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Bacteria immobilization for a bioluminescent biosensor

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Microbial biosensors provide detection of specific compounds with rapid and simple operations. In order to enhance the handling, the miniaturization and stability of the biosensor, bacteria immobilization is desirable. This paper proposes a suitable immobilization system for a microbial biosensor detecting pollutants in water. Agarose, alginate and poly-vinyl-alcohol were tested. As there is a lack of systematic studies in literature, this work tried to evaluate the effects of immobilization on biosensing performances, through comparison with suspended bacteria. Analyte detection, storage stability and the possibility of re-using immobilized bacteria were investigated.

Key words: **Bioluminescence - Immobilization - Membrane - Microbial biosensor.**

The worldwide biosensor market is quickly growing: at the end of 2003 it was 7.3 billion dollars and it is expected to grow of 10% in 2007.[1] In particular, microbial biosensors provide rapid measurements, no need for complex sample preparation and stability in wide operating conditions.[2] Bacteria immobilization is essential for the development and commercialization of a biosensor, since it provides a close proximity between the cell and the transducer, it enhances miniaturization, stability and handling. Bacteria may be immobilized using different methods, such as adhesion, adsorption, entrapment.[2-4] All these techniques are currently used in biosensor technology; the choice of the best one for the specific application depends on the microorganism, on the analyte, on the transducer and on the configuration employed.

The aim of this work was to set up an immobilization system suitable to bioluminescent biosensors for pollutant detection in water. For this purpose, a recombinant bioluminescent strain *Ec::lux*AB TBT3 was employed, for the detection of tributyltin chloride (TBT).[5, 6] In order to select the suitable immobilization system for the biosensor, a systematic characterization procedure, evaluating physical, chemical and biological properties, was carried out in a previous work.[7] Agarose, alginate and freeze-thawed poly (vinyl alcohol) were then chosen as immobilization matrices. This paper illustrates the results obtained with bacteria immobilized in microtiter plates, as a first step for the development of a real biosensor. Analyte detection, reuse and storage stability were tested and results were compared with those obtained with suspended bacteria.

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Materials and methods

Chemicals

Alginate solution was prepared by dissolving 2% w/w sodium alginate (Satialgine S170 Degussa texturant systems, France) in distilled water.

Agarose low melting point was supplied by SoBiGel (France). The solution was prepared by dissolving 2% w/w of agarose in distilled water at 70 °C. This solution has a jelling temperature of 25 °C (measured by Perkin Elmer DCE calorimeter).

PVA poly (vinyl alcohol) (98-99% hydrolyzed, M_w=85 000-146 000 kDa) was purchased from Sigma Aldrich (France) and it was dissolved in distilled water at 80 °C in order to obtain homogeneous solutions at different concentrations (5% and 10% w/w).

Calcium chloride 1% w/w solution for alginate gelation was prepared by dissolving CaCl2 dehydrated (Fluka chemicals) in distilled water.

Glucose medium for *Ec::lux*AB TBT3 [6] was prepared with tap water, filtered with 0.45 mm membranes (Millipore) and the following composition: 1.376 g/L D(+)glucose monohydrate, 0.192 g/L NH_4Cl, 0.028 g/L K_2HPO_4, 5 g/L NaCl, 0.5 g/L yeast extract, 1 g/L tryptone. pH was adjusted at 7 and the medium was sterilized at 100 °C for 30 min. The medium was then completed with 0.01% v/v of SL7 solution, having the composition: 10 mM HCl (25%), 0.5 mM $ZnSO_4 \cdot 7H_2O$, 0.5 mM $MnCl_2 \cdot 4H_2O$, 1 mM H_3BO_3, 0.8 mM $CoCl_2 \cdot 6H_2O$, 0.1 mM $CuCl_2 \cdot 2H_2O$, 0.1 mM $NiCl_2 \cdot 6H_2O$, 0.15 mM $Na_2Mo4 \cdot 2H_2O$. Antibiotics were sterilized by filtration through a 0.22 µm membranes (Millipore) and added to all media, to the following final concentrations: 10 mg/L Tetracycline (Fluka) and 40 mg/L Ampicilline (Sigma).

Decanal solution, required to start luminescence reaction, was prepared with 840 mM N-Decyl aldheide (Sigma Aldrich, France) in distilled water and 2.4% v/v isopropanol.

TBT stock solution (600 µM) was prepared with Tributyltin monochloride (Sigma Aldrich, France) with distilled water and 60% of ethanol at 70%, then serial dilutions between 0.015 µM and 15 µM were prepared with synthetic seawater at pH 8.2 (Instant Ocean, France).

