Feature Article

Engineered Bacteria Based Biosensors for Monitoring Bioavailable Heavy Metals

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Abstract
This work presents an integrated analytical system based on immobilized engineered microorganisms and bioluminescence measurements for monitoring of bioavailable heavy metal ions (Cu being chosen as a model ion). A strain of microorganisms from *Alcaligenes eutrophus* (AE1239) was genetically engineered by inserting a *luxCDABE* operon from *Vibrio fischeri* under the control of a copper-induced promoter. As a result, copper ions induce bioluminescence, which is proportional to the concentration of the triggering ions, representing the basis of the design of the hereby described heavy metal biosensor. Microorganisms grown in two different media (Luria Broth and a modified mineral reconstitution medium/RM) were optimized and characterized in solution with regard to the influence of growth media and cell density in order to obtain optimal bioluminescent signals. Next, the microorganisms were immobilized in polymer matrices, compatible with fiber optics and were characterized with regard to sensitivity, selectivity, detection limit and storage stability. The lowest detection limit (1 μM) was achieved with microorganisms cultivated from glycerol stock solutions in the RM media and immobilized in a calcium alginate matrix.

Keywords: Genetically engineered *Alcaligenes eutrophus*, Bioluminescence, Biosensor, Optical fibers, Heavy metal ions

1. Introduction

Analytical examination of heavy metals is needed when working with environmental problems since many polluted soils and wastes contain heavy metals at various concentrations. Selective and sensitive methods are therefore needed for both detection and quantification of these toxic compounds. However, toxicity is not always related to the total heavy metal concentration measured with various analytical methods (e.g., ion-selective electrodes). Presently, many analytical tests examine the total content of heavy metals and some of them are able to discriminate to a certain degree different heavy metals (e.g., MetPad) [1]. However, these methods can not discriminate between different bioavailable metal ions while simultaneously determining the total heavy metal ion content.

Recently, a marked interest has been shown for the use of microorganisms with fused *luxCDABE* genes for environmental monitoring and detection of toxicological contamination, e.g., heavy metal [1–3] nitrate [4] or oxidative hazards [5]. Biological cells offer several advantages over enzyme-based biosensors [6, 7]. Most importantly, analytical systems that require a sequence of biochemical reactions are greatly simplified by using cells, because all the reactions are conveniently packaged within the cells and thus, efficiently carried out. In addition, the enzymes necessary for the biorecognition are in their natural environment inside the cell. Whole cell biosensors are also able to distinguish pollutants that are available to biological systems from those that exist in the environment in an inert, precipitated or complexed, but not bioavailable form. This is of particular concern with respect to the toxic heavy metals [1, 2]. In most of the described cases, only suspensions of whole cells were used for biosensor construction [7–9]. Detection is made with commercially available luminometers but use of optical fiber has also been reported (see below).

An analytical system based on such engineered microorganisms integrated with highly sensitive optical fibers shows many practical advantages compared to measurements carried out with a luminometer, such as: most standard vials can not be placed in a conventional luminometer, while in contrast fiber optics allow i) measurements to be performed outside the sample compartment, ii) through the walls of a container of any size and shape, and iii) to analyze many vials simultaneously by making use of fiber bundles.

Immobilization of the cells is a crucial stage of a such optical biosensor design [1, 7, 9]. Alginate and agarose have previously been considered as suitable matrices for cell immobilization [10–12]. There is, however, only limited published work on the use of bioluminescence of immobilized genetically engineered bacteria [13–15]. The relationship between the viability of *luxCDABE* genes containing

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cells and their ability to produce light when immobilized, e.g., in alginate gel is not straightforward and seems to be very complicated [13]. The feasibility of using whole cells immobilized in matrices connected to a fiber optic was demonstrated for the detection of naphthalene and salicylate [16] but such systems were never reported for the detection of bioavailable heavy metal ions.

Therefore, the aim of the present work was, to show the principle and the feasibility of a heavy metal biosensor based on measuring the bioluminescence emitted by an engineered bacteria from *Alcaligenes eutrophus* with fiber optics. However, considering the novelty of the present work, the behavior of this engineered bacteria (both in solution and immobilized in various matrices) had to be investigated and optimized, in order to construct an integrated analysis system for monitoring of bioavailable heavy metals.

