DESIGN OF A PORTABLE INSTRUMENT FOR ENVIRONMENTAL APPLICATIONS BY AMPEROMETRIC MEASUREMENTS ON BIOLOGICAL MATERIAL

K.Buonasera[§*], I.Pezzotti[^], G.Rea[*], G.Pezzotti[^], M.T.Giardi[*]

[*] IC-CNR, National Research Council Institute of Crystallography, Research Area of Rome, Via Salaria Km.29.600 00016 Monterotondo Scalo (Rome), Italy;

[§] Corresponding author: katia.buonasera@mlib.ic.cnr.it.

[^] Biosensor S.r.l. V.le Tivoli Km.18.642 00018 Palombara Sabina (Rome) Italy
email: g.pezzotti@biosensor.it, www.biosensor.it

ABSTRACT

A portable instrument performing amperometric measurements for monitoring bioactive materials has been designed, manufactured and tested. It has been specifically designed to operate with a wide range of photoactive biosamples. The sensing chamber in the instrument features two different optical sources to detect the photosynthetical activity of plants (i.e. *spinacia oleracea*) and microorganisms (i.e. algae and cyanobacteria). The chamber is provided with screen-printed electrodes to measure the photogenerated current and with a fluidic system for the electrolyte transport. Photosynthetic electron transfer is activated by two LEDs (470nm and 660nm emission) in order to enable various excitation wavelengths and match several different biological materials. Target applications belong to the agro-food, pharmaceutical and biomedical fields. This paper describes some possible environmental applications.

Keywords: Amperometry, biosensor, screen-printed electrode, water monitoring

Recibido: 5 de Octubre de 2009. Aceptado 18 de Diciembre de 2009
Received: October 5, 2009 *Accepted: December 18, 2009*

DISEÑO DE UN INSTRUMENTO PORTATIL PARA APLICACIONES AMBIENTALES POR MEDICIONES AMPEROMETRICAS SOBRE MATERIAL BIOLOGICO

RESUMEN

El instrumento portátil optimizado para las medidas de amperometria, en el monitoreo de materiales bioactivos ha sido diseñado, fabricado y probado. Expresamente ha sido diseñado, para funcionar con una amplia gama de bio-muestras foto-activas. La cámara de medición del instrumento; destaca dos tipos de fuentes ópticas para detectar la actividad fotosintética de plantas (p. ej. spinacia oleracea) y microorganismos (p. ej. algas y cyanobacteria). En la cámara son inseridos los electrodos serigrafiados para medir la corriente fotogenerada, ademas cuenta con un sistema de flujo para el transporte del electrólito. La transferencia fotosintética de electrones, es activada por dos LEDs (470nm y 660nm de emisión), para permitir varias longitudes de onda de excitación para utilizarlos con diversos materiales biológicos. El objetivo de la aplicacion, es en campos como agroalimentario, farmacéutico y biomédico. Este Artículo describe algunas de las posibles aplicaciones ambientales.

Palabras claves: Amperometria, biosensores, Electrodos serigrafiados, Monitoreo de aguas

1. INTRODUCTION

Various types of transduction methods are used in biosensors to convert the biochemical signal into an electrical one: amperometric and potentiometric transducers are among the most commonly adopted [1]. The former have been widely used since the introduction of the Clark's oxygen electrode in 1953 [2], which is the base of the amperometric biosensors in use. Coupling enzyme-catalyzed electrochemical reactions to electrodes has been an attractive approach to develop sensors since the end of 1960s [3]. The first considerable commercial success was registered for glucose sensors with a market well past $\$10^8$/year for electrochemical sensors and $\$10^9$/year for all sensors in 1997 [3].

Portable electrochemical biodevices based on disposable screen-printed electrodes (SPE) technology have been developed and are available on the market for research, diagnostic and monitoring applications, such as the products offered by Alderon Biosciences (www.alderonbiosciences.com) and Palm Instruments (http://www.palmsens.com). They have some features in common with the bio-amperometer described in this article, with similarities on the use of SPE and photosynthetic biomediators for environmental monitoring applications.

