# DEVELOPMENT OF AN ENZYMATIC AMPEROMETRIC BIOSENSOR USING CYTOCHROMES $C_3$ FOR THE FAST QUANTIFICATION OF CHROMATE BIO-AVAILABILITY IN THE ENVIRONMENT

Ignatiadis I.*, Michel C.*, Battaglia-Brunet F.*, Bruschi M.**, Bianco P.**, Lojou E.**, Tran Minh C.***

* BRGM, Environment & Process Division, Biotechnology, BP 6009, 45060 Orléans Cedex 02, France.
** CNRS, Bioénergétique et Ingénierie des Protéines, 31, chemin de J. Aiguier, 13402 Marseille Cedex 20, France.
*** Ecole Nationale Supérieure des Mines de St-Etienne, Centre SPIN, Biotechnologie, 158, Cours Fauriel, 42023 Saint-Etienne Cedex 2, France.

## SUMMARY

The presence of toxic Heavy Metals and Metalloids (HMM) in the environment greatly affects the quality of water, soil and chain-food. The toxicity of the HMM depends on metal availability. Many analytical methods, such as sequential extraction or mathematical modeling, have been used for a long time for the assessment of HMM bioavailability. However, these techniques are very often difficult, expensive and long. The biosensors, like analytical tools, have advantages while bringing in addition to specificity, fast and quantitative measurement of a metal that reacts with the biomaterial. This principle is applied to detect the presence of bio-available concentrations of certain metals. The biosensor presented in this study is an amperometric one and its sensitive part is a hemo-protein, the cytochrome $c_3$ from *Desulfomicrobium norvegicum*. The cytochrome $c_3$ has been chosen for its better properties as a reducing agent of chromate ($CrO_4^{2-}$).

This study required instrumental developments or adaptations: the development of glassy carbon electrode with immobilized cytochrome $c_3$ and the implementation of electrochemical methods for the study of redox systems, i.e. cyclic voltammetry (CV) and chronoamperometry (CA). The performances of various configurations of biosensors, according to the mode of immobilization of the enzyme, are studied for the qualitative and quantitative determination of chromate. These tools made it possible to identify and follow the redox reactions taking place during the contact of the electrode without and with chromate in solution. The tests on the various configurations of electrode allowed us, for the moment, to choose two promising configurations: The first one is an immobilization of the enzyme with a dialysis membrane and the second is an immobilization with a cellulose nitrate filter. Chromate concentrations from 0.2 to 6.8 mg/L can be detected by the biosensors that were designed.

## INTRODUCTION

Biosensor is an analytical device, which correlates a biologic phenomenon to an electrical signal. For this, the biosensor is composed by two elements: the bio-receptor and the transducer. The bio-receptor can be an enzyme or a microorganism. Electrodes, transistors, optical fibbers are transducers. In contact with the bio-receptor, the substrate is transformed in products that directly or indirectly interfere with the transducer to produce an electrical signal. This principle is applied to detect the presence of bio-available concentrations of certain metals.

Chromate ($CrO_4^{2-}$), as major pollutant in the environment (underground and surface waters and soils), was selected to be the model metal. According to the chromate-reducing properties of the cytochromes $c_3$ from sulfate and metal reducing bacteria (Lojou *et al.*, 1998; Michel *et al.*, 2001), it has been thought to make cytochrome $c_3$ based biosensors, as an alternative to the classical techniques of $CrO_4^{2-}$ measurement. The aims of this work were the evaluation of various configurations of amperometric biosensors, and finally the development of the most valuable.

## MATERIALS AND METHODS

An amperometric biosensor consists in determining the intensity of a current at an applied potential. This intensity is a function of the concentration of the electroactive species being oxidized or reduced. Hence, after proper calibration, the intensity can be used to measure the concentration of the present species. Figure 1 presents the experimental set-up for amperometric biosensor measurements. There are 3 electrodes (working, reference and auxiliary) connected to the potentiostat, which is connected to the recorder.