2. Experimental

2.1. Material and Methods

2.1.1. Cell Cultivation

In all cases, if not otherwise mentioned, solutions were prepared using HPLC-grade water produced in a Milli-Q system from Millipore, Bedford, MA, and all measurements were carried out at room temperature. *Alcaligenes eutrophus* strains (AE1239 and AE104) were grown both in Luria-Broth (LB) and a modified mineral reconstitution medium (RM) with 20 mg/L tetracycline hydrochloride (tet) (Duchefa, Harlem, Netherlands) as a selective pressure. The LB medium was prepared by adding the following components to 1 liter water: 10 g tryptone (Duchefa), 5 g yeast extract (Duchefa) and 10 g sodium chloride (Merck, Darmstadt, Germany). The RM was prepared by mixing the following components (quantities per liter): 4.18 g MOPS-NaOH (Merck), 4.68 g NaCl (Merck), 1.49 g KCl (Merck), 1.07 g NH_4Cl (Merck), 430 mg Na_2SO_4 (Merck), 200 mg $MgCl_2 \cdot 6\ H_2O$ (Riedel de Haën, Hannover, Germany), 30 mg $CaCl_2 \cdot 2\ H_2O$ (Merck), 294 mg β-glycerophosphate (Merck), 4.8 mg $Fe(NH_4)$-citrate (Merck) and 2 g Na acetate (Merck). Trace elements were added to the RM by including the following components (quantities per liter): 100 mg $MnCl_2 \cdot 4\ H_2O$ (Merck), 62 mg H_3BO_3 (Merck), 190 mg $CoCl_2 \cdot 6\ H_2O$ (Merck), 17 mg $CuCl_2 \cdot 2\ H_2O$ (Merck), 24 mg $NiCl_2 \cdot 6\ H_2O$ (Merck), 36 mg $Na_2MoO_4 \cdot 2\ H_2O$ (Merck) and 144 mg $ZnSO_4 \cdot 7\ H_2O$ (BDH Chemicals Ltd. Poole, Dorset, England). Both media were autoclaved before use.

The freeze dried AE1239 (cultivated by VITO, Mol, Belgium) had been stored more than 12 months and showed long cultivation time; 50 h to reach an optical density (OD) at $\lambda = 600$ nm (OD_{600}) of 1 due to less viable cells. Therefore, glycerol stocks (20% v/v) were made by adding glycerol (Merck) to a bacterial culture (OD_{600} close to 1), that had been inoculated by freeze dried cells and cultivated in LB + tet (20 μg/mL) medium. One mL of the bacterial solution was dispensed in Eppendorf tubes and kept at $-80\,^{\circ}C$ until use.

The thawed bacterial solutions were used as inoculum in 100 mL of media (LB + tet and/or RM + tet) and showed a shorter cultivation time (18 h to reach $OD_{600} \approx 1$). The cells were aerobically cultured in baffled Erlenmeyer flasks closed with cotton stoppers, in a rotary shaker at 30 °C. The OD_{600} of the cultures was used as a measure of the cell density.

2.1.2. Noncomplexed Cu^{2+} Measurements

A sample of 25 mL was adjusted for ionic strength with 0.5 mL M $NaNO_3$ (p.a.). A copper(II) ion selective electrode (Model 9629, Orion, Cambridge, England) was immersed in the constantly stirred sample and the potential for nil Cu^{2+} concentration was measured. Next, 125 μL of 100 mM $CuSO_4 \cdot 5\ H_2O$ were added to the sample, measuring again the potential. All measurements were repeated twice, if not otherwise mentioned.

2.1.3. Cell Immobilization

Alginate and agarose were considered as suitable matrices for the immobilization of AE1239. When immobilizing in calcium alginate, first 50 mL of fresh bacterial suspension in LB + tet ($OD_{600} = 0.8$) were centrifuged at 6000 rpm for 10 min. Next, the supernatant was removed, the pellet was washed in 20 mL 0.9% NaCl and centrifuged again at 6000 rpm for 10 min. The supernatant was discarded again and the washing step was repeated. Finally, the pellet was resuspended in 20 mL of 0.9% NaCl and added to 20 mL of 4% sodium alginate (BDH). A syringe (ONCE, Pharma-Plast A/S, Rødby, Denmark) was filled with the cell/alginate suspension and droplets were created by injecting the suspension through a needle (ID 1.2 mm) into 200 mL of 0.2 M $CaCl_2$ to form beads of alginate gel. The beads were washed twice with 0.9% NaCl and kept in the same saline solution at 4 °C until use.

When immobilizing AE1239 in Seaplaque agarose (gelling temperature: 26 – 30 °C, Duchefa), first, 50 mL of a fresh bacteria suspension (LB + tet) were centrifuged at 6000 rpm for 10 min. The supernatant was removed, the pellet washed with 20 mL of 0.9% NaCl, and centrifuged once more at 6000 rpm for 10 min. Then, the pellet was resuspended in 9 mL of 0.9% NaCl. Next, agarose (2.22 g) was dissolved in 100 mL of 0.9% NaCl under heating. Finally, the solution was cooled to 30 °C using a water bath, and 5 mL of bacteria suspension were added under stirring. A 2 mm thick gel was produced. The gel was kept at 4 °C in 0.9% NaCl solution until use.