The portable amperometric system described here is designed for general electrogenic biomaterials, with special features for the employment of photoactive biosamples used as biomediators. The biodevice presented is a complete small size and weight analytical instrument, where the biological species have been immobilized on a screen-printed working electrode by a proper chemical deposition technique, and integrated with an innovative miniaturized measurement cell, an automatic fluidic and electronic control system and an optical module for light excitation. All components are described and shown in the following sections and the immobilization procedure adopted for the production of a stable biomediator layer is also presented. The targeted applications belong to the agro-food, pharmaceutical and biomedical fields. In particular, in this work, results in environmental detection are reported, with experiments conducted on polluted river water samples.

2. DESIGN AND FABRICATION

The miniaturized system realized is innovative, being designed to be flexible, modular and small, with two independent not-interfering sensing cells equipped with optical excitation, current measurement system and flow control system

2.1 Sensing Mechanism

For the present study, various biological materials based on the protein complex known as photosystem II (PSII) were used to prepare the biomediators. The core of the PSII is the reaction centre (RC). In the RC, the absorption of a quantum of light by the primary donor chlorophyll dimer P680 raises an electron from the ground state to the excited state, from which it can pass to the primary acceptor Q_A [4]. This fast and directive electron transfer inside the biomolecule can be transduced externally into an output current, measured between the screen-printed working electrode and the so called counter electrode. The electrical neutrality is guaranteed by an electrolyte buffer solution or by the sample itself according to the specific application: its ions migrate under the action of an electric field, produced by an applied small voltage polarization, and compensate the charge reduction locally induced by the electrochemical process. The current signal can eventually be amplified or converted into a voltage signal for easier processing and data analysis.

The sensing mechanism is based on the total or partial inhibition of the electron transfer due to the presence of a chemical or physical-chemical environmental conditions reacting either by direct binding to the RC-complexes or by changing the equilibrium of the local environmental chemistry. In particular, when pollutants such as pesticides are present in the sample solution and come into contact with the photosystem, they inhibit the primary acceptor along the photosynthetic chain and partially or fully block the electron transfer [4, 5]. Consequently, the current output level decreases according to the pollutant concentration.

2.2 Biosensor Description

The biosensor is based on an innovative measurement cell where biological material, excitation light source, electrodes and flow are integrated in a compact miniaturized sensor (30mm external diameter, 25mm height). Fig. 1 represents a schematic cross section of the biosensor, which helps to explain the main features. The biological

chamber dimension is given by the O-ring thickness, resulting in a volume of 4-6 µl. Such a small volume makes the flow exchange very fast and consequently the biochemical reactions are improved, thus increasing the response time of the sensor. The biosample is deposited onto a disposable screen-printed graphite electrode. This is held in place by a screw-type adjustment system. The measurement cells are designed to work with different sized SPEs and are made of an inert material (light acetal resin) featuring low friction and high temperature resistance (max 90°C).

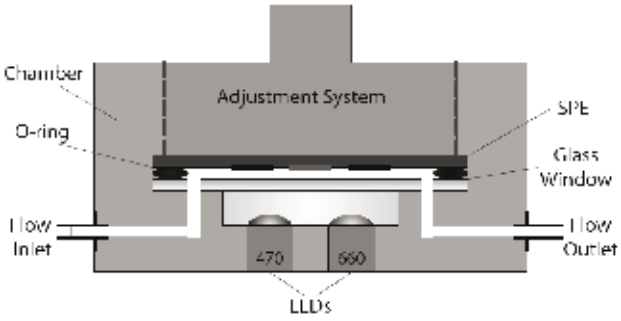

Fig.1. Schematic cross section of the biosensor equipped with two excitation LEDs at the bottom, screen-printed electrodes (SPE) deposited with the photosynthetic biomediator (green area) and a flow system.