A cytochrome $c_3$ biosensor takes part in the reduction of a metal by oxidation of the cytochrome. The regeneration is the reduction of the cytochrome by the mean of electrons. Practically the biosensor consists in the measurement of the regeneration current at an applied potential, the reduction potential. Amperometry may transfer the electron directly from the electrode if the cytochrome is in solution. A mediator may be necessary by acting as a bridge or a shuttle between the immobilized enzyme and the electrode. A conducting polymer allows both entrapment and electron transfer between the electrode and the enzyme. The sensitive part of this biosensor is a hemo-protein, the cytochrome $c_3$ (13 kDa) from *Desulfomicrobium norvegicum* and purified according to a procedure (Bruschi *et al.,* 1977). Practically, we operate with a cytochrome $c_3$ concentration of 65 µM in anaerobic electrolyte consisting in 0.1 M Tris-HCl pH 7.6.

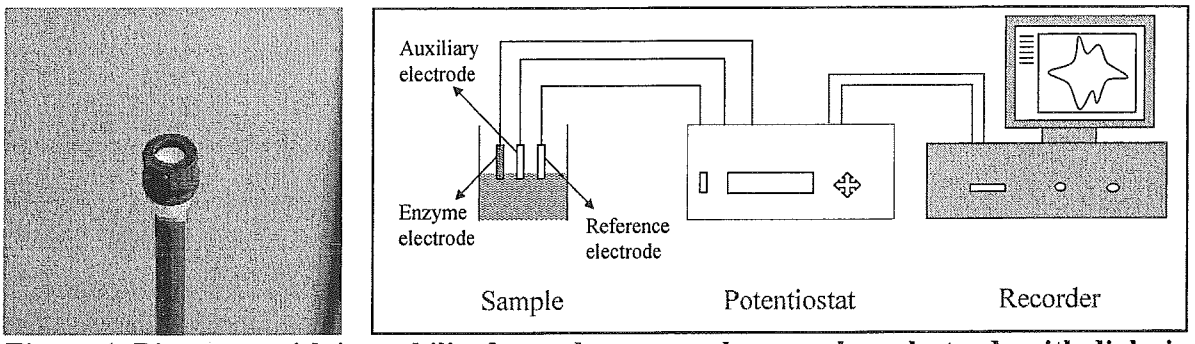


**Figure 1. Biosensor with immobilized cytochrome on glassy carbon electrode with dialysis membrane and experimental set-up for biosensor's testing.**

The study required instrumental developments or adaptations (fig. 1): The development of glassy carbon electrodes with immobilized cytochrome and the implementation of electrochemical methods for the study of the redox systems (cyclic voltammetry and chrono-amperometry).

The performances of various configurations of biosensors are studied according to the mode of immobilization of the enzyme. The enzyme immobilization on the glassy carbon electrode was obtained using either dialysis membrane (Spectra/Por, with a molecular weight cut off of 6-8 kDa, from Spectrum Laboratories, Inc, Rancho Dominguez, CA USA), or different filters (Sartorius AG, Goettingen, Germany, with a porosity ranging from 0.01 µm to µm), or by entrapment of cytochrome in polyethylene dioxy-thiophene films obtained (by electro-polymerisation) or by entrapment of cytochrome in functionalized polypyrrole films (obtained by adsorption of an aqueous mixture of amphiphilic enzymes and pyrroles on the electrode surface, followed by electro-polymerisation of the adsorbed monomers).

These tools made it possible to identify and follow the redox reactions taking place during the contact of the electrode without and with chromate in solution.

## RESULTS AND DISCUSSION

Different configurations of biosensors were made up with a cytochrome $c_3$ immobilized to a glassy carbon electrode. These biosensors were then immersed in a desaerated electrolyte (consisting in 0.1 M Tris-HCl pH 7.6) without chromate and cyclic voltametry experiments were used to evaluate the stability and the electrochemical properties of the immobilized cytochrome $c_3$ (fig. 2). These electrochemical tests allowed us, for the moment, to choose two promising configurations: the first one is an immobilization of the enzyme with a dialysis membrane (with a molecular weight cut off of 6-8 kDa) and the second is an immobilization with a cellulose nitrate filter (diameter of the pores 0.1 µm). For each configuration that has succeeded in the cyclic voltametry test (fig. 2), a chrono-amperometry test at the reduction potential of the enzyme was carried out (fig. 3). When the current as a function of time (I=f(t)) is stabilized, the chromate is added in the solution. Then, the enzyme reduces the chromate present in the vicinity of the electrode and simultaneously an increase in the current is observed. In fact, the first stage of the enzymatic activity is the reduction of the substrate by the cytochrome, being in its reduced form, according to the equation:

$$Cr^{VI} + \text{cytochrome red} \rightarrow \text{cytochrome ox} + Cr^{III} \quad (1)$$