Bacteria immobilization

*Ec::lux*AB TBT3 was grown in continuous culture with glucose medium in a special conceived bioreactor.[5] Since all the assays were performed in parallel with immobilized and free bacteria, two inocula of microbial suspensions were drawn at the same time from the reactor and centrifuged for 5 min at 5 000 g (Biofuge PrimoR Heraeus). Then the first set was suspended in the polymer solution at 35 °C, while the second set was suspended in $MgSO_4$ 0.1 M. After homogenization, both solutions were poured and let solidifying in microtiter plates (100 µL for each well). Analogously, 1 µL of the solutions were poured in spectroscopy cuvettes to measure optical density by UV spectroscopy at 620 nm. In this way the cellular density was evaluated, attaining results in agreement with the theoretical values calculated (15-20% error).

Microtiter plates containing bacteria in alginate were immersed in a $CaCl_2$ bath for gelification. Microtiter plates with agarose were left 10 min at 30 °C for solidification, while those with PVA were kept in the refrigerator at 4 °C for 1 h, then frozen at -20 °C and thawed after 16 h.

For some tests, immobilized bacteria were reacclimated adding 50 µL of glucose medium to each well and incubating the microtiter plate at 30 °C for 30 min.

Analyte detection

Fifty microliters of TBT solution at different concentrations were added to microtiter wells, then suspended bacteria were induced for 1 h and immobilized bacteria for 2 h, at 30 °C. For each set of tests control samples were prepared with 7% ethanol in synthetic seawater. Twenty-five microliters of N-Decyl aldheide solution was then added to every well, in order to start the luminescence reaction. Light signal was measured by microtiter plate luminometer (Microlumate L96V EGG Berthold). Every measurement was carried out on 12 wells and every assay was repeated in triplicates.

Stability

Storage tests: microtiter plates with immobilized bacteria were sealed and stored at 4 °C, then induced with TBT.

Reuse tests: after the first induction, the wells containing immobilized bacteria were regenerated, by washing with $MgSO_4$ 0.1 M solution, then induced again with TBT solutions.

Results

Analyte detection

In order to evaluate the performances of a luminescent biosensor, two parameters were investigated: the signal intensity and the induction ratio.

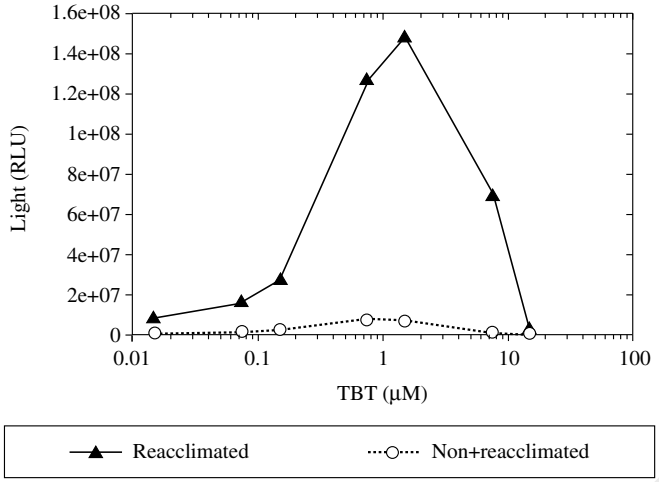

Figure 1.—TBT induction: light signal emitted by bacteria immobilized in agarose, acclimated and non acclimated.

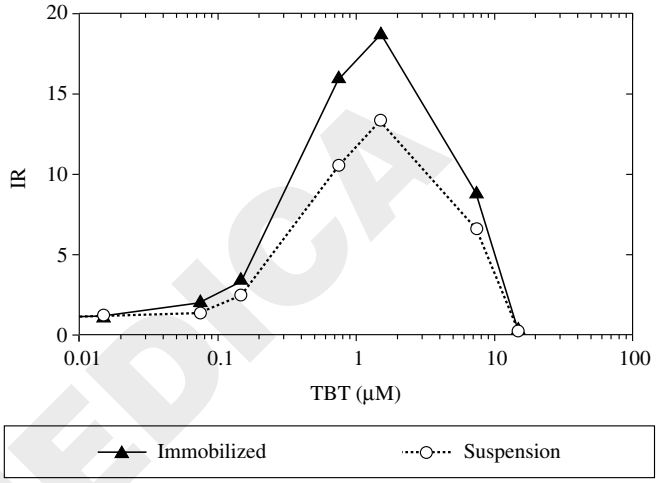

Figure 2.—TBT induction: Induction Ratio with bacteria in suspension and immobilized in agarose (acclimated).

The signal intensity is registered by the commercial luminometer as RLU/s (relative light unit/s) along time. In this study, the profile registered along time was integrated in the measurement interval (30 min), in order to quantify the total luminescence emitted by the bacteria (measured in RLU). This integral approach allows comparing the luminescence of different systems, independently from the process kinetics, which may be influenced by diffusion limitations through the polymeric matrix.