The optical module at the bottom is necessary for the electron transfer excitation, considering the photosynthetic nature of the biosamples. Fig. 2 shows a picture of the open cell before inserting the SPE. Two LEDs with emission wavelengths of 470 and 660 nm are used in order to provide a flexible excitation for different biologic compounds and to target the maximum photosynthesis rates corresponding to the light absorption peaks in the spectra of the antenna pigments (chlorophyll *a*, *b* and carotenoids) (Fig. 3) [10]. The intensity of the LEDs in the two cells of the instrument are shown in Table 1. They have been measured at room temperature by the LI-COR Biosciences LI-250A Light Meter, which uses a high stability silicon photovoltaic detector (blue enhanced) with 0.4% accuracy.

A glass window separates the optical compartment from the biological chamber. The electrolytic solution flows, into this chamber, not directly but through three small holes in the glass window in order to preserve the biomaterial from physical damage or detachment from the support medium and the electrode.

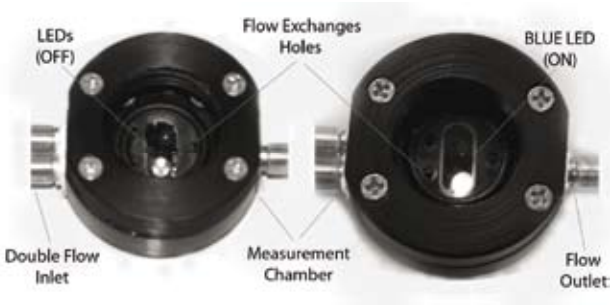

Fig.2. Image of the biosensor cell with one of the two LEDs on. Two flow inlets are provided for mixing solutions or administrating stimuli.

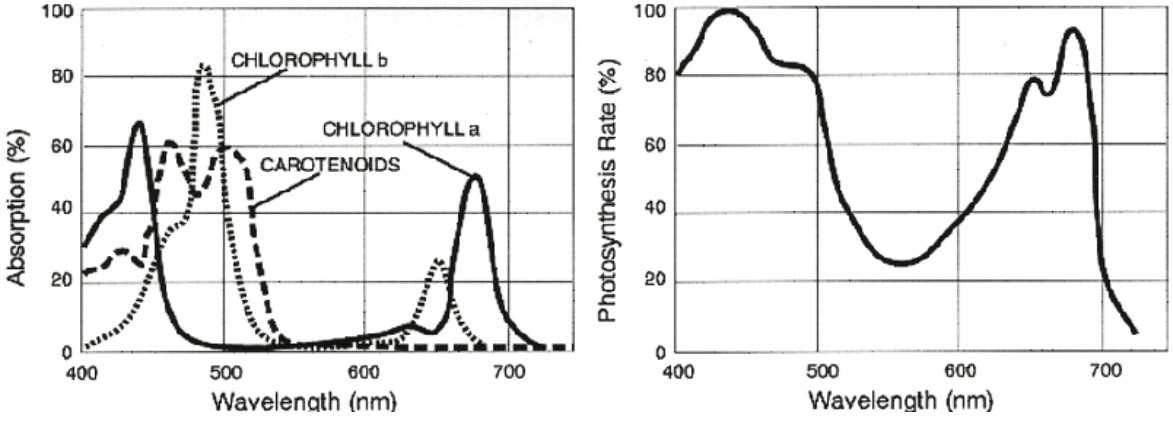

Fig.3. Left: light absorption spectrum of the most common antenna pigments of the photosystem; Right: Photosynthesis rate spectrum [9].

Table 1. LED specifications and measured characteristics

LED	Nominal intensity	Nominal peak emission wavelength	Measured quantum intensity	Measured photometrical intensity
	[mcd]	[nm]	[µmol/s·m^2]	[lux]
Blue	1500	470	4.47	18
Red	1400	660	52	220.1

The electrolyte solution and analyte flow are supplied in a parallel mode to the two measuring cells by a rotary peristaltic pump with adjustable speed: it can be modified from a minimum value of 200 µl/min up to a maximum of 350 µl/min. The fluidic system is completely automatic. In the,table 2 are describes the technical specifications of the bio-amperometer.

