf thymus DNA. Then a washing step was performed by immersion of the biosensor in a clean acetate buffer solution for 30 s, at open circuit condition. The interaction step was performed just by placing 10  $\mu$ L of the sample on the electrode surface of the DNA biosensor. After 2 min the biosensor was washed, immersed in acetate buffer and a square wave voltammetric scan was carried out to evaluate the oxidation of guanine peak on the electrode surface. The area of the guanine peak (around + 1 V vs. Ag screen printed pseudo-reference electrode) was measured. Potentially toxic compounds present in water were evaluated by changes of the electrochemical signal of guanine. We estimated the DNA modification with the value of the percentage of response decrease (S%) which is the ratio of the guanine peak area after the interaction with the analyte (GPAs), and the guanine peak area after the interaction with buffer solution (GPAb):  $S\% = [100 - (GPAs/GPAb)] \times 100$ .

The results of the test for one sample can be obtained within 8 min.

The supporting electrolyte for the voltammetric experiments and for any step in the biosensor set up was acetate buffer 0.25M pH 4.74, KCl 10 mM.

Square wave voltammetric parameters were: frequency = 200 Hz, step potential = 15 mV, amplitude = 40 mV, potential range 0.2 – 1.2 V versus Ag – pseudo-reference electrode.

## **Results and discussion**

### *a. PAH metabolites response in standard solutions*

The behaviour of some hydroxy-PAHs and dihydroxy-PAHs standard solutions was investigated with the DNA biosensor. A stock solution of each compound was previously prepared in ethanol or acetone.

Preliminary analysis of some PAH metabolites standard solutions were performed, in order to establish their behaviour with DNA-based biosensor.

In the fig. 5 the comparison between one metabolite for each PAH class is reported. S(%) values decreased increasing the standard concentration and increasing the number of the condensed aromatic rings. An exception is chrysene which, even if has 4 rings is less stronger than phenantrene (3 rings): this behaviour could be explained with different positions of fused rings that permits a less or strong interaction with DNA.

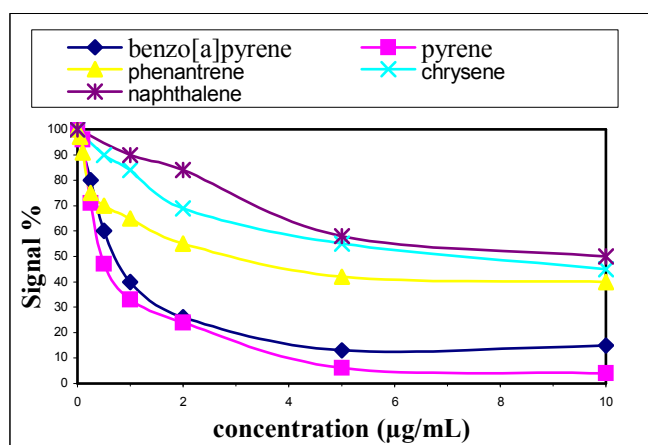


Fig. 4. Comparison of the DNA biosensor response for one metabolite standard solutions for each PAH class.

b. Analysis of fish bile samples with single PAH intraperitoneal injection dose before and after a deconjugation process.

A simulation experiment of acute exposure to PAH was performed in fish. A single PAH compound was injected intra peritoneally in Atlantic cod (*Gadus morhua*) in different doses depending to the ring number of the compound and the fish weight. The fish were sacrificed five days after the exposure and the bile was sampled for the analysis.

The bile of fish exposed with single PAHs did not show very strong genotoxic effects on the DNA biosensor. In order to highlight the differences between the not exposed bile and the injected bile, a deconjugation (or hydrolisis) process of the bile has been performed.

It seems that the not strong genotoxic effect could be due to the metabolic pathways of the PAHs in fish. In the bile after the fish metabolism it didn't find that PAH or the hydroxy-PAHs and dihydroxydihydro-PAHs in a high percentage, but the PAHs conjugated with glucuronic acid and sulphatase that are not toxic and so they don't interact DNA.

