Amperometric cytochrome c3-based biosensor for chromate determination

Caroline Michel, Fabienne Battaglia-Brunet, Canh Tran-Minh, Mireille Bruschi, Ioannis Ignatiadis

To cite this version:

Caroline Michel, Fabienne Battaglia-Brunet, Canh Tran-Minh, Mireille Bruschi, Ioannis Ignatiadis. Amperometric cytochrome c3-based biosensor for chromate determination. Biosensors and Bioelectronics, 2003, 19 (4), pp.345-352. 10.1016/S0956-5663(03)00191-X . emse-00519585

HAL Id: emse-00519585

https://hal-emse.ccsd.cnrs.fr/emse-00519585

Submitted on 21 Sep 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Biosensors and Bioelectronics, 2003, 19(4), 345-352 , doi:10.1016/S0956-5663(03)00191-X

Amperometric cytochrome c_3-based biosensor for chromate determination

Michel Caroline[(1)], Battaglia-Brunet Fabienne[(1)], Tran-Minh Canh[(2)], Bruschi Mireille[(3)], Ignatiadis Ioannis[(1)] *

(1) *BRGM, Service Environnement Industriel et Procédés Innovants, Unité Biotechnologies, 3 Avenue Claude Guillemin, BP 6009, 45060, Orléans Cedex 02, France*

(2) *Ecole Nationale Supérieure des Mines de Saint Etienne, Centre SPIN ; Département PMMC ; 158 Cours Fauriel ; 42023 Saint-Étienne Cedex 2, France*

(3) *CNRS, IBSM, Laboratoire de Bioénergétique et Ingénierie des Protéines, 31 chemin Joseph Aiguier, 13402, Marseille Cedex 20, France*

Abstract

The chromate reductase activity of cytochrome c_3 (Cyt c_3, M_r 13 000), isolated from the sulfate-reducing bacterium *Desulfomicrobium norvegicum*, was used to develop an amperometric biosensor to measure chromate (CrO_4^{2-}) bioavailability. The performance of various biosensor configurations for qualitative and quantitative determination of Cr(VI) was studied. Biosensor properties depend on the technique used to immobilize the enzyme on the electrode (glassy carbon electrode). Immobilization of Cyt c_3 by entrapment in poly 3,4-ethylenedioxythiophene films denatured the enzyme, while application of an adsorption technique did not affect enzyme activity but the detection range was limited. The best results were obtained with dialysis membranes, which allowed the determination of Cr(VI) from 0.20 to 6.84 mg l^{-1} (3.85–132 μM) with a sensitivity of 35 nA mg^{-1} l (1.82 nA μM^{-1}). No interference was observed with As(V), As(III) and Fe(III). Only a small amount of Cyt c_3 (372 ng of protein) was needed for this biosensor.

Keywords:

Enzymatic sensor; Polyheme cytochrome c_3; Chromate quantification; Sulfate-reducing bacteria; Environmental biosensor; Chronoamperometry

I. Introduction

Most heavy metals and metalloids (HMM) are toxic for plants, animals and humans. Chromium, which can contaminate soils, ground- and surface waters, is one of the major environmental pollutants. The toxicity of chromium, like other HMM, depends on its oxidation state and bioavailability. For example, Cr(VI) is a highly toxic and soluble oxyanion, whereas Cr(III) is cationic, less toxic and less mobile because it tends to form insoluble hydroxides. The assessment of HMM bioavailability with analytical methods such as sequential extraction and mathematical modeling is difficult, expensive and time-consuming. The redox speciation of chromium can be accomplished by separate preconcentration of Cr(III) and Cr(VI) fractions using chelating resins, coprecipitation, ion chromatography, and solvent extraction, but such procedures are obviously complicated. The colorimetric analytical method at 543 nm for chromium using diphenylcarbazide as reagent is known to be selective for Cr(VI) but not Cr(III). However, 72 foreign ions are known to interfere by clouding or

* Auteur à qui la correspondance devait être adressée : i.ignatiadis@brgm.fr

discoloring the sample. The measuring range of this method is 0.05–2.00 mg l−1 Cr or 0.11–4.46 mg l−1 CrO42− (Kit MERCK spectroquant® 1.14552.0001). Cr(III) can be indirectly determined by analyzing the total chromium (in the form of Cr(VI)) after high acidification and oxidation of the solution that leads to Cr(III) oxidation in Cr(VI). Biosensors, however, in addition to their specificity, enable rapid, quantitative and in situ measurement of the metallic derivatives that interact with the biomaterial.

Several studies have shown that it is possible to make biosensors to quantify metals with genetically engineered microorganisms (D'Souza, 2001). Recombinant luminescent microorganisms such as Alcaligenes eutrophus (Peitzsch *et al.*, 1998) and *Saccharomyces cerevisiae* (Lehmann *et al.*, 2000) have been used to determine chromium (Peitzsch *et al.*, 1998), cadmium, zinc, mercury and chromium (Ivask *et al.*, 2002), arsenic and antimony (Ramanathan *et al.*, 1997), cadmium and lead (Tauriainen *et al.*, 1998). Other micro-organisms, such as sulfate-reducing bacteria (Michel *et al.*, 2001 and Tebo and Obraztsova, 1998), Enterobacter cloacae (Rege *et al.*, 1997), and *Deinococcus radiodurans* (Fredrickson *et al.*, 2000), are able to reduce Cr(VI) into Cr(III) and could, therefore, also be used in biosensors to differentiate between Cr(VI) and Cr(III). Engineered microorganism-based biosensors are able to detect low metal concentrations, but these whole-cell biosensors have several disadvantages: (1) long induction period (1–2 h before the signal is detected), (2) low specificity (detection of other metals) and (3) interference of other physiological metabolites (sulfate metabolism, metal oxidation–reduction) (Peitzsch *et al.*, 1998, Tauriainen *et al.*, 1998 and Cai and Dubow, 1997). These drawbacks can be avoided with enzymatic biosensors.

