

Transduction methods used on biosensors: amperometry and fluorescence

Métodos de transducción usados en biosensores: amperometría y fluorescencia

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Abstract

Biosensor devices have applications in a variety of fields as environmental analysis, biomedical, bio-defense, food and agriculture. On this kind of sensors, a biological material (known as biomediator) reacts with target analytes and an appropriated transduction system converts that reaction to an electrical signal that can be processed, saved and transmitted by using electronic systems.

In this article, two transduction methods used for biosensing applications are described: amperometry that is based on the measurement of the electron transfer occurring inside the biomediator and fluorescence, that is based on the measurement of the re-emitted light. Emphasis has been done on the electronics design, including component selection, useful circuit topologies and common problems and solutions. Electronics has been validated for the development of biosensor-based instruments characterized by low production costs and portability.

-----**Keywords:** Biosensors, amperometry, fluorescence, transduction methods, electronic design

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Resumen

Los biosensores tienen aplicación en una gran variedad de campos, incluyendo el análisis ambiental, biomedicina, biodefensa, alimentación y agricultura, entre otros. En este tipo de sensores, un material biológico (conocido como biomediator) reacciona con el analito y un sistema de transducción apropiado transforma dicha reacción en una señal eléctrica que puede ser procesada, almacenada o transmitida usando sistemas electrónicos.

En este artículo, se describen dos métodos de transducción usados en aplicaciones bio-sensoriales: la amperometría que consiste en la medida del transferencia electrónico (corriente) del biomediator y fluorescencia que es basada en la medida de la luz re-emitida. Se enfatiza en el diseño electrónico (selección de componentes, topología de los circuitos, problemas comunes y soluciones). Estos diseños han sido utilizados en el desarrollo de instrumentos comerciales para biosensores, caracterizados por bajos costes de producción y portabilidad.

-----*Palabras clave:* Biosensores, amperometría, fluorescencia, métodos de transducción, diseño electrónico

Introduction

Bio-sensors devices use a biological recognition element known as bio-mediator. This biomediator can be composed by isolated enzymes, immunosystems (antibodies), tissues, cells or cell organelles capable to produce an output signal in response to a biochemical variation caused by particular environmental, chemical, or physical conditions [1].

Biomediator's output signal can be of different types. Some biomediators produce an optical signal (fluorescence, chemiluminescence), others change their mass or their electrical properties (voltage, current, impedance), or produce temperature or pH changes. The output signal must be converted by a suitable transducer into an electrical signal that can be easily processed, registered or transmitted by an electronic system.

Bio-sensors application includes different fields, such as environmental protection, medical analysis, alimentary and safety, and are becoming commercial realities in applications where low cost and ease of use are important [2]. This article focuses on two types of biosensors: those based on amperometry and those based on fluorescence detection.

Amperometric biosensors are very often found on literature. Glucose sensor for diabetes control (using an enzyme as bio-mediator) is the most known biosensor of this type [1]. Amperometric sensors has been also proposed for herbicide detection on water by using enzymes [3] or photosynthetic material (algae) [4, 5].

Fluorescence markers are used on DNA-based biosensors [6], or to detect the enzyme activity. Sometimes auto-fluorescent substances are also used on fluorescence biosensors, as is the case of photosynthetic organisms [4] and some bacteria [5].

The two transduction systems here investigated are described focusing on electronics. The two systems were used on the development of real, commercial biosensor instruments, characterized by low production cost, small size and portability.

Amperometric transduction

Amperometric detection consists on the measurement of current generated by the reduction/oxidation processes of electro-active substances in intimate contact with an electrode system. In order to allow this processes to happens, an appropriated potential must be set

between the electrodes, and the correct potential depends on the characteristics of the reaction occurring and the electrode's materials.

Two types of configurations are commonly used in amperometry for the electrodes: two and three electrodes systems. A two electrodes system is composed by a reference (RE) and a working (WE) electrode, and, in this case, the current is measured on the working electrode (I_{rw}) whereas the potential is set between working and reference electrodes (V_{rw}) (Figure 1, up).

