

# COMPARISON OF TWO PHOTOSYNTHETIC BIOMEDIATORS FOR HERBICIDE DETECTION

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## Abstract

Monitoring programs require rapid, simple and low-cost screening procedures for the detection of harmful chemicals in the environment. Photosynthetic biosensors better reflect the impact of toxic compounds by measuring the alteration of a physiological process [14,17]. Under illumination, photosynthetic materials are able to transfer electrons to an electron acceptor changing its redox state; the electric current generated can be measured amperometrically. The present work focuses on the construction and comparison of two photosynthesis-based biosensors using either thylakoid membrane or photosynthetic algae. This setup resulted in two reusable amperometric biosensors for the detection of herbicides both with limits of detection in the nanomolar range. The algal biosensor, as it was expected, presented an increased lifetime.

**Keywords:** Immobilization, biosensor, algae, photosynthesis, herbicides, biomediator.

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## COMPARACION DE DOS BIOMEDIADORES FOTOSINTETICOS PARA LA DETECCION DE HERBICIDAS

### Resumen

*Los programas de monitoreo requieren procedimientos de detección rápida, sencilla y a bajo costo para la detección de productos químicos nocivos en el medio ambiente. Los biosensores fotosintéticos son los que mejor reflejan el impacto de los compuestos tóxicos mediante la medición de la alteración de sus procesos fisiológicos [14,17]. Cuando son expuestos a la iluminación, los materiales fotosintéticos son capaces de transferir electrones a un aceptor de electrones que cambia su estado en reducción, en el cual la corriente eléctrica generada puede ser medida. El presente trabajo se centra en la construcción y la comparación de dos biosensores basados en la fotosíntesis usando la membrana tilacoide o de algas basadas en fotosíntesis. Esta disposición dio lugar a dos biosensores amperométricos reutilizables para la detección de herbicidas, con límites de detección en el rango nanomolar. El biosensor de algas, como era de esperarse, presentan un tiempo de vitalidad mayor.*

**Palabras clave:** Inmovilización, biosensor, alga, fotosíntesis, herbicidas, biomediator.

## 1. INTRODUCTION

Water pollution by herbicides is a very important environmental problem. Herbicides are highly toxic for human and animal health, and the enormous increase in the application of herbicides in agriculture during recent decades has resulted in the herbicide pollution of both soil and water. This can result in serious harm to the environment, thus the importance of herbicides monitoring in runoff water and soils is evident. Further, it is important to have systems available for a rapid analysis of aqueous systems in order to monitor the presence of increased amounts of herbicides.

Detection of environmental hazardous chemicals using biosensor technology is ever increasing. Biosensors are analytical devices incorporating a biological material as biorecognition element (biomediator), utilized in conjunction with a physicochemical transducer. These usually yield a digital electronic signal that is related to the concentration of a specific analyte or a particular physical-chemical condition.

Unicellular algae and cyanobacteria have been already used for the construction of biosensors capable of real time testing the presence of pollutant emissions in water [1-6]. Chloroplasts, thylakoid membranes, photosystem II (PSII), and photosynthetic reaction center based biosensors have already been reported [7-11]. Several classes of herbicides that are used at present in agriculture inhibit photosynthesis competing with plastoquinone for binding to the  $Q_B$  pocket of the PSII reaction centre protein D1. The high binding affinity of these herbicides to D1 offers a unique possibility to use photosynthetic materials for herbicide detection.

The major drawback using photosynthetic material is the short life-time. To circumvent this limitation, various immobilization techniques have been designed to improve the stability [12]. The photosynthetic material was often immobilised in a bovine serum albumin-glutaraldehyde cross-linked matrix (BSA-Glu). This method gives a good preservation of the native thylakoid activity and provides good protection against aging and strong illumination.

Thylakoid membranes and photosynthetic algae owing to their sensitivity to a number of pollutants they are frequently utilized for the construction of biosensors. These devices are based on the

electrochemical detection of the inhibiting effect by some pollutants on the photosynthetic activity of algae. In those cases, the reducing equivalents produced by the electron transport chain were detected amperometrically, and a decrease of electric signal indicated the presence of inhibitors [8,13-15].