Enzymatic sensors for metal detection have already been described (Kormos and Lengauer, 2000 and Krawczynski vel Krawczyk *et al.*, 2000). Potentiometric urease-based sensors sensitive to metals have been developed since some metals are urease inhibitors. Indirect detection of metals such as Pb^{2+}, Fe^{2+}, Sn^{2+}, Zn^{2+} and Hg^{2+} has been achieved by reduction of the biosensor response to urea. Other proteins such as those involved in metal resistance mechanisms could be used to develop biosensors for direct detection of metals. For example, ChrA, which is involved in chromate resistance, can discriminate between Cr(VI) and Cr(III) (Cervantes *et al.*, 2001). Several bacterial redox proteins that have metal-redox properties could also be of interest. For example, cytochromes c_3 and hydrogenases from sulfate- and sulfur-reducing bacteria (Battaglia-Brunet *et al.*, 2002a and Chardin *et al.*, 2002) have bioremediation properties because they can reduce various metals such as U(V) and Cr(VI) (Assfalg *et al.*, 2002, Lojou *et al.*, 1998, Lovley and Phillips, 1994 and Michel *et al.*, 2001). While most hydrogenases are sensitive towards oxygen (Vignais *et al.*, 2001), cytochromes c3 are not affected by oxygen, temperature, pH, denaturing agents or ageing (Bianco *et al.*, 1986 and Florens *et al.*, 1995). They also have a good Cr(VI)-reductase activity (Lojou *et al.*, 1998, Lovley and Phillips, 1994 and Michel *et al.*, 2001). Cytochromes c3 are multiheme redox proteins with very negative redox potentials and are involved in the respiratory metabolism of sulfate- and sulfur-reducing bacteria (Bruschi, 1981 and Haser *et al.*, 1979). They are, therefore, well suited for the development of enzymatic biosensors for Cr(VI) quantification. This type of biosensor could be of great interest for measuring Cr(VI) concentrations in surface or ground waters polluted by chromate (tannery effluents, chromium plating effluents, etc.) and assessing the efficiency of water (bio)treatment processes such as bioreactors.

We developed an amperometric biosensor to measure Cr(VI) bioavailability using cytochrome c_3 from *Desulfomicrobium norvegicum*, a sulfate-reducing bacterium. Various techniques to immobilize the enzyme on the working electrode were tested by applying cyclic voltametry. Biosensor parameters such as sensitivity, specificity, linearity and detection limit were studied using chronoamperometry.

II. Materials and methods

II.1. Purification of Cyt c_3

Cyt c_3 (M_r 13 000) from *Desulfomicrobium norvegicum* (DSM 1741) was purified as described by (Bruschi *et al.*, 1977). Various concentrations of purified cytochrome c_3 (2.85, 14.3, 28.5, 47.5, 60, 71.25 and 114 µM in water) were used to optimize the biosensor response to Cr(VI).

II.2. Immobilization of Cyt c_3 on the working electrode

Four methods were tested to immobilize the enzyme on the working glassy carbon electrode (GCE):

a Entrapment with a dialysis membrane (Haladjian *et al.*, 1994). A Spectra/Por® regenerated cellulose dialysis membrane (6–8.000 MWCO) was used to maintain a 2 µl Cyt c_3 solution in contact with the electrode.

b Adsorption: 2 µl of enzyme solution were deposited on the electrode and aerated for 30 min.

c Entrapment of 5 µl of a Cyt c_3 sample with cellulose nitrate filters (Sartorius AG (Goettingen, Germany), porosity 0.01–0.1 µm).

d Entrapment in poly 3,4-ethylenedioxythiophene (PEDT) films obtained by electropolymerization (at +1400 mV/SCE for 10 s) of a 10 µl solution containing Cyt c_3 (10% v/v) and PEDT (10 mM).

II.3. Apparatus

Cyclic voltametry and chronoamperometry techniques were done with an EG&G 273A potentiostat controlled by a microcomputer with an EG&G M270 software program (Princeton Applied Research, Oak Ridge, TN). The electrochemical cell consisted of a working electrode (a polished GCE with immobilized Cyt c_3), an auxiliary electrode (platinum wire), and a reference saturated calomel electrode (SCE). Potential values with respect to the normal hydrogen electrode can be obtained by adding 240 mV. Measurements were done in 20 ml of trishydroxymethyl aminomethane (Tris–HCl) buffer (0.1 M, pH 7.6), deoxygenated under N_2 atmosphere. All experiments were carried out at 25 °C under stirring conditions

II.4. Biosensor characterization

II.4.1. Stability of the immobilized Cyt c_3

Once the Cyt c_3 was immobilized on the GCE, cyclic voltametry was used to evaluate the stability of the immobilized protein.

II.4.2. Characterization of the biosensors

Chronoamperometry was used to determine biosensor characteristics (response time, calibration curve, sensitivity, specificity and detection range). Experiments were conducted at −570 mV/SCE (the reduction potential of the enzyme) (Figure 2, black line), so that all of the 4 hemes except Cr(VI) were reduced at the electrode. When the potential was applied, a current was observed showing the reduction of Cyt c_3 at the electrode. When the current had stabilized, indicating that the cytochrome was totally reduced, Cr(VI), As(V), As(III) or Fe(III) metal ions were added. The linear relationship between metal concentration (x) and the intensity of the current produced (y) was used ($y=Ax$ with A being the sensitivity of the biosensor (nA mg^{-1} l or nA M^{-1})).