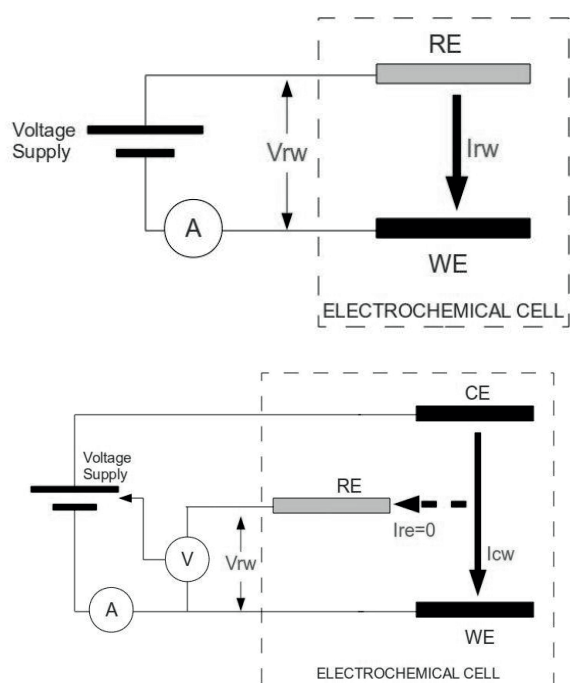


Figure 1 Two and three electrodes amperometric systems

This configuration has problems on potential stability. The current flowing through the reference electrode leads to ohmic voltage drops and material polarization problems. To overcome those issues, the three electrodes configuration is commonly used (Figure 1, down).

On three electrodes systems, an auxiliary electrode (also known as counter electrode, CE) is used. The current will flow between the counter and working electrodes (I_{cw}), while

the reference electrode circuit presents a high impedance to block the current flow ($I_{re}=0$). To set the reference electrode potential a special control circuit is necessary (potentiostat circuit on Figure 2).

The electrodes systems for biosensors are normally implemented on screen-printed electrodes (S.P.E) [7]. These are built by the deposition of different "ink" layers over a PVC, ceramics or aluminum base. There are one or more layers of electrical conductive surfaces for electrodes conformation and a contact line for external electronics interface. S.P.E's main advantages are low cost (intended to be disposable), easy automated production and small size.

Figure 2 shows the electronics circuit implemented for amperometric detection on three electrodes S.P.E. This circuit is discussed on the next paragraphs.

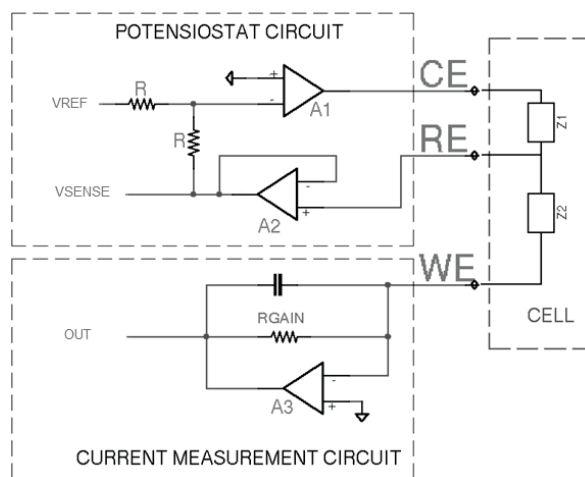


Figure 2 Circuit for amperometric measurements

Current Measurement

The current measurement circuit for the working electrode has been implemented by using an operational amplifier on transconductance amplifier configuration (Figure 2, down, A3 amplifier). This configuration guarantees a potential near to zero on the working electrode (affected only by the operational amplifier input

offset voltage and finite gain) that is important for precision of electrochemical potential (V_{rw}) [8].

Considering that the input current on to the amplifier inputs is zero, the measurement current travels through the RGAIN resistor, giving an output voltage of minus RGAIN times measurement current. A feedback capacitor, in parallel with RGAIN is necessary for stability reasons (especially if large gains are desired) and forms a single pole low pass filter.

In biosensor applications, the intensities are normally in the range of some nano-ampere up to tenths of micro-ampere. These low currents require special considerations to obtain accurate measurements:

- The operational amplifier must be characterized by low input bias current (I_{bias}), this bias is subtracted (or added depending on his sign) from the WE current before being amplified, becoming an important error source. By this, F.E.T (field effect transistor) based amplifiers are recommended for this type of applications. It's also important to control the I_{bias} variation with temperature when choosing the right amplifier.
- As already discussed, low input offset amplifiers are required. The electrochemical potential can be low as few tents of millivolts for some applications. By this amplifiers with input offset lower than one millivolt or with an offset adjustment mechanism are recommended.
- Special attention must be taken on the printed circuit board (P.C.B) layout. Current losses trough board isolation are possible if "high" voltage signals crosses near to the amplifier input (depending on measurement range, isolation between signals and distance is possible to have considerable losses with voltages as low as five volts). Use of guard rings is recommended. Proper P.C.B distribution is necessary, digital and switching signals must be keep away from low current signals. Supply line

decoupling, star ground and ground planes are important [9, 10].