2.1.4. Selectivity Measurements

Only immobilized cells were investigated for selectivity as follows: alginate beads (approximately 0.5 g) were resuspended in a certain volume of RM + tet to which

calculated volumes of stock solutions of $ZnSO_4 \cdot 7\ H_2O$ and $CuSO_4 \cdot 5\ H_2O$ were added.

2.1.5. Analysis Systems-Biosensor Design

Cells in solution: A certain volume of cells cultured either in LB + tet or in RM + tet (both 20 μg/mL) was added to different stock solutions of $CuSO_4 \cdot 5\ H_2O$. The bioluminescence signal was registered with a Luminometer for 10 seconds and light intensities were expressed in mV units.

Immobilized cells: When measuring bioluminescence of immobilized cells, alginate beads (approximately 0.5 g) or a disk of agarose gel (diameter 4 mm, height 2 mm) were placed in each tube and suspended in a certain volume of media (LB + tet used for alginate and agarose gels and RM + tet used only for alginate gel). Then, volumes of different stock solutions of copper were added to the tubes and the signals expressed as arbitrary units (bioluminescence per gram gel, mV/g) were measured using a luminometer.

Immobilized cells integrated with fiber optics-biosensor: A cylindrically shaped in-house built flow cell (diameter 10 mm, height 2.2 mm), was modified by creating a little chamber in which the immobilized microorganisms were packed. Cu^{2+} solutions in RM + tet media were pumped through the cell at a flow rate of 1 mL/min using a peristaltic pump. Photons were collected by a fused quartz optical fiber bundle (8 mm diameter) and were evaluated in a fiber optic photon counter connected to a computer. The bioluminescent intensities (photon counts) were measured by the optical detector every minute and expressed as arbitrary units (a.u.).

2.2. Instrumentation

A commercial LKB luminometer (type 1250, LKB Wallac, Bromma, Sweden) was used to quantify the bioluminescence. In-house made soda glass test tubes (ID 8.9 mm; height 45.2 mm) were used as reaction vials for experiments with bacteria both in suspension and immobilized in gel. Light intensities were expressed in mV, and were proportional to the intensity of the inner standard. OD measurements were carried out using a spectrophotometer (type UV-120-02, Shimadzu, Kyoto, Japan). The peristaltic pump used in the integrated fiber optic experiments was of type C-4V (Alitea, Gothenburg, Sweden), the optical fiber was from Livany glass factory, Latvia, and the fiber optic photon counter was purchased from PreSens (Precision Sensing, Regensburg, Germany).

Average values obtained for three equally prepared samples are presented throughout this work, if not otherwise mentioned.

3. Results and Discussion

Resistance to several heavy metal ions was reported for *Alcaligenes eutrophus* CH34 (AE CH34) which is a facultative chemolithotropic, gram-negative soil bacterium and carries two megaplasmids, named pMOL28 (165 kb) and pMOL30 (240 kb), containing the genes for multiple resistance to heavy metals [17, 18]. The pMOL28 megaplasmid ensures the resistance to cobalt, nickel (*cnr* operon), chromate (*chr* operon) and mercury (transposon Tn4378), while pMOL30 is responsible for the resistance to cadmium, cobalt and zinc (operon *czc*), mercury (transposon Tn4380), copper, lead and tallium [19].

To operate as a selective biorecognition unit, the multiresistant strain had to be modified to produce a detectable signal when exposed to individual heavy metals. For this reason, the observed multiresistance had to be divided in separate derivatives of AE CH34 [17, 18] and provided with a reporter system able to produce a detectable signal [1, 2]. The fusion of heavy metal resistant genes with the *luxCDABE* operon, involving luciferase genes, was used as a reporter system. A strain named AE1239 was genetically engineered to emit light in the presence of Cu^{2+}. The work presented here is reporting on experiments based on AE1239, the structure of which is schematically presented in Figure 1.

The bacteria was genetically constructed with a megaplasmid (pMol 90), which carries copper inducible genes (see Fig. 2A), and a transposon (Tn 4431), containing a gene encoding for an antibiotic (tetracycline) resistance and a *luxCDABE* operon (see Fig. 2B). This operon descends from the marine bacteria *Vibrio fisheri* [1, 2, 7]. Its five structural genes are responsible for encoding luciferase (*luxAB*) and the three companion enzymes: reductase (*luxC*), transferase (*luxD*), and synthetase (*luxE*). The companion enzymes catalyze the biosynthesis of a long-chain fatty aldehyde [6]. The light-emitting reaction in bacteria with fused *luxCDABE* genes involves the oxidation of reduced flavin mononucleotide ($FMNH_2$) and of a long-chain fatty aldehyde in the presence of molecular oxygen [20, 21]:

$$FMNH_2 + RCHO + O_2 \xrightarrow{luciferase} FMN + H_2O + RCOOH + h\nu\ (490\ nm)$$