II.5. Chemical reagents

Cr(VI) (as Na_2CrO_4) and As(V) (as As_2O_5) were dissolved in H_2O, As(III) (as As_2O_3) was dissolved in a NaOH solution, as described by (Battaglia-Brunet *et al.*, 2002b.), and Fe(III) (as $FeCl_3$) was dissolved in 15% HCl.

Cr(VI) was quantified using the Merck Spectroquant® kit 1.14758.0001 based on the oxidation of diphenylcarbazide to the purple-colored Cr(III)-diphenylcarbazone complex. As(V) and

As(III) concentrations were determined as described by (Battaglia-Brunet et al., 2002b). Fe(III) was quantified using the Merck Spectroquant® kit 1.14761.0001.

III. Results and discussion

III.1. Principle of the Cyt c_3-based biosensor

The Cyt c_3-based biosensor for Cr(VI) determination is based on the reduction of the metal by oxidation of the immobilized cytochrome that is electrochemically regenerated (Figure 1). The biosensor signal comes from the cytochrome regeneration current at an applied reduction potential when Cr(VI) is added to the solution. Electrons may be transferred directly from the electrode to the cytochrome in the working solution.

Chronoamperometry allows the current to be measured as a function of time, $I=f(t)$. When the current has stabilized for an applied potential, an increase in Cr(VI) concentration in the solution causes an increase in the current through the amperometric electrode. Cr(VI) can then be determined with a calibration curve of current change as a function of Cr(VI) concentration.

III.2. Comparison of immobilization modes—characterization of the biosensors

The first step in constructing a biosensor is determining how to immobilize the bioreceptor (Cyt c_3) on the transducer GCE. We tested five immobilization modes: entrapment in PEDT, physical entrapment with a dialysis membrane, adsorption, adsorption+dialysis membrane and entrapment with a filter (Table 1). The ability of each mode to prevent Cyt c_3 denaturation was studied using cyclic voltametry. The cyclic voltammograms obtained for all of these modes except PEDT entrapment were typical of Cyt c_3 since the immobilized cytochrome was still a rapid and reversible system, and the redox potentials were characteristic of this cytochrome (Figure 2, black line). The catalytic wave observed in the presence of chromate demonstrates the ability of the immobilized enzyme to catalyze the reduction of chromate (Figure 2, grey line). Only in the case of entrapment in PEDT, therefore, does immobilization lead to protein denaturation and loss of Cr(VI)-reductase activity.

Amperometric biosensors were therefore constructed with ‘dialysis membrane’, ‘adsorption’, ‘adsorption+dialysis membrane’ and ‘filter’ immobilization techniques. Figure 3 shows the biosensor response achieved with one of these (the ‘dialysis membrane’ electrode). Its detection range, linear domain and sensitivity are given in Figure 4, and the characteristics of all of the biosensors are given in Table 1.

The very short response time (a few seconds) obtained with all of the cytochrome c_3-based biosensors is remarkable. This is much shorter than the response times obtained with other enzymatic sensors for metal measurement such as the urease-based biosensor, whose response time varies from 10 min to 1 h (Krawczynski vel Krawczyk *et al.*, 2000).

Experiments with ‘filter’ immobilization showed that, of all the filters tested, only a 0.1 µM pore size filter enabled the detection of Cr(VI), probably because filters with smaller pore sizes acted as insulators (Table 1). However, experiments with a 0.1 µm pore size filter did not give reproducible results. This mode of Cyt c_3 immobilization is therefore unsatisfactory for the development of a biosensor.

The ‘adsorption’ immobilization technique (Table 1 and Figure 5) had the highest sensitivity, possibly because the dialysis membrane and the filter act as physical barriers, reducing the rate of Cr(VI) ion diffusion from the bulk solution to the Cyt c_3 layer. This might explain the difference in sensitivity observed between the two immobilization techniques using adsorption with and without the dialysis membrane (Table 1).

The ‘adsorption’ biosensors did not, however, have a detection range (0.20–0.80 mg l^{-1} Cr(VI)) as broad as that of the ‘dialysis membrane’ biosensors (0.20–6.84 mg l^{-1} Cr(VI)). They are, therefore, of less interest (Table 1). While there was no difference in sensitivity between the ‘dialysis membrane’ and ‘adsorption+dialysis membrane’ modes (Figure 5), the ‘adsorption+dialysis membrane’ biosensor had a wider Cr(VI) detection range.

The lowest detection limit of the 'adsorption+dialysis membrane' biosensor is 0.2 mg l^{-1}, which is greater than the regulatory standard for Cr(VI) (0.05 mg l^{-1}). Efforts should therefore be made to decrease the detection limit of the Cyt c_3-based biosensor. This could be done by (1) testing other techniques that might produce better results than 'dialysis membrane' immobilization, (2) using mediators that optimize electron transfer, and/or (3) decreasing the signal/noise background ratio, in particular by improving the electrochemical device.

III.3. Amount of enzyme

Various Cyt c3 concentrations, ranging from 2.85 to 114 µM in 2 µl, were used to test the different biosensor configurations (Table 1). We were not able to obtain good sensitivity and reproducible results with 2.85 µM of Cyt c3 (S.D.±4.1, Table 1). The Cyt c3 concentration giving the best sensitivity was determined for each mode of immobilization (Figure 5).

For 'dialysis membrane' immobilizations, a concentration of 14.3 µM (372 ng) of Cyt c3 was the lowest concentration that allowed the best sensitivity and a satisfactory detection range (Figure 5). This is a very small amount of enzyme and is, therefore, very interesting for the development of low-cost biosensors. The urea-sensitive enzymatic sensor constructed by (Krawczynski vel Krawczyk *et al.*, 2000) for Pb^{2+}, Fe^{2+}, Sn^{2+}, Zn^{2+} and Hg^{2+} requires 2.5–15mg of enzyme.