Fig. 4 shows a picture of the complete assembled bio-instrument (dimensions 22×15×10 cm, weight 1409 g).

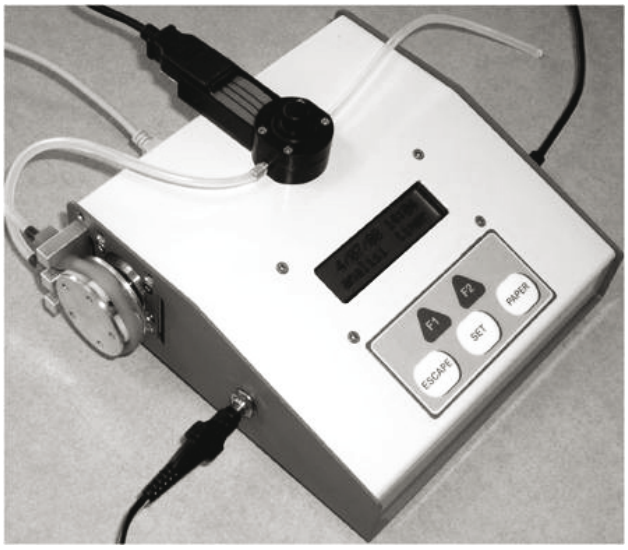

Fig.4. Photograph of the assembled bio-amperometer showing the flow cell with the electrode lodged in to perform the analysis.

Table 2. Additional technical specifications of the bio-amperometer.

Display	LCD, 2 rows × 16 columns
Keyboard	Digital, five-keys equipped
Light source excitation	Time-controlled (precision of 1 second)
Reading system	Two indipendent channels
Reading velocity	1 byte/s
Reading time	1 s
Interface	USB
Results	Output signals are converted into frequency domain for a better onboard data management; real-time readable on PC-display
Date/Timer	Date/time associated with the running analysis; photoperiod function (to modulate and set the excitation light interval); pump and measurement timer to set the flow and analysis time interval
Power supply	230/115 VAC, 50-60 Hz, 110 W

3. IMMOBILIZATION PROCEDURE

RC-complexes used in this study were extracted from cyanobacteria (*Synecococcus elongatus*), algae (*Chlamydomonas reinhardtii*) and higher plants (*Spinacea oleracea* and *Medicago sativa*). The aim of using different organisms was to consider any different responses to oxidants in terms of resistance. In fact, although the RC of green plants, algae and cyanobacteria appear to use the same fundamental mechanisms of energy transfer, primary charge separation, electron transfer and charge stabilization, the resistance to oxidants can be partially different. One purpose of this study was to increase the stability of the biomediator towards oxidants through the use of various immobilization procedures summarized in Table 3.

The production of RC hybrids with organic and inorganic compounds has been implemented by oriented immobilizations of RCs onto solid surfaces. In order to evaluate the suitability of the immobilization procedures in terms of stability and

lifetime on the SPE, the PSII oxygen evolution was measured with a Clark's electrode immediately after immobilization and after 15 days storage in the dark. The best techniques allowing most of the PSII activity to be preserved, involved the use of magnetic beads with polymers and BSA with glutharaldeide. After keeping the hybrid components for 360h in the dark at 5°C, the PSII activity strongly decreases till becoming absent in 50% of the reported cases. An effective storage can only be achieved through freezing.

Table 3. PSII activity measured as oxygen production in the Clark's electrode after immobilization and after 360 h of storage in the dark at 5°C (Nr: not revealed).

Immobilization procedure	***PA → After immobilization**	***PA → After storage**
BSA-glutaraldehyde	68±5	16.3±2
Urethane polymer -BSA	50±6	15±3
Gelatine-glutaraldehyde	45±3	Nr
Alginate gel	22±4	Nr
Magnetic beads-polymers	70±3	14.6±4
Photo-crosslinkable resin	19±3	Nr

*PA: % PSII activity

The used SPEs feature an Ag/AgCl reference and graphite working and counter-electrode. Fig. 5 shows a picture of the SPEs.