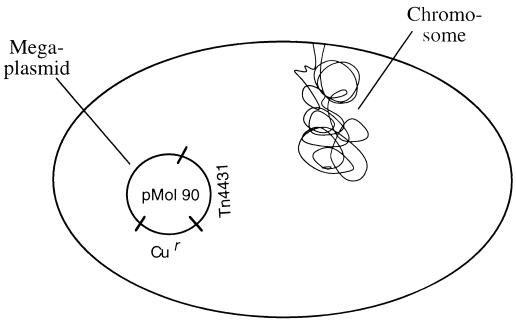

Fig. 1. Schematic structure of the Alcaligenes eutrophus 1239 cell.

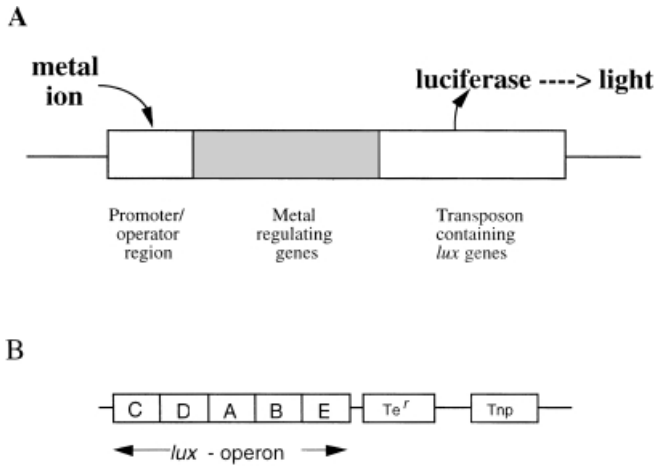

Fig. 2. Schematic presentation of the A) detection principle using the engineered plasmid with the transposon 4431 which contains the B) *luxCDABE* operon from Vibrio fisheri.

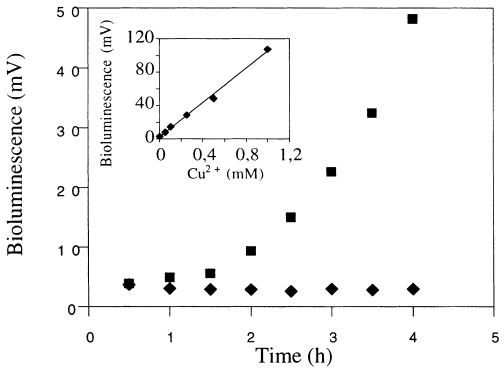

Fig. 3. Bioluminescence obtained for AE1239 in LB + tet suspension (◆) in the absence Cu^{2+} and (■) in the presence of 0.5 mM Cu^{2+}. Insert: calibration plot recorded after 4 h incubation.

In order to construct an analysis system comprising the engineered bacteria-based biosensor integrated with fiber optics, the biosensor design had to be optimized first. Therefore, the hereby reported work focused on the following aspects: i) comparison between engineered and non-engineered microorganisms, ii) study of the influence of the cultivation media and optical density, iii) screening of various matrices for bacteria immobilization in order to obtain an integrated analysis system, iv) characterization of the developed copper biosensor, and v) integration of this sensor with optical fibers. Cu^{2+} was chosen as a model heavy metal ion for all experiments.

3.1. Influence of the *luxCDABE* Genes on the Bioluminescence Signal

In order to confirm the decisive role *luxCDABE* genes are playing in the light emitting process upon addition of Cu^{2+}, the bioluminescence emitted by AE 1239 and AE104 (plasmid-free host) was measured. AE 104 lacking the *luxCDABE* genes, did not display any bioluminescence, while increasing signals with increasing time were recorded for AE 1239 (genetically fused with *lux* genes) in the presence of Cu^{2+} (results not shown).

3.2. Influence of the Cultivation Media and Optical Density

It was expected that the cultivation media containing different nutrients will greatly affect the bioluminescence signal. Therefore, two different media, LB and RM were considered in this work also studying the effect of the addition of some extra nutrients, such as riboflavin and tryptophan, to the much less nutrient containing RM media. The chemical composition of the RM media was previously optimized [2] and the use of MOPS as buffer agent and acetate as energy supply resulted in the best compromise between a sufficient energy supply for the cells and the absence of metal chelation.