III.4. Specificity for Cr(VI)

(Lojou et al., 1998) reported that cytochromes c3 can also reduce several other metals in addition to Cr(VI). Furthermore, (Assfalg et al., 2002) have demonstrated that the interaction between Cr(VI) and cytochromes c_3 could be electrostatic since CrO_4^{2-} ions interact with the protein's lysines (positively charged amino acids). Other heavy metals, in particular, negatively charged ions, could therefore interfere with Cr(VI) detection by Cyt c_3-based sensors. We therefore studied the possible interference of one metal (Fe(III)) and two metalloids (As(V) and As(III)). In the presence of each of these three ions, as shown in Figure 6 for As(V), no signal was observed, which suggests that there was no electron transfer between the Cyt c_3-based sensors and these ions. Addition of Cr(VI) caused a response of the biosensor (Figure 6). This strongly indicates that the biosensor was still able to detect Cr(VI) with the same sensitivity as in the absence of As(V), As(III) or Fe(III). As(V), As(III) and Fe(III) do not, therefore, interfere in Cr(VI) detection in the concentration range studied here. However, other HMM must be tested, in particular, Mo, as (Turyan and Mandler, 1997) have reported that MoO_4^{2-} interferes with Cr(VI) determination, probably because of its similar structure and size. If a given metal appears to interfere with Cr(VI) detection, selective complexing agents could be used for the determination of individual concentrations.

III.5. Influence of Tris–HCl buffer concentration

Tris–HCl buffer has often been used in electrochemical studies with Cyt c3 (Lojou *et al.*, 1998) to provide an optimal pH value (7.6) for enzyme activity and an optimal ionic strength for electrons transfer between the electrode and the solution. The effect of the Tris–HCl buffer concentration on signal intensity was, therefore, studied by chronoamperometry. We found that the main characteristics (sensitivity and concentration range) of Cyt c_3-based biosensors were not affected by Tris concentration (Table 2). We therefore used the usual Tris concentration for electrochemical experiments with cytochromes c_3 (0.1 M, (Lojou *et al.*, 1998) in this study.

III.6. Loss of enzyme activity at high Cr(VI) concentrations

There is no correlation between Cr(VI) concentration and signal intensity for high concentrations of chromate (>4.5 mg l^{-1} Cr(VI)) (Figure 3). This indicates the loss of enzyme activity. NMR experiments with cytochrome c7, a c3-type cytochrome, have shown that Cr(VI) reduction is due to (1) an interaction between CrO_4^{2-} and some lysines located near heme IV, (2) an intramolecular electron transfer from all of the hemes to heme IV and (3) an electron transfer from heme IV to Cr(VI) (Assfalg *et al.*, 2002). An accumulation of Cr(III) ions near heme IV (due to Cr(VI) reduction) might, therefore, prevent Cr(VI) access to the reduction

site, decreasing the Cr(VI) reduction capacities of Cyt c_3. In this case, the enzyme might be able to recover its activity and it would therefore be possible to regenerate the biosensor by eliminating Cr(III). Another explanation for the loss of enzyme activity could be that high Cr(VI) concentrations denature the enzyme. In this case, regeneration would not be possible. In our experiments with Cyt c_3-based biosensors, no recovery of enzyme activity was observed after incubation of the biosensor in a Tris buffer solution or after application of a very negative potential (−1000 mV/SCE). This suggests a denaturation of the Cyt c_3. However, this result should be confirmed with other regeneration techniques (such as pH variation, EDTA and thioacetamide in a Tris–HCl buffer, cysteine in a phosphate buffer, NaI and EDTA, NaI alone, and any other reagents that could complex Cr(III) (Krawczynski vel Krawczyk *et al.*, 2000)).

Conclusion

Enzymatic sensors based on Cyt c_3 immobilized with a dialysis membrane are suitable for the detection of Cr(VI) bioavailability using an amperometric method. The principal characteristics and main advantages of the Cyt c_3-based biosensor developed in this study are: rapid Cr(VI) quantification (a few minutes), a detection range of 0.20–6.84 mg l^{-1}, good sensitivity (35 nA mg^{-1} l or 1.82 nA μM^{-1}), small amount of enzyme needed, and specificity for Cr(VI) (no detection of As(V), As(III) and Fe(III)). Interference with other metals or metalloids should nevertheless be studied. Loss of enzyme activity at high Cr(VI) concentrations and sensor regeneration also require further investigation. Undeniably, cytochromes c_3 isolated from sulfate-reducing bacteria are attractive enzymes for the development of biosensors for Cr(VI) quantification because of their selective Cr(VI)-reductase activity, chemical stability and high production rate in the bacterial cells.

Acknowledgements

This work was carried out under the European Commission's Fifth Framework Programme, Contract no EVK1-CT 1999-00033, project METALBIOREDUCTION. It also received financial support from the BRGM Research Directorate, who authorized the publication of this paper under reference No. 02496.

References

M. Assfalg, I. Bertini, M. Bruschi, C. Michel and P. Turano, The metal reductase activity of some multiheme cytochromes c: NMR structural characterization of the reduction of chromium (VI) to chromium (III) by cytochrome c(7). Proc. Natl. Acad. Sci. USA **99** (2002), pp. 9750–9754.

F. Battaglia-Brunet, S. Foucher, A. Denamur, I. Ignatiadis, C. Michel and D. Morin, Reduction of chromate by fixed films of sulfate-reducing bacteria using hydrogen as an electron source. J. Ind. Microbiol. Biotechnol. 28 (2002), pp. 154–159.

F. Battaglia-Brunet, M.-C. Dictor, F. Garrido, C. Crouzet, D. Morin, K. Dekeyser, M. Clarens and P. Baranger, An arsenic(III)-oxidizing bacterial population: selection, characterization, and performance in reactors. J. Appl. Microbiol. 93 (2002), pp. 656–667.