- Residues of soldering process can lead to current losses, appropriated cleaning is advised [8].
- Induced noise from AC power and other electromagnetic devices is always present. Shorter cable (and PCB tracks) length and proper cable shielding (in some cases even amplifier shielding) helps to mitigate this.

Potensiostat

Potensiostat circuit must keep the required reference potential on three electrodes systems (Fig, upper, A1 and A2 amplifiers) [11]. The potenziostat circuit uses the high input impedance of operational amplifier (A2 on figure 2) to block the current flow through the reference electrode. The A2 amplifier is configured as voltage follower and his output can be used to monitor the actual status of the reference potential (V_{sense} signal).

The V_{ref} input is used for setting the desired reference potential (from a digital to analog converter). If the A1 amplifier is on his linear region (possible only if exists a feedback path between RE and CE electrodes $-Z_1-$, due to the chemical substances on contact with the electrodes) is possible to demonstrate that the voltage on RE electrode is kept equal (with opposite sign) to the V_{ref} signal.

Proper potenziostat operation strongly depends on the conditions of the substances on contact with the electrodes (on Figure 2 modeled as Z_1 and Z_2 impedances). A poor conductivity between CE and RE or/and an excessive WE current will require a higher CE to RE voltage and can put the A1 amplifier on saturation condition. Also amplifiers stability is not guaranteed because feedback impedances are unknown. By this a constant monitoring is necessary on V_{sense} signal, warning the user when the potenziostat is out of operation in order to modify the analysis conditions.

Considerations done for current measurement are also applicable to this circuit. The RE electrode signal is particularly sensible to coupled noise due to the higher input impedance, by this additional shielding on this signal is recommended.

System Integration

Development of an integrated, low cost, portable instrument for amperometric detection has taken as basis the circuits described on Current Measurement and Potentiostat.

A digital to analog converter has been used to generate the voltage reference for the potentiostat circuit. This converter can be configured (by the central processing unit) to generate a constant DC voltage (necessary on current vs time analysis –chronoamperometry-) or a different waveform (by example a triangle waveform used on cyclical voltammetry analysis). This converter has 12 bits resolution and a conversion rate of up to 1M samples/second.

Instead, an analog to digital converter is used to sample the Vsense signal and the output of the current amplifier. This converter is 16 bits resolution and up to 64ksample/s (Figure 3).

Finally, the control and data processing is carried out by an 8-bit Atmel microcontroller (C51 family). A friendly user interface -trough graphical LCD and keyboard- and a PC communications interface were also provided. The interconnection of the different electronic systems is shown on figure 3.

From a mechanical point of view, the instrument consist in two measurement cells, each one with space for S.P.E insertion and flow lines for sample and measurement buffers. The cells are also provided with light sources (Light Emitting Diodes, LEDs at 670nm and 470nm wavelengths) for stimulation of photo-sensible materials.

Transduction system validation

Validation of the potentiostat and current measurement circuits was conducted by connecting these to a dummy load circuit (Figure 2). This circuit guarantees the feedback path necessary for proper potentiostat operation. It consists of two impedances: Z1 connected between CE and RE and Z2 connected between RE and WE. Z1 is a resistor in parallel with a capacitor (capacitance is necessary for stability reasons) while Z2 is pure resistive.