The cytochrome which is in its oxidized form collects electrons coming from the electrode to regenerate itself in order to establish initial balance according to the equation:

$$\text{cytochrome ox} + e^- \text{red} \rightarrow \text{cytochrome red} \quad (2)$$

That results in an increase of the intensity of current. The number of electrons having been used to regenerate the cytochrome is thus proportional to the quantity of cytochrome having reacted in (1) and thus to the quantity of chromate. There is thus a direct relationship between the current increase and the concentration of chromate in solution. The enzyme is always regenerated because of the positioning of the applied potential at its reduction potential value. If previously a denaturation of the enzyme took place (probably due to a high concentration of chromate in the solution), the enzyme is definitively not regenerated, and the current is decreasing. At this moment a cyclic voltametry experiment can demonstrate that the enzyme has lost its properties. Chromate concentrations from 0.2 to 6.8 mg/L can be detected by the biosensor. Figure 4 shows a calibration curve for a biosensor with 71 µM of cytochrome c3 immobilized by a dialysis membrane and a chromate concentration ranging from 0.2 to 3.5 mg/L. Chromate concentrations near the upper detection limit cause a rapid denaturation of the enzyme, which is characterised by a decrease of the intensity in chrono-amperometry (or by an absence of characteristic peaks in cyclic voltammetry). The reliability of the correlation of concentration - signal is not yet satisfactory as well as the stability of the biosensor. The problem of the interference in complex medium was not studied yet. These first results are very promising for the validation of this concept of biosensor for the quantification of chromate in groundwaters.

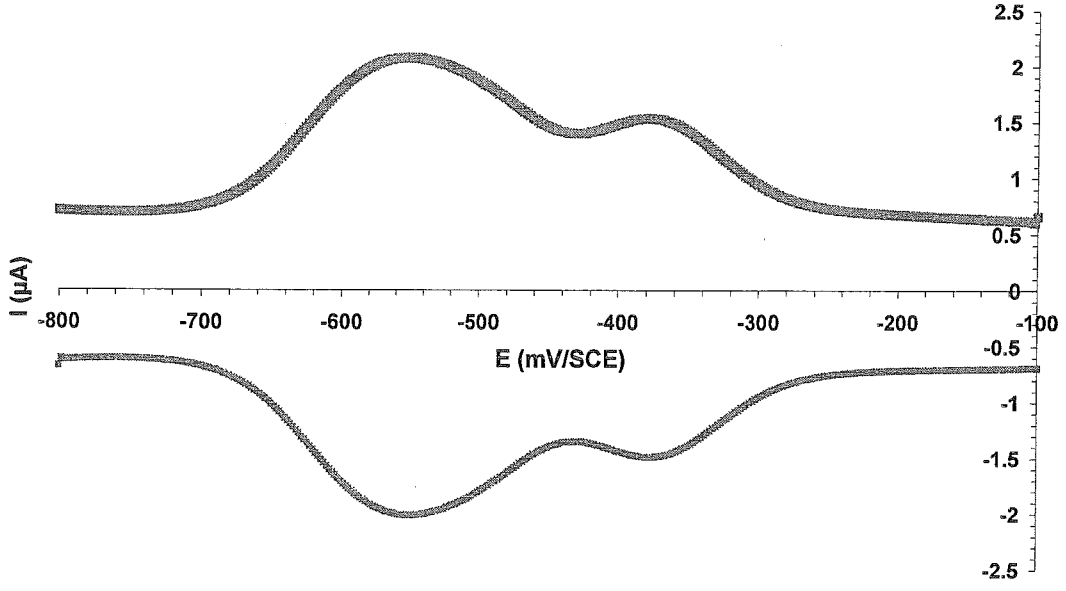


**Figure 2. Evolution of the current as a function of the potential applied to the electrode-biosensor during a square wave cyclic voltammetry (direct mode from -100 to -800 mV/SCE and inverse mode from -800 to -100 mV/SCE).**

The four hemes of the cytochrome present two peaks in oxidation and two in reduction. The biosensor is made up with a cytochrome $c_3$ immobilised to a glassy carbon electrode by a dialysis membrane (with a molecular weight cut off of 6-8 kDa). The evolution of the current shows that the cytochrome is a fast and reversible system.