P. Bianco, J. Haladjian and M. Bruschi, An electrochemical study of the stability of cytochrome c3 from Desulfovibrio desulfuricans Norway strain. Bioelectrochem. Bioenerg. **15** (1986), pp. 57–66.

M. Bruschi, The primary structure of the tetrahaem cytochrome c3 from Desulfovibrio desulfuricans (strain Norway 4). Description of a new class of low-potential cytochrome c. Biochim. Biophys. Acta **671** (1981), pp. 219–226.

M. Bruschi, C.E. Hatchikian, L.A. Golovleva and J. LeGall, Purification and characterization of cytochrome c3, ferredoxin, and rubredoxin isolated from Desulfovibrio desulfuricans Norway. J. Bacteriol. **129** (1977), pp. 30–38.

J. Cai and M.S. Dubow, Use of a luminescent bacterial biosensor for biomonitoring and characterization of arsenic toxicity of chromated copper arsenate (CCA). Biodegradation **8** (1997), pp. 105–111.

C. Cervantes, J. Campos-Garcia, S. Devars, F. Gutiérrez-Corona, H. Loza-Tavera, J. Torres-Guzman and R. Moreno-Sanchez, Interactions of chromium with microorganisms and plants. FEMS Microbiol. Rev. **25** (2001), pp. 335–347.

B. Chardin, A. Dolla, F. Chaspoul, M.L. Fardeau, P. Gallice and M. Bruschi, Bioremediation of chromate; thermodynamic analysis of the effects of Cr(VI) on sulfate-reducing bacteria. Appl. Microbiol. Biotechnol. **60** (2002), pp. 352–360.

S.F. D'Souza, Microbial biosensors. Biosens. Bioelectron. **16** (2001), pp. 337–353.

L. Florens, P. Bianco, J. Haladjian, M. Bruschi, I. Protasevich and A. Makarov, Thermal stability of the polyheme cytochrome c3 superfamily. FEBS Lett. **373** (1995), pp. 280–284.

J. Fredrickson, H. Kostandarithes, S. Li, A. Plymale and M. Daly, Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by Deinococcus radiodurans R1. Appl. Environ. Microbiol. **66** (2000), pp. 2006–2011.

J. Haladjian, P. Bianco, F. Nunzi and M. Bruschi, A permselective-membrane electrode for the electrochemical study of redox proteins. Application to cytochrome c552 from Thiobacillus ferrooxidans. Anal. Chim. Acta **289** (1994), pp. 15–20.

R. Haser, M. Pierrot, M. Frey, F. Payan, J.P. Astier, M. Bruschi and J. Le Gall, Structure and sequence of the multihaem cytochrome c3. Nature **282** (1979), pp. 806–810.

A. Ivask, M. Virta and A. Kahru, Construction and use of specific luminescent recombinant bacterial sensors for the assessment of bioavailable fraction of cadmium, zinc, mercury and chromium in the soil. Soil Biol. Biochem. **34** (2002), pp. 1439–1447

F. Kormos and A.N. Lengauer, Studies concerning the biomonitoring of the degree of environmental pollution using urea-sensitive enzymatic sensor. Lab. Robotics Automat. **12** (2000), pp. 27–30.

T. Krawczynski vel Krawczyk, M. Moszczynska and M. Trojanowicz, Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosens. Bioelectron. **15** (2000), pp. 681–69

M. Lehmann, K. Riedel, K. Adler and G. Kunze, Amperometric measurement of copper ions with a deputy substrate using a novel Saccharomyces cerevisiae sensor. Biosens. Bioelectron. **15** 3–4 (2000), pp. 211–219.

E. Lojou, P. Bianco and M. Bruschi, Kinetic studies on the electron transfer between bacterial c-type cytochromes and metal oxides. J. Electroanal. Chem. **452** (1998), pp. 167–177.

D.R. Lovley and E.J.P. Phillips, Reduction of chromate by Desulfovibrio vulgaris and its c3 cytochrome. Appl. Environ. Microbiol. **60** (1994), pp. 726–728.

Michel, M. Brugna, C. Aubert, A. Bernadac and M. Bruschi, Enzymatic reduction of chromate: comparative studies using sulfate-reducing bacteria. Key role of polyheme cytochromes c and hydrogenases. Appl. Microbiol. Biotechnol. **55** (2001), pp. 95–100.

N. Peitzsch, G. Eberz and D.H. Nies, Alcaligenes eutrophus as a bacterial chromate sensor. Appl. Environ. Microbiol. **64** (1998), pp. 453–458.

S. Ramanathan, W. Shi, B.P. Rosen and S. Daunert, Sensing antimonite and arsenite at the subattomole level with genetically engineered bioluminescent bacteria. Anal. Biochem. **69** (1997), pp. 3380–3384.

M. Rege, J. Petresen, D. Johnstone, C. Turick, D. Yonge and W. Appel, Bacterial reduction of hexavalent chromium by Enterobacter cloacae strain H01 grown on sucrose. Biotechnol. Lett. **19** (1997), pp. 691–694.

S. Tauriainen, M. Karp, W. Chang and M. Virta, Luminescent bacterial sensor for cadmium and lead. Biosens. Bioelectron. **13** (1998), pp. 931–938.

B. Tebo and A. Obraztsova, Sulfate-reducing bacterium grows with Cr(VI), U(VI), Mn(IV) and Fe(III) as electron acceptors. FEMS Microbiol. Lett. **162** (1998), pp. 193–198.

I. Turyan and D. Mandler, Selective determination of Cr(VI) by a self-assembled monolayer-based electrode. Anal. Chem. **69** (1997), pp. 894–897.