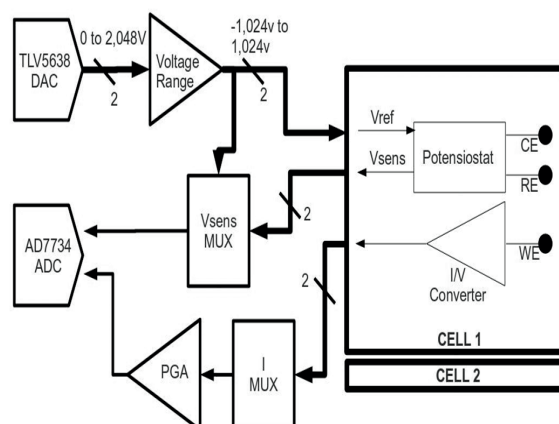


Figure 3 Portable amperometric instrument and diagram of his electronic systems

Validation of the potentiostat circuit was done by setting -on the user interface menus- a desired potential and measuring the true voltage between the reference and working electrodes. That measurement was conducted using a Keithley 2700 Digital Multimeter (DMM)/data acquisition system. Figure 4 (up) shows the data points obtained and his linear regression. Offset and gain calibration factors were software implemented on the instrument to diminish the effect of electronics tolerances.

Validation was also conducted on current measurement. To do this, Z2 was replaced by a variable resistor (in order to vary the working electrode current while maintaining a constant reference electrode potential) and the current was measured using the Keithley DMM (in series with the working electrode) and the instrument itself. Figure 4 (Down) shows the plot of the obtained data, with the measurement of Keithley DMM on X axis and instrument on Y axis.

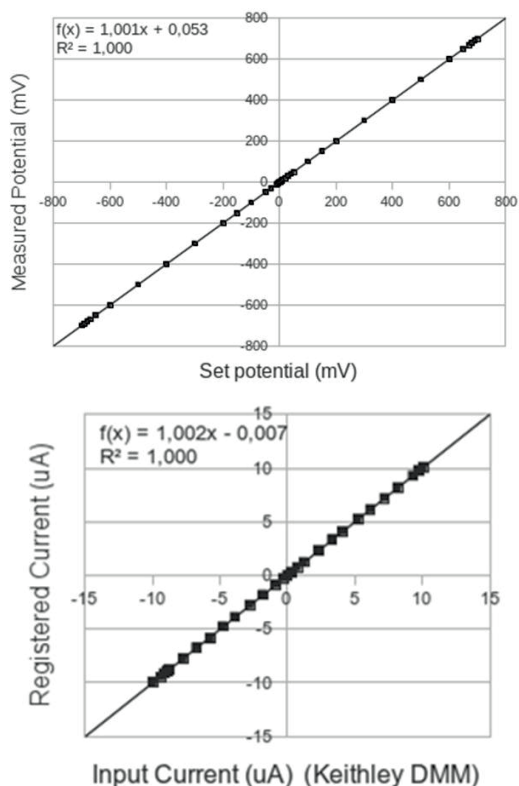


Figure 4 Potential and current measurement calibration data

Linearity and accuracy shown on instrument potential and current allows using it on real sample measurements, as illustrated on next paragraphs.

Real sample instrument validation

Instrument operation was validated using photosynthetic bio-mediators immobilized on screen printed electrodes, an unicellular green algae (*Chlamidomonas reinhardtii*) was used [12].

Operation whit this type of bio-mediator is based on successive dark/light periods. During the dark period the biological material enters on relaxation status, after that it's stimulated by a light pulse producing an electronic transport due to the photosynthesis process. This process is repeated cyclically.

In the dark period exists a constant "base line" current on the working electrode. During the light time, the current rises to a peak value and then decreases until returning to the base line level when the light is turned off. That light-induced current peak depends on photosynthesis efficiency and if an external factor (by example a photosynthesis blocking herbicide) is stressing the bio-mediator the current peak will be lower.

Thus, measuring the magnitude of the current peak (peak minus baseline) when the bio-mediator is in non stress conditions (I_{buffer}) and when is in contact with a sample (I_{sample}), then calculating the inhibition percentage (Eq. 1) is possible to obtain an estimate of herbicide presence on sample [12].

$$INH\% = \frac{I_{buffer} - I_{sample}}{I_{buffer}} \cdot 100\% \quad (1)$$

Tests consisted on a mutant of *C. reinhardtii* algae exposed to different concentrations of linuron and simazine herbicides [4]. Results are shown on the table 1; it shows how the inhibition percentage increases with herbicide concentration as expected.