Cells grown in LB medium were found to emit light exceeding the background level approximately 1 – 1.5 h after addition of the inducer Cu^{2+} (see Fig. 3). Light emission continued to increase gradually for at least 24 hours (not shown). Bioluminescence values obtained after 4 h growth were chosen for the evaluation of the response of the microbial biosensor [1], as a compromise between the intensity of the recorded signals and analysis time. The observed signals strongly depended on the concentration of the inducer ion. The correlation between copper concentration and light emission is depicted in the insert of Figure 3, showing an increase of the signal with increasing copper concentration. The suspension based copper biosensor exhibited a linear range up to 1 mM Cu^{2+} and a detection limit (calculated as twice the signal-to-noise ratio) of 40 μM.

As previously mentioned, whole cell biosensors are sensitive to bioavailable metal ions. The high concentration levels of copper (up to millimolars) detected in LB might be attributed to the higher complexation of Cu^{2+} in LB medium compared to the RM medium. The bacterial behavior in the less nutrient RM medium (containing only 1% of LB nutrients, such as amino acids, vitamins, etc.) supported this assumption. Figure 4 shows the signals obtained for cells cultivated and incubated in LB + tet and in RM + tet, respectively. At copper concentrations of 1 – 30 μM cells grown in RM displayed an approximately 3-fold higher signal, attributed to a less complexation level of copper ions in the RM medium, resulting in higher sensitivities at lower concentrations of copper but also an inhibition at copper concentrations above 30 μM.

To check the complexing ability of the different media, additional Cu ion measurements were done using ion-selective electrodes. 6 different LB + tet solutions were prepared with tryptone and yeast extract from 3 companies (Difchamb, Difco and Oxoid) and signals were recorded after addition of $CuSO_4$. No significant difference was

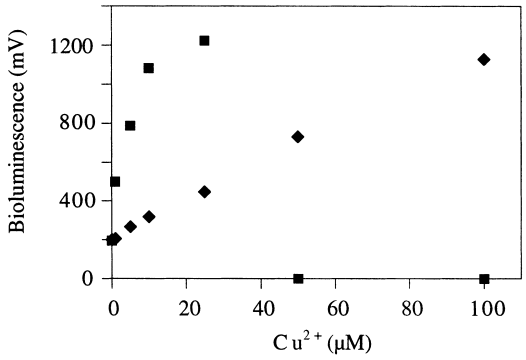

Fig. 4. Bioluminescence obtained for AE1239 in (◆) LB + tet and in (■) RM + tet; the signal being recorded after 4 h incubation with different concentrations of Cu^{2+}.

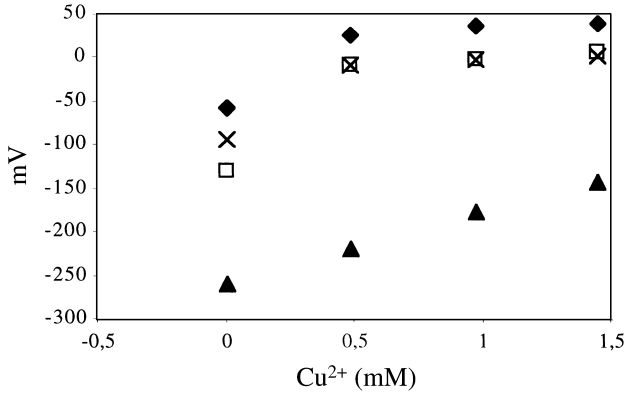

Fig. 5. Signals recorded for Cu^{2+} in (◆) water, (×) RM, (□) RM + tet, and (▲) the mean of 6 signals obtained in different LB + tet. All signals were detected with a copper selective electrode at room temperature.

obtained between the Cu^{2+} signals from the different batches (results not shown), therefore, the average of the results was compared with the responses obtained for water, RM and RM + tet (see Fig. 5). As seen the signal was 25-fold lower in LB than in RM, thus, supporting the assumption that less soluble copper ions were present in LB, probably due to chelating of copper ions with components present in the LB medium.

The effect of riboflavin and tryptophan addition to the RM medium was considered only for immobilized bacteria, since it was previously shown that responses of the *luxCDABE*-based biosensors depended on the endogenous concentration of flavins present in the yeast extract [22]. The dependence of the signal, recorded for the biosensors based on bacteria immobilized in alginate beads (exposed to 10 μM Cu^{2+}) on the concentrations of riboflavin and tryptophan (amino acids present in the yeast extract) added to RM is shown in Figure 6. As seen, the bioluminescence intensity is highly dependent on the concentration of these two components, especially on the concentration of riboflavin. Thus, the necessity of using a standardized composition of nutrient medium is evident.