P. Vignais, B. Billoud and J. Meyer, Classification and phylogeny of hydrogenases. FEMS Microbiol. Rev. **25** (2001), pp. 455–501.

Figures

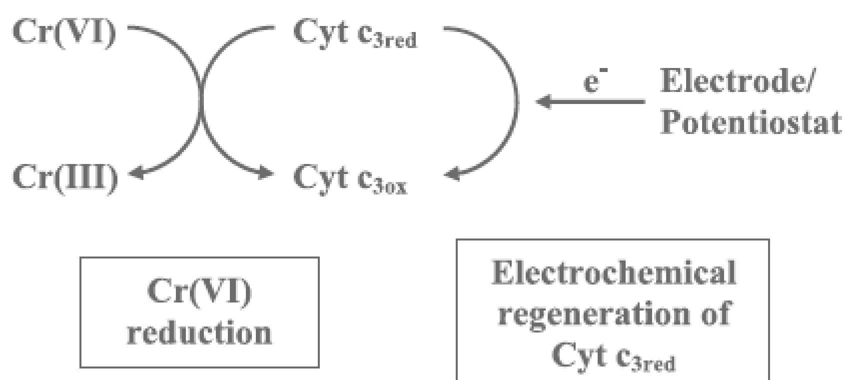

Figure 1: Principle of Cyt c_3-based biosensor recognition of Cr(VI): the Cyt c_{3red} regeneration current is proportional to the Cyt c_{3ox} content and therefore to the Cr(VI) content. The measured signal corresponds to the intensity of the regeneration current.

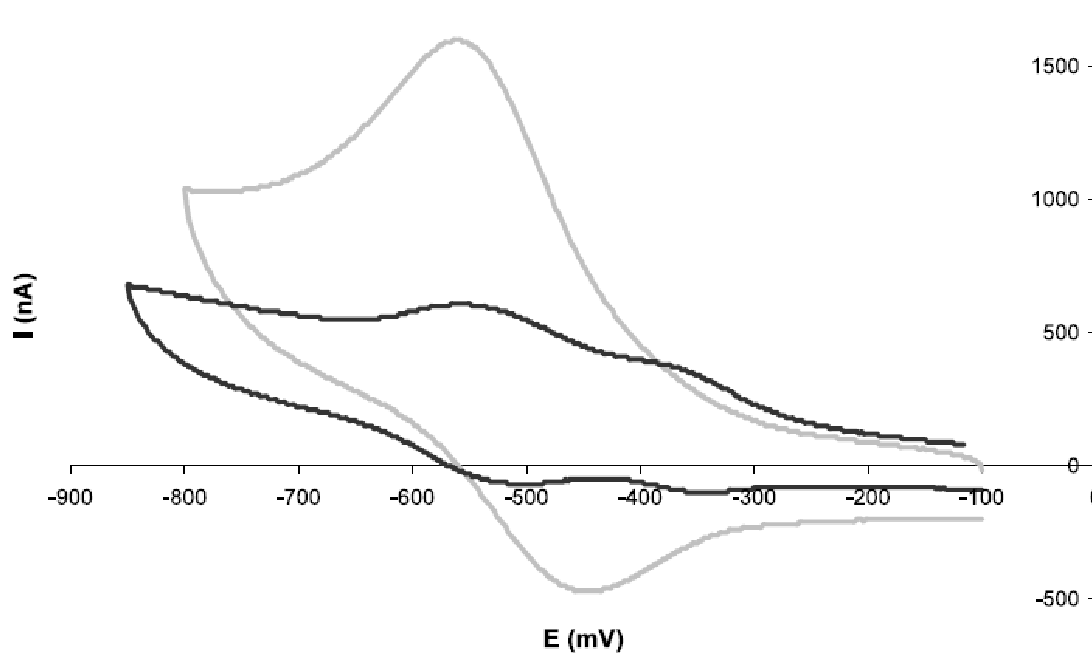

Figure 2: Cyclic voltammograms of the Cyt c_3-based biosensor in the absence (black line) and in the presence (grey line) of Cr(VI) (8.3 mg l^{-1}). Cyt c_3 was entrapped between a dialysis membrane and GCE. Scan rate: 20 mV s^{-1}.