In this study we present the use of two photosynthetic materials as biomediators for the realization of an amperometric biosensor suitable for herbicide detection. The approach is suggested by the fact that although the photosystems of green plants and algae appear to function using the same fundamental mechanisms, the molecular constituents are in part different. The photosynthetic materials used for this study were thylakoid membranes extracted from *Spinacea oleracea* and the *Chlamydomonas reinhardtii* algae cells.

## 2. METHODS

### 2.1 Photosynthetic material

*Spinacea oleracea* plants were obtained from the local market. *Chlamydomonas reinhardtii* cells were grown in liquid cultures acetate-phosphate medium. Liquid cultures were grown under continuous illumination from white fluorescent lamps ( $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) at  $25^\circ\text{C}$ . When required, this media was solidified with 1.5 % w/v agar.

### 2.2 Thylakoid membranes extraction

Thylakoid membranes were isolated from plant deveined leaves. Leaves were homogenised in a medium containing 20 mM tricine pH 7.8, 300 mM sucrose, 5 mM  $\text{MgCl}_2$ , 1 mM ethylenediaminetetraacetic acid and 0.2% w/v bovine serum albumin. The mixture was filtered through four layers of cheesecloth and centrifuged 20 min at 7500 g. The pellet was resuspended in a medium containing 20 mM tricine pH 7.8, 70 mM sucrose and 5 mM  $\text{MgCl}_2$  and centrifuged 20 min at 7500 g. The new pellet was resuspended in the same medium. The preparation was kept at a Chlorophyll concentration of 3 mg/ml and stored in liquid nitrogen. All steps were performed at  $4^\circ\text{C}$ .

### 2.3 Photosynthetic material characterization

Isolated thylakoid membranes and *Chlamydomonas reinhardtii* cells were tested by using the Plant Efficiency Analyser (Hansatech Instruments Ltd, UK) at room temperature to measure their activity. The  $F_v/F_m$  parameter is the maximum quantum yield

of the photosystem II and reflects the potential quantum efficiency as a sensitive indicator of plant photosynthetic performance. Chlorophyll content was calculated according to Porra et al. [16].

### 2.3 Immobilization of the photosynthetic material

The electrochemical biosensor has been prepared by immobilizing 2.5  $\mu\text{g}$  of the thylakoid membranes on the graphite (working) electrode's surface of a screen printed electrode. The immobilization was based on protocol of Touloupakis et al [8].

*Chlamydomonas reinhardtii* cells were immobilized on the working electrode using the following protocol; 13.5  $\mu\text{l}$  BSA 20% w/v, 13.5  $\mu\text{l}$  glutaraldehyde 1.5% w/v, plus Algae (50  $\mu\text{g}$  Chlorophyll) were mixed in a final volume of 50  $\mu\text{l}$ . 5  $\mu\text{l}$  of the mix were putted on the electrode surface.

### 2.4 Biosensor set-up

The experiment was realised under the following conditions: the graphite electrode has been polarized at +0.2 V vs reference electrode Ag/AgCl. The current intensity on the graphite electrode has been registered with a potentiostat (Metrohm 791 VA Detector, Herisau, Switzerland). The biosensor was mounted into a custom-made flow cell with illumination provided by a single red light-emitting diode (LED) (peak wavelength 650 nm, with light intensity of about 100  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Amperometric detection of the electric current generated by the photosynthetic material was measured upon 5 seconds of illumination by the red LED in the presence of an artificial electron acceptor (Fig 1).

The electrode has been continuously washed with the measuring buffer; 20 mM Tricine pH = 7.8, 70 mM sucrose, 15 mM NaCl, 5 mM  $\text{MgCl}_2$ , and 30  $\mu\text{M}$  2,6-dichlorophenol-indophenol (DCPIP) as electron acceptor [8]. The flow of the buffer (0.2 ml/min) was driven by a peristaltic pump (Watson Marlow 101U/R). All measures were made at room temperature.

The principle of herbicide detection is based on a decrease of the biosensor signal (decrease of electron transport rate). Firstly, the activity in the absence of herbicide is recorded. Then, the sample containing herbicide is loaded to the cell and the residual activity is registered after 10 min. The ratio of the signals in the presence and absence of herbicide was plotted against herbicide content in

the sample. Then, the herbicide was washed out using the measuring buffer and the biosensor used for a new measurement. Each concentration was tested in 2-4 independent measurements.