Table 1 Inhibition percent obtained in test with different pesticide concentrations

<i>Sample</i>	<i>Inhibition</i>	<i>Sample</i>	<i>Inhibition</i>
Pure water	0%		
Water+Linuron 10-8M	20.2%	Water+Simazine 10-8M	9.8%
Water+Linuron 10-7M	49.2%	Water+Simazine 10-7M	18.1%
Water+Linuron 10-6M	78.5%	Water+Simazine 10-6M	36.5%

Fluorescence

A fluorescent element absorbs light from an external source and re-emits part of it. The re-emitted light has a longer wavelength than excitation light. Different elements are fundamental on a fluorescence measurement system:

Excitation light source: This element must generate light of appropriated intensity and wavelength in order to stimulate the fluorescence process. It's important that the light intensity be as constant as possible and (for some applications) fast turn-on times are desired.

Optical filter: A long-pass filter is put above of the light measuring element. It must block light of lower wavelengths (the excitation light) while allows to pass light of higher wavelengths (the fluorescent material's emitted light).

Detector: This is the transduction element that converts the incident light on an electrical variable.

Cell body: A suitable cell body must isolate the material under measurement of the environmental lights interference (non necessary on pulsed fluorescence applications [13]). Also sample loading / unloading mechanisms (as micro-fluid systems) are necessary.

The parameters of interest on fluorescence measurements can vary depending on application. On some application the interest is only on the presence / absence of fluorescence, in others the steady state or final value [14], sometimes is necessary to capture the entire fluorescence transient from start to end of the light pulse [15].

Fluorescence Transduction

Light measurement on fluorescence applications requires a careful selection of detector device and design of associated electronics.

Different types of photodiodes (conventional, avalanche (APD), PIN), photo-transistors and photo-multiplier tubes are valid options as light transducers. Selection with depend on allowed fabrication costs, size and required response speed and sensitivity of the system.

Photo-multiplier tubes (PMTs) provides a very high sensitivity, internal gain, low noise and fast response, but his main drawbacks are high cost, large size and specific operational conditions (high voltage bias, sensibility to magnetic and electric fields, requires special handling considerations). By this are more suitable for high precision laboratory applications than for low cost portable applications.

Avalanche Photo-diode has also an excellent sensitivity and internal gain but requires a high voltage supply. Conventional photo-diodes (PN and PIN types) doesn't have such larger sensitivity and don't provides internal gain, but are easy to operate with low voltage supplies, are low cost and small size making them ideal for portable equipment.

Photo-diodes acts by converting incident light on current. The transfer relationship (sensitivity: amperes per watt of incident light) depends on light wavelength. During design, the emission wavelength of the bio-mediator must be taken into account for the selection of the right photo-diode.

Photo-diode response speed is an important factor on applications where interest is on fluorescence transient. On this cases use of PIN photo-diodes is recommended. Speed depends on internal diode capacitance that can be further reduced by using a reverse bias [16].

Figure 5 shows a circuit used for photo-diode conditioning based on an operational amplifier in trans-resistance configuration. This configuration has already been discussed on Current Measurement title.

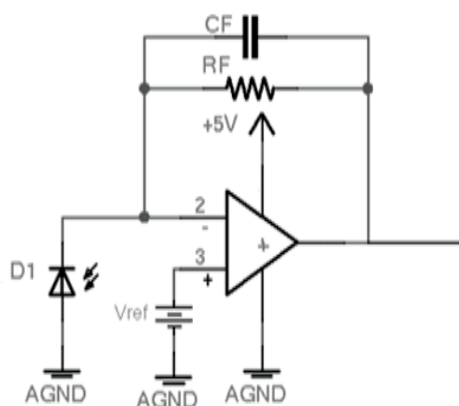


Figure 5 Photo-diode's signal conditioning circuit

The Vref voltage supply function provides a reverse bias on the photo-diode, given the fact that the voltage on the inverting and not inverting inputs of the operational amplifier are the same while on linear operation. Reverse bias is mandatory when using PIN type photo-diodes.

Stability is a critical issue on figure 6's circuit. Photo-diode internal capacitance acts on the inverter input of the operational amplifier and is a potential source of instability. Stability analysis must take into account the dynamic properties of the operational amplifier (normally modeled as a one pole low pass system) the feedback path (R_f and C_f) and the photo-diode capacitance [17].

High values of feedback capacitance helps to stabilize the system, but decreases the bandwidth producing slower responses. This is an issue on

transient fluorescence analysis. Figure 6 shows PSPICE simulation results for the figure 5 circuit. It shows the transient response at a photo-diode current step (ie: a light suddenly turning on) at time 1 ms. Simulation has been built using LTC6081 operational amplifier model from Texas Instruments, a gain of one million (1Mohm), and a feedback capacitance varying from 1pF to 15 pF. The photo-diode simulated was the Osram BPW34 which model was built using data-sheet information.