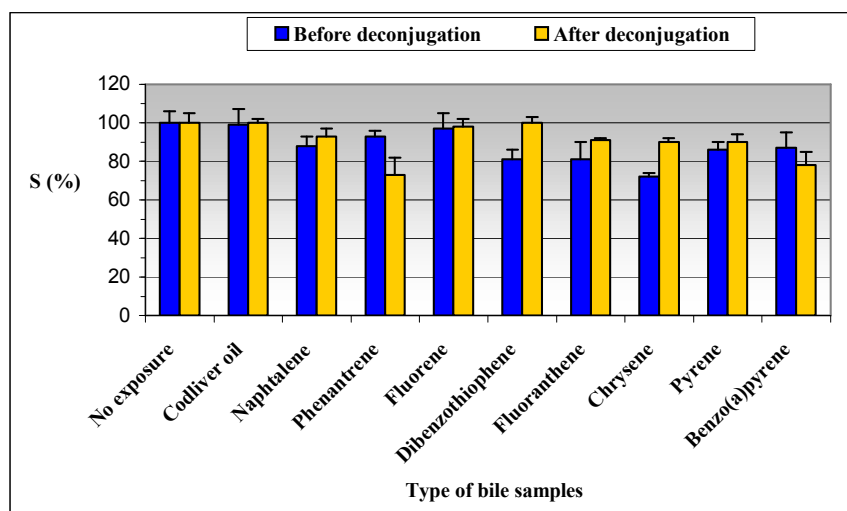
Therefore, the next step of the study was the determination of the influence of deconjugation process of fish bile samples on the signal of guanine. To obtain this, the deconjugation process was performed and all the parameters were optimised. Different volumes of crude bile sample were mixed with different volumes of  $\beta$ -sulphatase enzyme (from *helix pomatia*, Sigma) or glucuronidase enzyme (from *helix pomatia*, Sigma) in a solution of 0.25M acetate buffer (pH 4.74) with 10mM KCl. The mixture was kept for 2 or 4 hours at 40°C. Then samples were centrifugated for 5 minutes at 12.000 r.p.m. and the supernatant was used to prepare final concentration 1:5000 with 0.25M acetate buffer (pH

4.7) with 10mM KCl, containing 1% of ethanol. Supernatants were analysed during the same day.

The results obtained with electrochemical DNA biosensor analysing fish bile samples with a single PAH injection dose using the deconjugation process are presented in the table 1 and in the fig. 5. Samples were diluted 1/5000 with acetic buffer 0.25M (pH 4.74) containing 1% EtOH. A normalisation of the signal related to the no exposure bile sample was performed:  $S_n = (S_i \times 100)/S_{no\ exposure}$ , where  $S_i$  is the signal of different samples.

**Table 1. The results for fish bile samples**

Type of bile	Before deconjugation		After deconjugation with sulphatase	
	Signal %	sd	Signal %	sd
No exposure	100	6	100	5
Codliver oil	99	8	100	2
Naphtalene	88	5	93	4
Phenantrene	93	3	73	9
Fluorene	97	8	98	4
Dibenzothiophene	81	5	100	3
Fluoranthene	81	9	91	1
Chrysene	72	2	90	2
Pyrene	86	4	90	4
Benzo(a)pyrene	87	8	78	7



**Fig. 5.** Comparison between before and after deconjugation process using normalisation method

The simulation experiments of acute exposure showed that PAHs genotoxic effects were statistically different. This different carcinogenic power could be due to the ring number of compound, the molecular conformation and the presence of heteroatom. They were divided into two groups depending of the S% level. The compounds of the first group (naphtalene, phenantrene and fluorene) produced S% levels around 93%. The second group includes fluoranthene, chrysene, pyrene and benzo(a)pyrene and showed higher genotoxic effects

with S% levels around 82%. The dibenzotiofene effect cannot diffrence to the control sample; the presence of heteroatom could not facilitate the interaction with dsDNA.

## Conclusions

In conclusion it can be seen that electrochemical DNA based biosensor permits the measurements of the overall PAH metabolites content of a sample. Moreover DNA biosensor give the global content of pollution and can be used as markers of recent exposure at contminated sites.

Nevertheless the DNA biosensor could be a very good test since can give rapid and easy informations to evaluate the presence of small compounds with the affinity for nucleic acids.

Electrochemical DNA based biosensors offer several advantages like an easy immobilization of the DNA layer, fast measurement, portable instrumentation suitable for measurements in situ, in field screening analysis of toxic compounds and cost effective, moreover the disposable single use biosensor avoids contamination among samples and allows constant sensitivity and reproducibility.

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