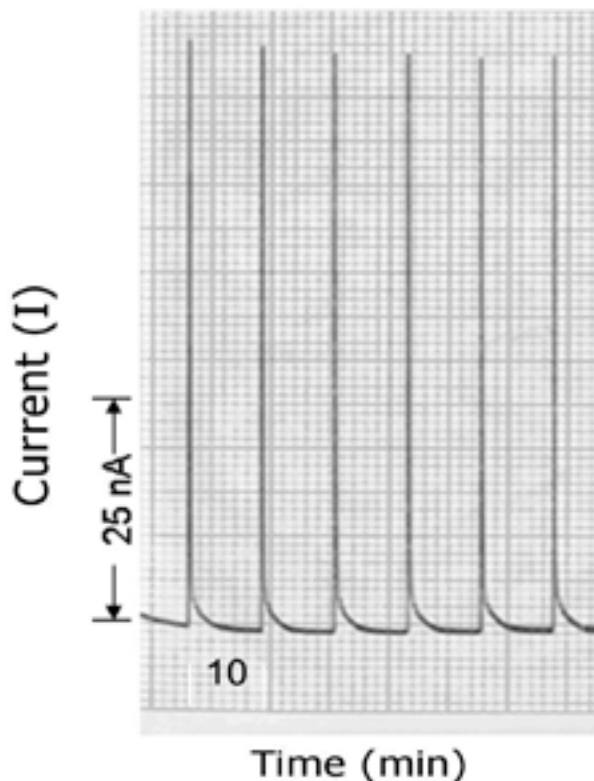


Fig. 1 Typical chronoamperometric measurements of the thylakoids membranes immobilized onto the screen printed electrode. The duration of light pulses was set to be 5 seconds with a frequency of 10 min.

## 3. RESULTS

Electron-transfer activity is detected by illuminating thylakoid membranes or unicellular algae in the presence of the artificial electron acceptor DCPIP. The reducing equivalents produced by the electron transport chain were detected amperometrically. The signal transduction process is accomplished by controlling the potential of the working electrode at a fixed value (relative to a reference electrode) and monitoring the current as a function of time. The applied potential serves as the driving force for the electron transfer reaction of the electro-active species. The resulting current is a direct measure of

the rate of the photosynthetic electron transfer reaction.

### 3.1 Detection of herbicides with immobilized thylakoid membranes

A ureic herbicide 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron) was analysed in the presence of 30  $\mu\text{M}$  DCPIP. Initially, the activity of the biosensor was recorded in the absence of the herbicides, then 4 ml of herbicide solution were loaded and the activity was recorded again. The ratio between these two measurements was determined. After each measurement, the herbicide was removed by washing the system with measuring buffer. Then the biosensor was ready to test another sample (Fig. 2).

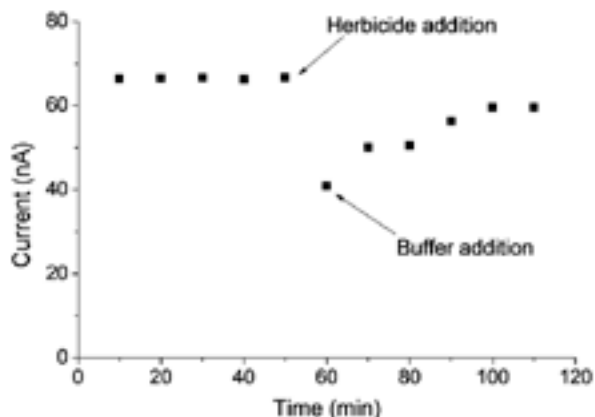


Fig. 2. Principle of herbicide detection. Firstly, the activity of the thylakoid membranes in the absence of herbicide is recorded. Then, the sample containing diuron ( $10^{-7}\text{M}$ ) is loaded to the flow-cell and the residual activity is registered after 10 min. Final, regeneration of the biosensor is obtained after washing with measuring buffer.