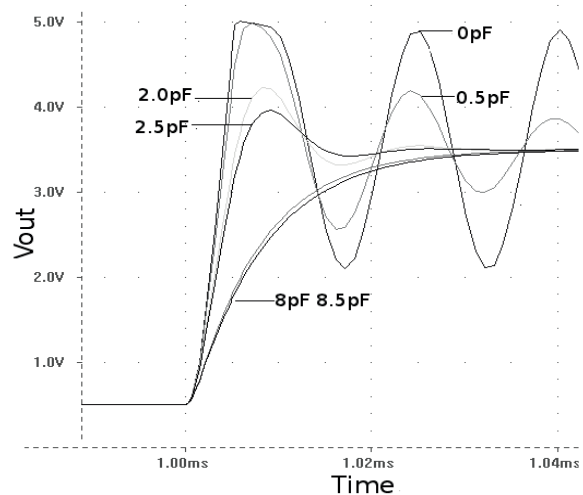


Figure 6 Simulation results, transient response at a current step (for different C_f values)

Simulation confirms critical importance of feedback capacitance on stability and response time. At lower values of C_f the system is near to instability and tends to oscillate showing also a considerable over-peak, at higher values the system response is slower but over-peak is not present.

System integration

Circuit described on previously had been implemented on a multi-cell instrument for transient fluorescence detection (Figure 7) [18].



Figure 7 Prototype Instrument for fluorescence measurements, single six cells module

This instrument is composed by up to four modules of six cells each. Each cell uses four leds as excitation light sources (red led 660nm) and the optical filters are set for fluorescence detection at the 730nm spectral range. Target biomediators are photosynthetic as explained on next paragraph.

The photo-diode conditioning circuits (one by each cell) are connected at a multiplexing system in charge of measurement cell selection. The multiplexers output feeds (through appropriated ADC drivers) the input of a high speed / high

resolution analog to digital converter (up to 1MSPS and 16 bits).

Also, controlled current, programmable led drivers are used to manage the excitation light intensity. A SD memory card allows results data saving. An USB communications port allows PC control and a user interface is available through a graphical LCD and alphanumeric keyboard. The instrument control is on charge of a 32 bits microcontroller, STMicroelectronics STM32 series, ARM CortexM3 core. This microcontroller provides enough computing power for fast data acquisition and real time data compression.

Instrument validation

Instrument has been validated on applications with photosynthetic bio-mediators. Transient fluorescence on photosynthetic materials is also known as Kautsky curve [19].

When this type of material is left on darkness by a given period of time (dark adaptation) and then suddenly exposed to a light, a fluorescence emission begins. Fluorescence (Figure 8) quickly increases to an initial level (named F0, stable after some nanoseconds of light pulse) and then begins a multi step rise (with different intermediate plateau named Fi, FJ, Fk) until arriving at a maximum level (Fm) and then begins to decrease [19].

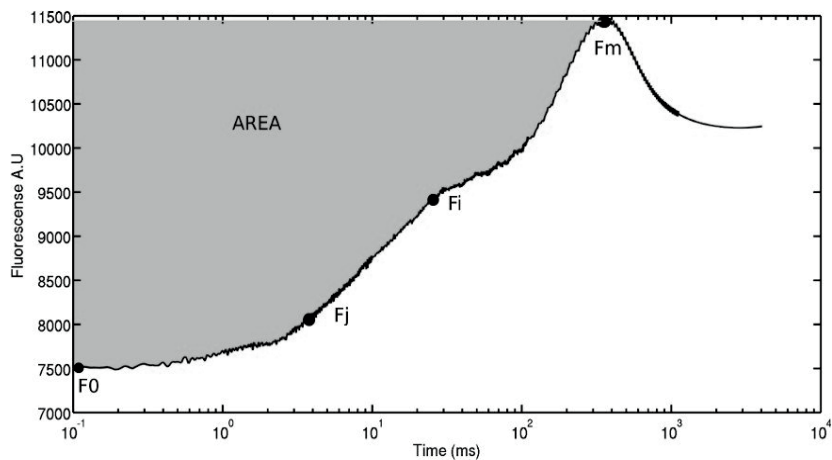


Figure 8 Example of fluorescence transient (Kautsky curve), X axis is time in ms (log) and Y axis is fluorescence on arbitrary units

This transient fluorescence is due to a series of electron transfer process internal at the chloroplasts (tilakoid membrane). When the bio-mediator is on contact with a photosynthesis blocking substance (by example some types of herbicides) the shape of this transient is changed.