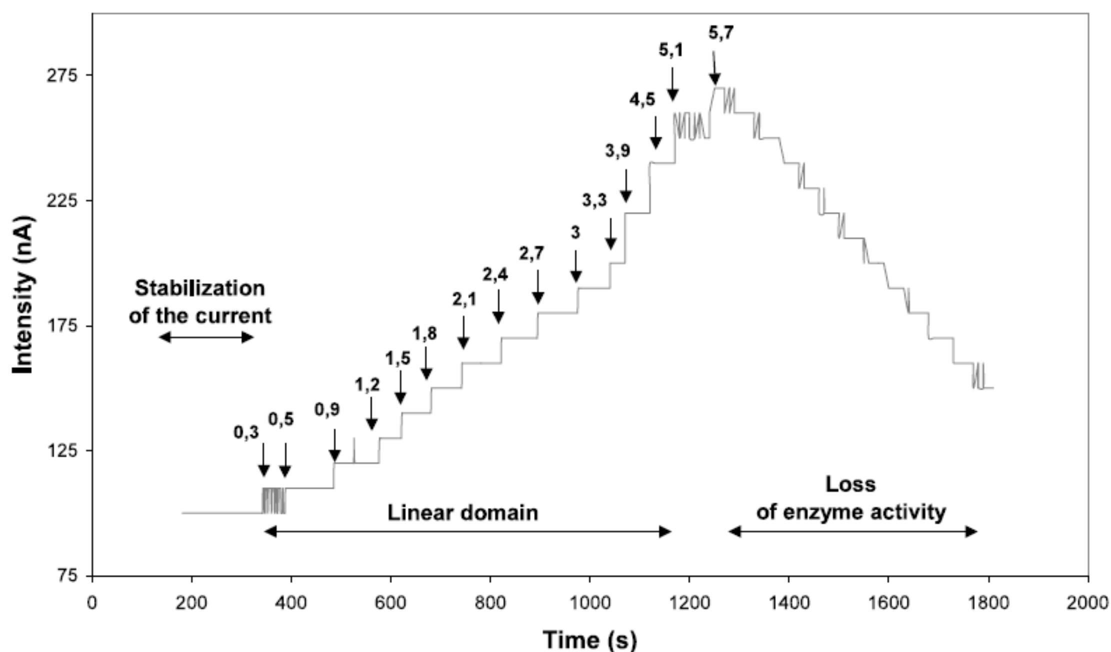

Figure 3: Chronoamperometry experiments showing the response of a Cyt c_3-based biosensor (47.5 µM Cyt c_3 entrapped with a dialysis membrane electrode) to Cr(VI) concentration changes. Variation of the current was recorded at −570 mV (the reduction potential of the enzyme). Arrows indicate chromate addition (milligram per liter).

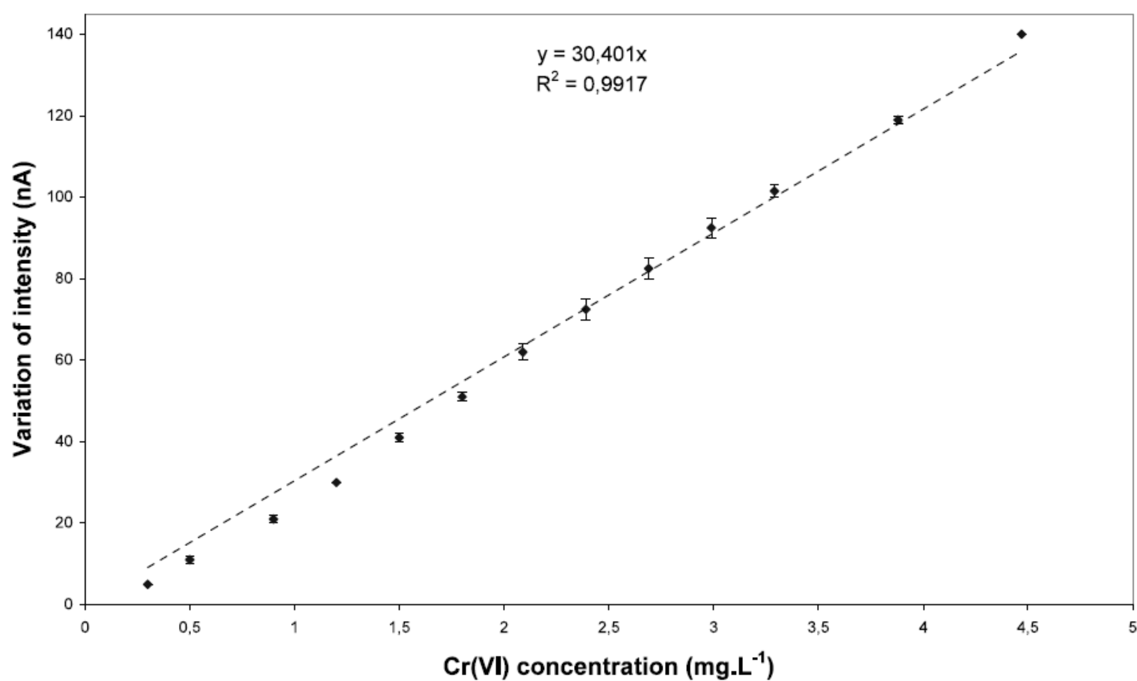

Figure 4: Calibration curve for a Cyt c_3-based biosensor (47.5 µM Cyt c_3 entrapped within a dialysis membrane electrode). The curve was obtained from the linear domain of the chronoamperometry experiments in Figure 3. A linear expression was calculated and the sensitivity determined for this biosensor was 30.40 nA mg^{-1} l.

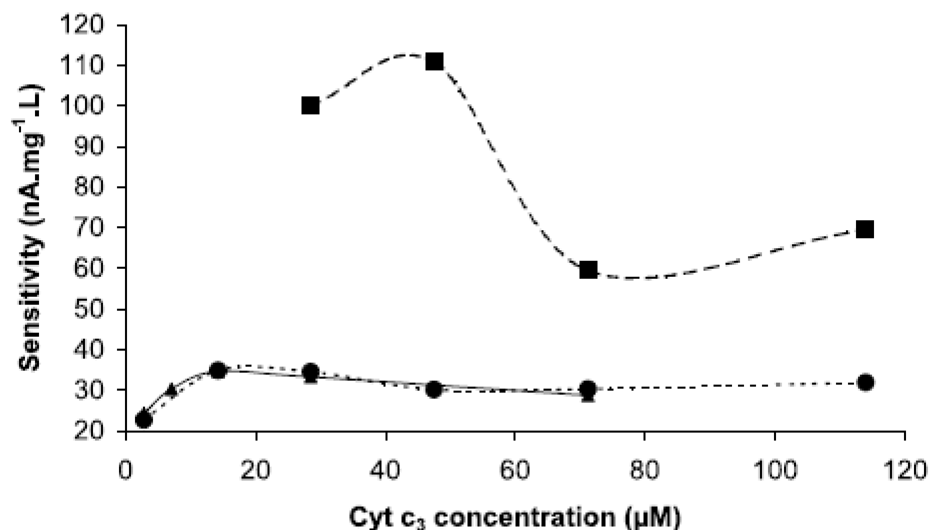

Figure 5: Sensitivity of biosensors as a function of Cyt c_3 concentration and various types of Cyt c_3 immobilization techniques: dialysis membrane (●), adsorption (▪) and adsorption+dialysis membrane (▲).