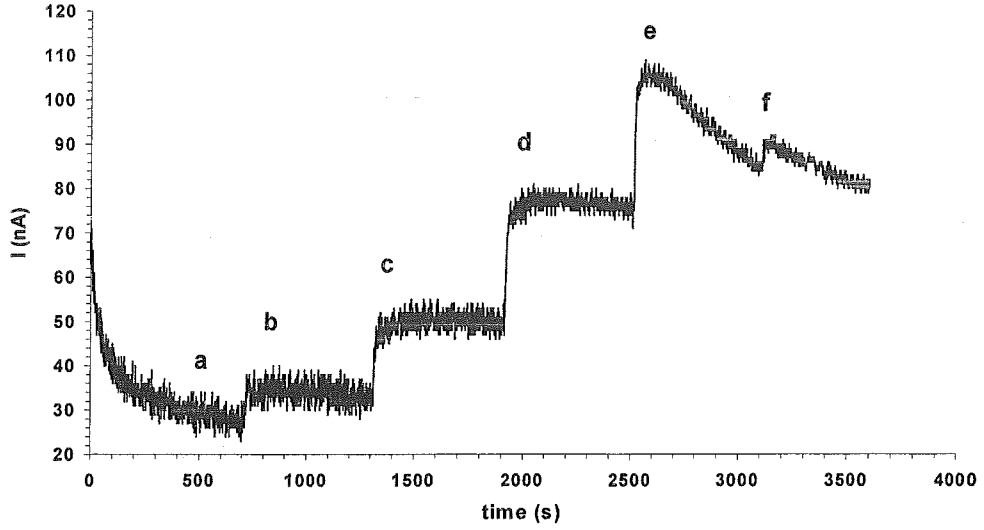


**Figure 3. Evolution of the current as a function of time (chrono-amperometry) at -570 mV/SCE (the reduction potential of the enzyme), after stabilisation (a) and successive injections (from b to f) of various quantities of chromate (b: 0.425; c: 1.275; d: 2.550; e: 4.250; f: 4.675 mg/l). The current variation is proportional to the quantity of chromate in water. The evolution of the current after the two last injections (e and f) reveals a denaturation of the enzyme. The biosensor is the same as in Fig. 2.**

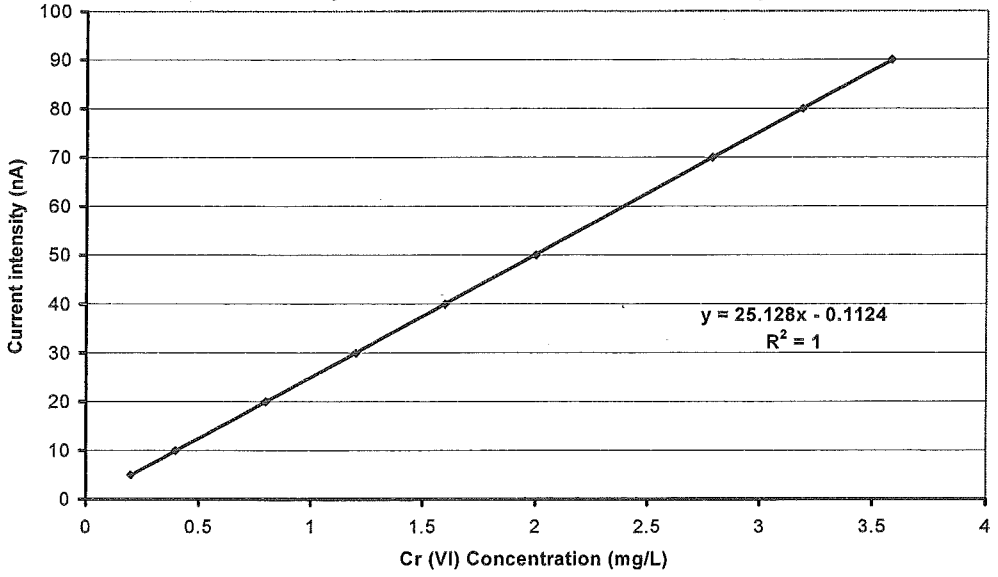


**Figure 4. Calibration curves of a biosensor (71 µM of cytochrome $c_3$ immobilized by dialysis membrane) with various concentrations in chromate.**

## AKNOWLEDGMENTS

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