The  $I_{50}$  value (herbicide concentration causing 50% inhibition of the initial activity) for diuron and the detection limit are summarized in table 1. The detection limit was calculated for 10% inhibition. The half-life of the biosensor using was  $23.5 \pm 2.0$  hours.

Table 1.  $I_{50}$  dose and LOD determined for diuron utilizing immobilized spinach thylakoid membranes.

Herbicide	$I_{50}$ (M)	RSD (%)	LOD (M)
Diuron	2.6E-07	12.0%	7.0E-09

### 3.2 Detection of herbicides with immobilised *Chlamydomonas reinhardtii* cells

The first step was to test the stability and to confirm the possibility to detect photosynthetic herbicides with the immobilised algae cells. Immobilized algae are kept in a stable physiological state under biosensor operating conditions for at least 2 days (Fig. 3). The half-life of the biosensor was  $42.0 \pm 6.9$  hours. The immobilised algae cells kept at  $-80^{\circ}\text{C}$  are stable for up to 60 days. The  $I_{50}$  value (herbicide concentration causing 50% inhibition of the initial activity) for diuron and the detection limit are summarized in table 2. The detection limit was calculated for 10% inhibition.

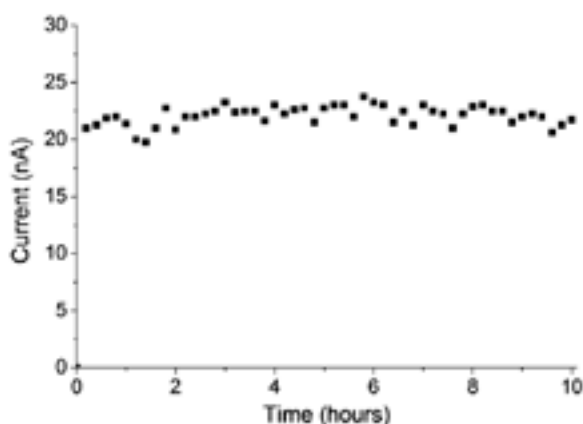


Fig. 3. Measurements of electron transfer of *Chlamydomonas reinhardtii* cells immobilized by BSA-GA on printed electrodes. The duration of light pulses was set to 5 s every 10 min.

Table 2.  $I_{50}$  dose and LOD determined for diuron utilizing immobilised *Chlamydomonas reinhardtii* cells.

Herbicide	$I_{50}$ (M)	RSD (%)	LOD (M)
Diuron	1.5E-06	15.0	1.9E-09

## 4. CONCLUSION

There is a big interest for the generation of rapid, inexpensive assays to screen the presence of herbicides. Only samples giving positive results would require laboratory analysis, and overall cost per sample would be significantly reduced. The most frequently-used biosensing systems for monitoring photosynthetic herbicides are based on photosystem II preparations and have been limited

by their instability, particularly upon illumination. A significant stabilization of photochemical activity has been achieved by the immobilization of cells or thylakoid membranes on the screen printed electrode surfaces.

In this work we have tested a sensitive biosensor for detection of classical photosynthetic herbicides. The biosensor was constructed using screen-printed electrodes with immobilized thylakoid membranes as the biorecognition element. Also immobilized *Chlamydomonas reinhardtii* cells were tested to assure higher stability of the biological part of the biosensor and for comparison with the immobilized thylakoid membranes. The activity was measured as a rate of the photoreduction of an artificial electron acceptor (DCPIP), which was registered as an increase in the response to light pulses. The close coupling of the biosensing element (thylakoids, algae cells) and the transducer (electrode) improved the measured signal and required smaller amount of the biological material in comparison with standard batch detection systems. The use of the flow-through set-up made it possible to re-use the biosensor for several analyses after washing out the inhibitor.

The obtained biosensor is able to reveal the presence of an inhibition effect on electron transport due to the photosynthetic herbicides in a concentration range which is useful for environmental analysis. The immobilized thylakoid membranes are stable for 23 hours and they show a good sensitivity towards different photosynthetic herbicides. Immobilized *Chlamydomonas reinhardtii* cells assured higher stability of the biological part of the biosensor showing a half-life of 42 hours.

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