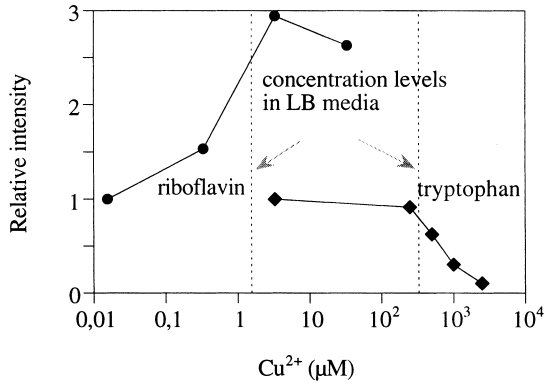

Fig. 6. Dependence of the bioluminescence signal on the concentration of (●) riboflavin and (◆) tryptophan recorded for AE1239, immobilized in calcium alginate, re-suspended in RM + tet and incubated with 10 μM Cu^{2+}. The relative intensity is calculated as the ratio between the intensities recorded for microorganisms with and without additions of riboflavin or tryptophan.

The bioluminescence signal is strongly dependent on the concentration of the cells (OD_{600}) in both media, the optical density being measured at 600 nm. Maximum light signals were obtained in the range of 0.4 – 0.5 and at 0.1 of OD units for AE 1239 in LB + tet and RM + tet, respectively.

It was concluded, accordingly, that the less nutrient RM medium is optimal when developing biosensors with a lower detection limit and hence, used for further experiments focusing on the integration of the copper sensor with fiber optics.

3.3. Screening of Various Matrices for Bacteria Immobilization

Several matrices, such as polyethylene imine (PEI), polyacrylate, agarose and calcium alginate were considered. Bacteria immobilized in PEI (1%) yielded bioluminescence signals but only for copper concentrations exceeding 0.5 mM, and a linear range up to 2 mM (results not shown). Bacteria immobilized in polyacrylate did not yield any signal. Both matrices can complexate copper ions, which might explain the above mentioned observations. Since both calcium alginate and agarose were previously found to be useful matrices, our attention was further focused only on these two matrices.

Figure 7 shows the signals obtained for bacteria immobilized in A) agarose and in B) calcium alginate, re-suspended in LB + tet and 0.5 mM Cu^{2+}, as a function of time, the inserts showing the signal dependence on the concentration of the triggering ion. Both biogels exhibited a linear response in the range of 0 – 250 μM and detection limits of 50 and 20 μM for alginate beads and agarose disks, respectively (2S/N). Figure 7C shows the signal obtained in RM + tet, incubated with 25 μM of Cu^{2+} as a function of time, the insert presenting the signal-concentration dependence. The alginate-immobilized biosensors exhibited a more

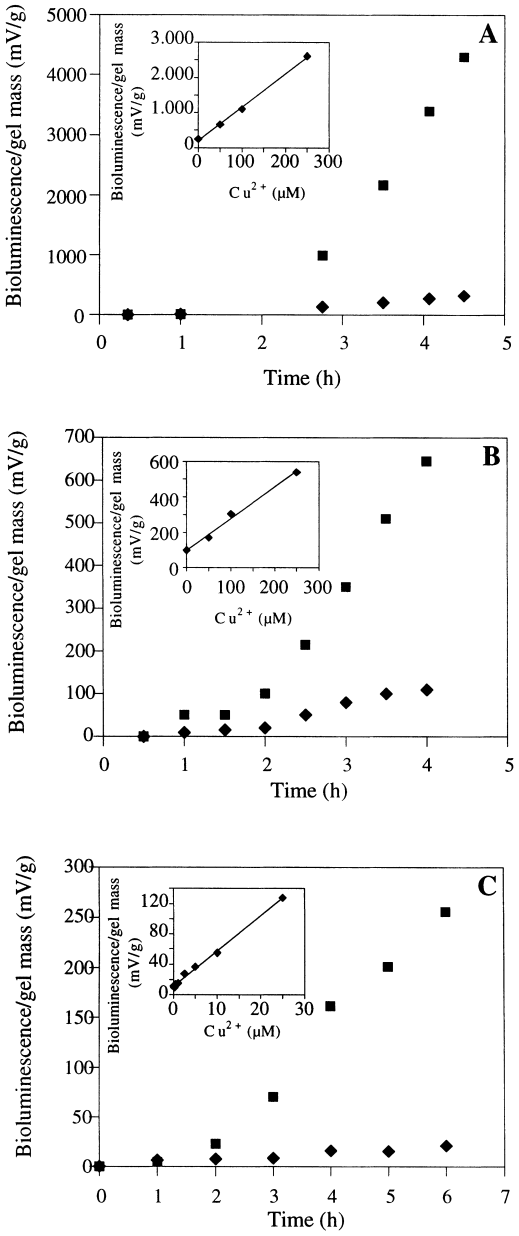

Fig. 7. Bioluminescence obtained for AE1239, immobilized in A) agarose re-suspended in LB + tet (◆) in the absence of Cu^{2+} and (■) in the presence of 0.5 mM Cu^{2+}; B) alginate re-suspended in LB + tet (◆) in the absence of Cu^{2+} and (■) in the presence of 0.5 mM Cu^{2+}; and C) alginate re-suspended in RM + tet (◆) in the absence of Cu^{2+} and (■) in the presence of 25 μM Cu^{2+}. Inserts show the obtained calibration plots after 4 h incubation.