The fluorescence instrument has been tested by measuring the fluorescence transient of *C. reinhardtii* algae. These measurements were conducted after fifteen minutes of dark

adaptation period, using the maximum light intensity possible by the instrument (aprox. 550Umol/m2/s2). The algae were in solution in a volume of 500uL, Optical Density (OD) = 1. Measurements were done with and without presence of Linuron pesticide.

Figure 9 (left) shows results. The fluorescence curves (measured on arbitrary units) were normalized by subtracting the F0 level and dividing by the Fm level.

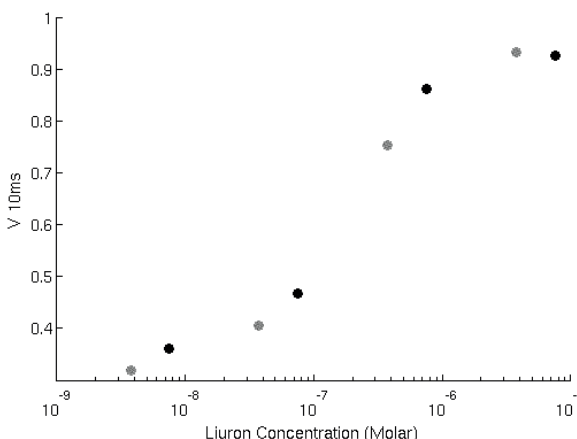
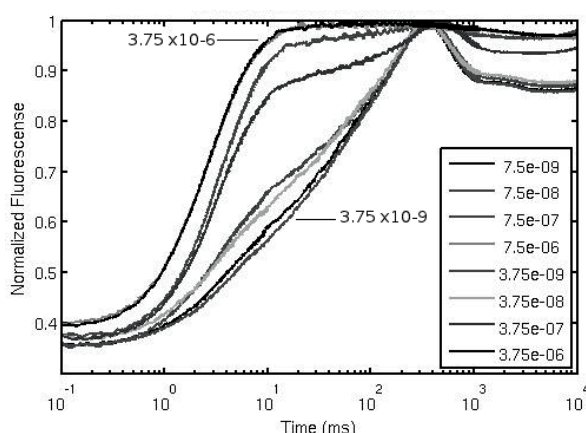


Figure 9 Left: Kausky curves of the IL mutant with linuron pesticide. Each curve corresponds at a pesticide concentration (molar). Right: V10m parameter vs pesticide concentration

Is evident the change (increase) on the fluorescence levels near 10ms when the bio-mediator is exposed to increasing concentrations of Linuron pesticide. This change can be quantified by defining a parameter (V_{10m} , Equation 2). It depends on the fluorescence intensity at 10ms (F_{10ms}), the initial fluorescence (F_0) and the maximum fluorescence (F_m).

$$V_{10ms} = \frac{F_{10ms} - F_0}{F_M} \quad (2)$$

Figure 9 (right) shows the evolution of the V10 value vs concentration of pesticide using data from the previously described tests. Notice how the parameter increases with the pesticide concentration following a sigmoid shape as expected.

Conclusions

Biosensors are becoming commercial realities on applications where low costs, easy to do, on site measurements are required. This is the case on environmental monitoring applications.

Many biosensor applications are based on fluorescence or amperometry transduction. The electronic systems required for these techniques can be implemented using commercial electronics, making them suitable for applications on developing countries. However is necessary to take into account the design problems related at working with low level and or fast changing signals.

The instruments that have been developed were capable of detecting the biomediator response during testing. This demonstrates the correct design and implementation of the transduction stages. It is important to notice that the use of different biomediators can lead to more exigent design parameters.

Photosynthetic biomediators (as the ones used on instrument testing) produces responses for both types of transduction methods (fluorescence and amperometry). This creates interest on the development of multi-transduction instruments that can measure contemporary these two variables in order to obtain complementary data to improve analyte estimations.

Acknowledgements

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