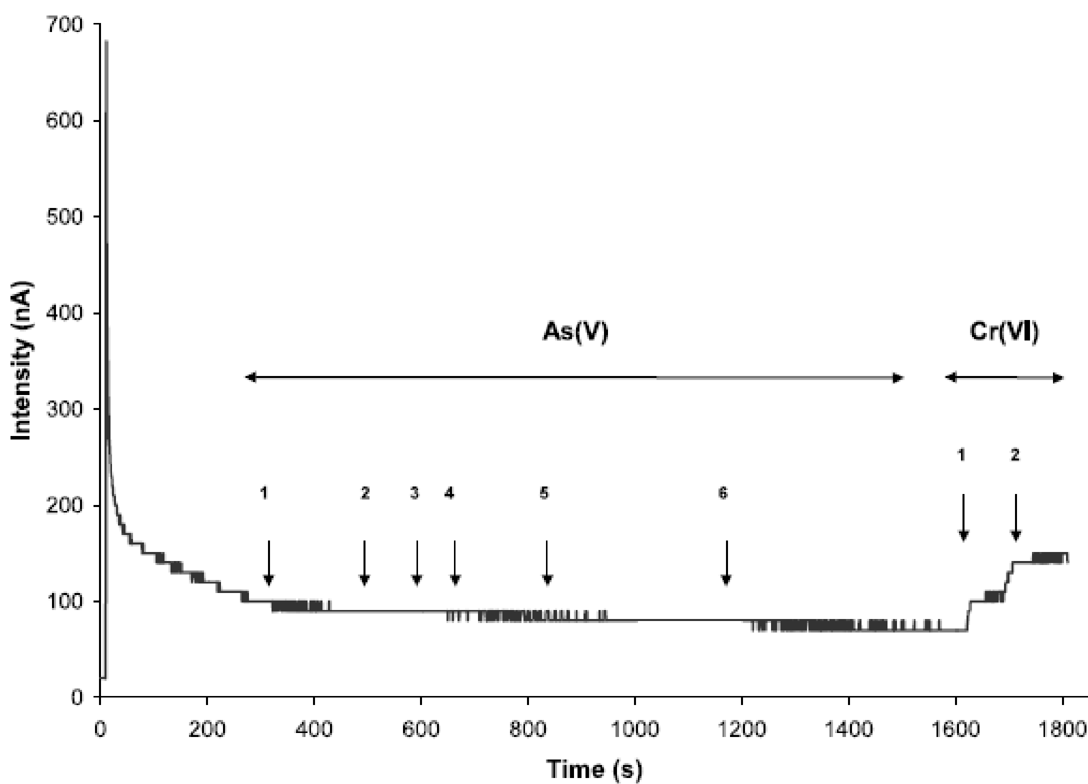

Figure 6: Specificity test. Chronoamperometry experiments with 71.25 µM Cyt c_3 entrapped with a dialysis membrane electrode. Variation of the current was recorded at −570 mV/SCE. Values above the vertical arrows indicate As(V) and Cr(VI) concentrations (mg l^{-1}).

Biosensors and Bioelectronics, 2003, 19(4), 345-352 , doi:10.1016/S0956-5663(03)00191-X

Tables

Table 1: Characteristics of biosensors according to Cyt c_3 immobilization techniques

Immobilization technique	[Cyt c_3] (µM)	Cyt c_3 stability	Sensitivity (nA mg^{-1} l)	Detection range (mg l^{-1})	Response time (s)
PEDT					
	14-114	No	[a]	[b]	[b]
Dialysis membrane					
	2.85	Yes	22.70	0.28-3.86	0-5
	14.3	Yes	34.90	0.28-3.74	0-5
	28.5	Yes	34.50	0.28-3.74	0-5
	47.5	Yes	30.20± 0.21	0.30-4.47	0-5
	71.25	Yes	30.30	0.20-4.38	0-5
	114	Yes	31.90±2.62	0.20-4.47	0-5
Adsorption					
	28.5	Yes	100	0.20-0.40	0-5
	47.5	Yes	110.70	0.20-0.60	0-5
	71.25	Yes	59.50	0.20-0.80	0-5
	114	Yes	69.50±0.52	0.20-0.80	0-5
Filter (0.01µM)					
	60	Yes	[b]	[b]	[b]
Filter (0.05µM)					
	60	Yes	[b]	[b]	[b]
Filter (0.1µM)					
	60	Yes	[c]	0.35-1.60	0-5
Adsorption + dialysis membrane					
	2.85	Yes	24.30±4.10	0.28-5.17	0-5
	7.1	Yes	30.30	0.20-4.18	0-5
	14.3	Yes	34.70±0.60	0.40-5.56	0-5
	28.5	Yes	33.30	0.80-6.35	0-5
	71.25	Yes	28.70	0.90-6.84	0-5

Experiments were performed in a Tris/HCl buffer (0.1 M, pH 7.6).
[a] *No S.D. means that the experiment was unique.*
[b] *No result could be obtained.*
[c] *Non reproducible experiment.*

Table 2: Influence of Tris concentration (pH 7.6) on biosensor properties (Cyt c_3 (71.25 µM) immobilized with a dialysis membrane)

Tris buffer pH 7.6 (M)	Sensitivity (nA mg^{-1} l)	Detection range (mg l^{-1})	Response time (s)
0.05	25.13	0.20-5.96	0-5
0.1	30.0	0.20-4.38	0-5
0.2	32.75	0.20-4.77	0-5
0.5	24.60	0.80-5.56	0-5