restricted linear range of 0–25 μM, and a detection limit of 1 μM (2 S/N)

The main conclusion arising from experimental data obtained for bacteria in suspension and immobilized in the two kinds of gels is the similarity of the biosensor characteristics, which can be explained by the similar ability of the microorganism to get nutrition and oxygen under the given experimental conditions. It is known, that diffusion coefficients for hydrogels with immobilized bacteria are almost the same as for those obtained in water [12, 14]. Thus, the use of gels instead of suspensions was considered in biosensor design aiming integration with fiber optics. The use of alginate gel was also preferable due to its higher storage stability.

3.4. Biosensor Characteristics

Alginate and agarose immobilized AE1239 where characterized with regard to relative signal intensity, linear range, detection limit, storage stability, and selectivity.

The obtained results (see Table 1) showed that the relative intensity (the ratio between the signal obtained for 0.5 and 0 mM Cu^{2+}, respectively) of the microorganisms in suspension were higher than those observed for the immobilized ones in agarose (30% higher) and alginate (100% higher) using the LB medium. Opposite, when using RM medium, the relative intensity (the ratio between the signal obtained for 25 and 0 μM Cu^{2+}, respectively) obtained for the alginate immobilized microorganisms were twice as high as the one noticed for suspended cells. Generally, the signals obtained from cells in suspension were almost three fold higher when using LB, while the ones obtained in RM media showed an opposite trend, the alginate immobilized microorganisms yielded signals twice as high as the ones in suspension. The relative standard deviations (RSD) calculated at 0.5 mM Cu^{2+} concentration and after 4 h incubation time, were higher for the microorganisms in LB medium (suspension; 19.5, alginate; 18.4, and agarose; 6.5) than the ones obtained for the RM medium (suspension; 0.2 and alginate; 7.5).

The largest linear range was displayed by the bacteria in suspension (LB media, up to 1 mM) while the immobilized ones (both in alginate and agarose, LB) displayed 4 times lower ones (up to 250 μM). When RM media was used, interestingly, the linear range of the sensor was extended (up to 25 μM) compared to the one for cells in suspension (up to 10 μM).

Concerning the detection limits displayed by the various sensor configurations, the RM media re-suspended ones (both in suspension and immobilized in alginate) displayed the lowest detection limit, 1 μM (2S/N), while the LB based ones were ten fold higher (40 μM for suspension, 20 μM for alginate and 50 μM for agarose, respectively).

Table 1. Relative signals obtained in different matrices where the relative signals are the ratio between the bioluminescence responses obtained at 0.5 and 0 mM Cu^{2+}, respectively. Both signals were measured after 4 hours incubation at room temperature.

Matrix	*Relative signal*	
	LB + tet	*RM + tet*
In LB/RM media	16	6.3
Immobilized in agarose	13	no data
Immobilized in alginate	7	12

The activity of the bacteria immobilized in alginate was almost unchanged for six days, while the activity of those immobilized in agarose (both re-suspended in LB + tet) decreased about seven fold during the same period. However, a two week long study of the storage stability showed a total loss of activity for both type of biogels.

Accordingly, alginate was chosen optimal immobilization matrix considering the better storage conditions and RM + tet was chosen optimal re-suspension media for the biosensor monitoring Cu^{2+} at low concentrations. On the other hand, the use of alginate as an immobilization matrix may have some disadvantages when measuring bivalent ions for a longer period of time. An exchange of Ca^{2+} and bivalent metals within the matrix may lead to either a destabilization of the alginate beads or an accumulation of bivalent metals within the alginate, being thus biologically unavailable for the bacterial sensor.

The selectivity of the copper biosensor was also considered. AE1239, immobilized in calcium alginate and re-suspended in RM + tet, was exposed to different concentration of Cu^{2+} or Zn^{2+}. (see Fig. 8). With copper as a trigger ion the relative signal showed an increase up to about 25 μM followed by a decrease, whereas the use of Zn^{2+} as a trigger ion showed no significant change in signal within the range of the assay (0–50 μM). This indicates that AE1239 is selective for Cu^{2+} in the mentioned concentration range. A full characterization of the biosensor with regard to its selectivity to other heavy metal ions, such as Cd, Pb, Hg, and Ni is beyond the scope of this work. Current experiments are considering the full examination of the hereby presented biosensors, and also the study of other strains selectively engineered for the individual heavy metal ions.