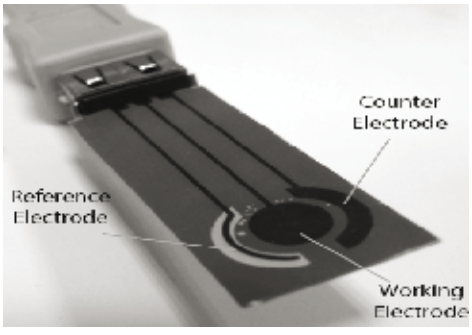

Fig. 5. Photograph of the SPE used in this work.

4. EXPERIMENTAL RESULTS

This work employed photosynthetic protein complexes as biomediators. Photosynthetic proteins exhibit the ability of binding and specifically recognizing environmental chemicals able to produce deep changes in their physiological activities, such as a variation in the light-induced electron transfer across lipid membranes occurring during the photosynthesis process [4]. In nature, many chromophore molecules, including chlorophylls, pheophytins and quinones, are arranged in reaction centres which maintain the same properties exhibited by the whole photosynthetic photosystem (PS), like the rapid and unidirectional electron transfer. They are able to generate supramolecular and self-assembling structures and, hence, are natural nanostructures. Therefore, even an individual protein molecule in the RC is a sophisticated molecular device. The possibility of changing and monitoring the photosynthetic activity of photosystems and RCs' subcomponents promoted the concept of innovative biosensors based on amperometric transduction where the biological material is coupled with electrodes. Previous works from some of this paper authors report the fabrication of biosensors employing photosynthetic organelles or cells deposited on screen-printed electrodes for pesticide detection in environmental applications [5, 6, 7] Tamura et al. in 1991 explored the feasibility of photosynthetic electrochemical cells based on the above mentioned electron transfer occurring in chromatophores extracted from *Rhodopseudomonas viridis* and deposited onto SnO_2 electrodes [8]. For the same application, Lam et al. in 2006 developed a technique for immobilizing thylakoids membranes onto a functionalized gold electrode with the objective of improving the electron transfer efficiency [9].

The application chosen to validate the sensor through experimental testing, and here described, belongs to the field of environmental monitoring for measuring pollutant content in water samples. By adding the pollutant agent, the photosynthesis electron transfer will decrease or will be totally inhibited by the reaction with the pollutant molecule and consequently the output current will decrease with respect to the calibration current observed in presence of the buffer solution. The amount of the registered current variation can be directly correlated to the pollutant concentration: the bigger the variation the higher the concentration (Fig 6).

The experimental set-up is constituted by the bioamperometer, a DC voltage supply and a computer. A voltage of 250mV is applied between the central working and the reference screen printed electrodes. The output current is measured between the working and the counter-electrodes through two independent recording channels (one per cell) and a data acquisition system controlled by the central microcontroller unit (Atmel AT89C51ED2). The microcontroller is also in charge of driving the LEDs and the pump and adjusting light and flow intensity. A small keyboard and display are provided to enter the measurement settings and read data in real time. A RS232 serial gate with conversion to USB provides the interface to the computer, where data are displayed and elaborated.

Experimental measurements aiming at calibrating the system with known concentrations of pollutants have been performed with different PSII-based biomediators. The experimental procedure measured and calculated the ratio of the current signals in the presence and absence of different pollutant concentrations in the sample. Sensitivity and detection limit have been calculated for the biomediators employed that exhibit different sensitivity and selectivity. It is possible to regenerate the electrode by washing it out using the measuring buffer.

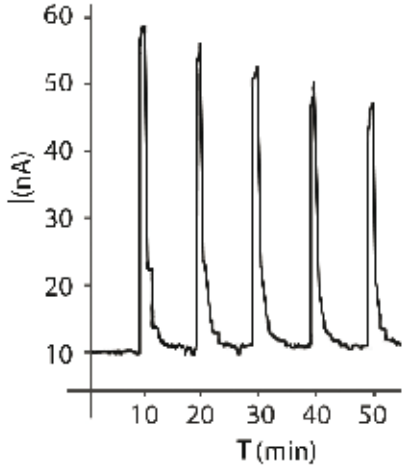

Fig 6. Amperometric measurement performed in static mode on thylakoids from *S. oleracea* immobilized with BSA-GA on a screen-printed electrode. Atrazine is added at intervals of 10 min at 4 different concentration (10^{-9}, 5×10^{-9}, 10^{-8} and 5×10^{-8} M) starting from 20 min. *Buffer* (pH 7.2)*:* tricine (20 mM), $MgCl_2$ (5 mM), sucrose (70 mM), NaCl (50 mM); *Mediator:* DCPIP (30 µM); *Dark time:* 10 min*; Light time:* 5 sec (by red LED at 650 nm, 1500 mCd); *Instrument:* AMPBIO-SPE SS LIGHT*; Potential:* + 0.200 V; *Current:* 100 µA.

5. CONCLUSIONS

There is an increasing demand worldwide for low cost, fast and reliable methods for monitoring chemical species. Biosensors offer all these advantages since they can be easily used both in laboratory and field applications. The objective of this work was to build a biosensor which can be easily adopted for the electrochemical analysis of real-world samples containing compounds able to interfere with the photosynthesis. The most significant innovations brought about are in the small volume measuring chamber, the integration of a compact light excitation module and the system automation, fluidics included. The novelty of this miniaturized instrument is its high versatility since, depending on the biomolecules used and immobilized on the electrode (e.g. DNA, RNA, proteins), different biodevices can be developed for different applications.

6. ACKNOWLDGEMENTS

The portable instrument construction was supported by MIUR (Italian Ministry of University and Research) within the “Biodiserba” Project for agricultural monitoring applications.

7. REFERENCES

[1] M. Chaplin, C. Bucke, *Enzyme Technology*, Cambridge University Press, 1990.

[2] L.C. Clark, R. Wolf, Granger D., Z. Taylor. Continuous recording of blood-oxygen tensions by polarography *Journal of Applied Physiology* 6 (1953). p.189-193.

[3] P. Kissinger**,** *Report Bioanalytical Systems Inc.*, 16 (3) (1997).

[4] M.T. Giardi, E. Pace. Photosynthetic proteins for technological applications *TRENDS in Biotechnology*, 23 (5) (2005). p.257-263.

[5] M. Koblizek, et al. A biosensor for the detection of triazine and phenylurea herbicides designed using Photosystem II coupled to a screen-printed electrode *Biotechnology and Bioengineering* 78 (1) (2002). p.110-116.

[6] E. Touloupakis, et al. A multi-biosensor based on immobilized Photosystem II on screen-printed electrodes for the detection of herbicides in river water *Biosensors and Bioelectronics* 20 (2005) p.1984-1992.

[7] K. Buonasera, G. Pezzotti, V. Scognamiglio, A.Tibuzzi, M.T. Giardi. New Platform of Biosensors for Pre-screening of Pesticide Residues To Support Laboratory Analyses. *J. Agric. Food Chem.*, (DOI:10.1021/jf9027602) enable online since Dicember 18 2009.

[8] T. Tamura, et al., *A photocell based on a high concentration of chromatophore Bioelectrochemistry and Bioenergetics* 26 (1991) p.117-122.

[9] K.B. Lam et al. Bioelectrocatalytic self-assembled thylakoids for micro-power and sensing applications *Sensors and Actuators B* 117 (2) (2006). p.480–487.

[10] G.S. Singhal, G. Renger, S.K. Sopory, K-D. Irrgang, Govindjee *Concepts in Photobiology: Photosynthesis and Photomorphogenesis*, Narosa Publishers and Kluwer Academic.