3.5. Integration with Optical Fibers

The experimental set-up is shown in Figure 9. The biogels were placed in the chamber of the flow cell, and copper solution was pumped through the cell, recording the bioluminescence signals. The change in signal was very similar

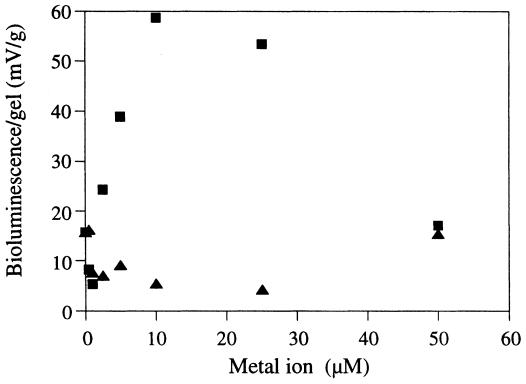

Fig. 8. Selectivity of AE1239 cells immobilized in alginate, re-suspended in RM + tet and incubated with different concentrations of (■) copper and (▲) zinc. The bioluminescence signals were recorded after 5 h incubation with the metal ions.

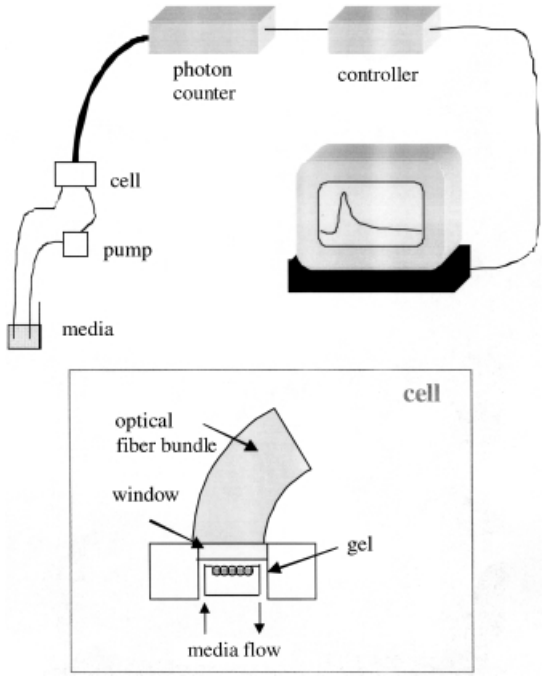

Fig. 9. Experimental set up of the integrated biosensor-fiber optics system.

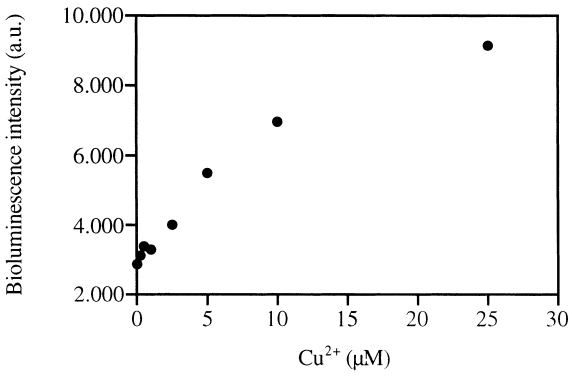

Fig. 10. Typical calibration plot obtained for AE1239, immobilized in alginate and re-suspended in RM + tet, after 4 h incubation in the integrated system. Other experimental conditions: flow rate 1 mL/min, room temperature, pH 7.0.

to the one observed for the biosensor design based on immobilized cells and re-suspended in RM + tet (data not shown). A typical calibration plot derived from bioluminescence signals collected by the optical fiber is shown in Figure 10.

The obtained results proved the possibility of measuring bioavailable heavy metals with an integrated analytical system containing the immobilized engineered microorganisms and fiber optics. Such an integrated system is expected to be very useful for field analysis. Considering the progress made in the field of genetical engineering the feasibility of the described sensor is obvious, opening up the possibility of developing an entirely novel family of biosensors.

4. Conclusions

The described engineered bacteria-based biosensors were not developed to detect the total amount of metal ions present in a sample. Dissolved ions can be easily detected using ion-selective electrodes. The main advantage of the biosensor presented in this work is that they sense the biologically available fraction of the metal present in a sample. This fraction may be higher than the dissolved fraction since the bacterial cells are readily able to take up metal ions by an active process. The feasibility and potential to measure the bioavailable heavy metal fraction has been clearly demonstrated in this work. The biosensors based on microorganisms immobilized in calcium alginate and agarose displayed almost the same sensitivities and linear ranges as their suspensions. The use of gels instead of intact cell suspension facilitates, however, handling and hence, is desirable when designing an integrated analytical system of practical use. The composition of media, is critical, in terms of additives such as vitamins and amino acids, and hence, must be standardized.

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6. References

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