The Induction Ratio (IR), defined as $IR=RLU_{ind}/RLU_0$, where RLUind is due to the induced sample and RLU0 is due to the control sample, not induced.[5, 8] As both samples were always drawn from the same batch of bacteria, this ratio is practically independent from the physiological state of the cells and it quantifies their response to the analyte. IR is employed for the semi-quantitative assessment of the analyte.

Both parameters were herein studied to assess the effects of immobilization on the biosensor operation and to optimize the procedures in order to enhance the performances. Experiments for TBT detection were firstly carried out with bacteria immobilized in agarose. The effects of induction time, reacclimation step and cell concentration were investigated.

It was observed that an acclimation step with glucose medium enhanced the luminescence activity of immobilized bacteria. As it is shown in Figure 1, 30 min acclimation at 30 °C resulted in a 20-fold increase of signal intensity, while the induction ratio kept almost unchanged.

This phenomenon may be due to rehydration phenomena or simply to substrate requirement for luminescence reaction. Immobilized bacteria thus acclimated were compared with suspended bacteria: the former attained RLU values four times lower than free bacteria (data not shown), probably because of diffusion limitation through the matrix, which may be limited by reducing the thickness. However, light intensity emitted by immobilized bacteria was still far above luminometer detection limits, and induction ratios were comparable with those from suspended bacteria (Figure 2). The same experiments were carried out with the other polymers: alginate and freeze-thawed PVA. Some of the results, obtained with the same cellular density, are resumed in Figure 3. The induction profile of bacteria in alginate was shifted towards higher TBT concentrations, resulting in a detection limit of 150 nM, higher than bacteria in agarose. This may be due to a hindered diffusion of the analyte, because of adsorption phenomena on the anionic polymer. Bacteria in PVA showed a flat induction profile, with a detection limit between 75 nM and 150 nM and low values of induction ratio. This may be due to insufficient reacclimation of the immobilized bacteria after the thawing and/or to insufficient induction time with TBT. A large difference among the three matrices was also observed in terms of reproducibility: for agarose standard deviation values varied between 9% and 20%, for alginate

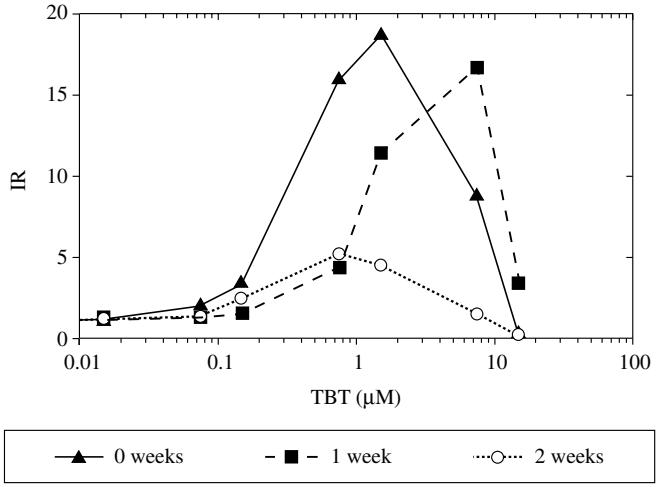

Figure 3.—Comparison of induction profile due to bacteria immobilized in three different polymers: agarose, alginate and PVA.

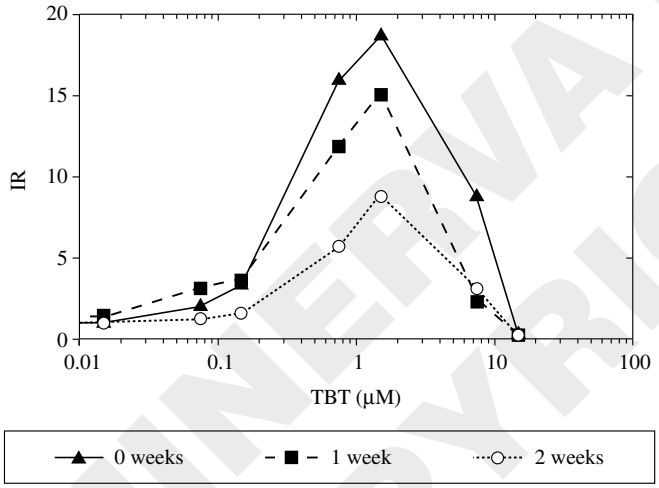

Figure 4.—Induction profile of bacteria immobilized in agarose, after 0, 1 and 2 weeks storage.

between 9% and 35% and for PVA between 20% and 40%. However reproducibility should be further improved.

Stability

In order to be widely employed and commercialized, biosensors should show high operational and storage stability. These characteristics are specific for immobilized bacteria, since free bacteria may neither be re-used for repetitive measurements, nor be stored

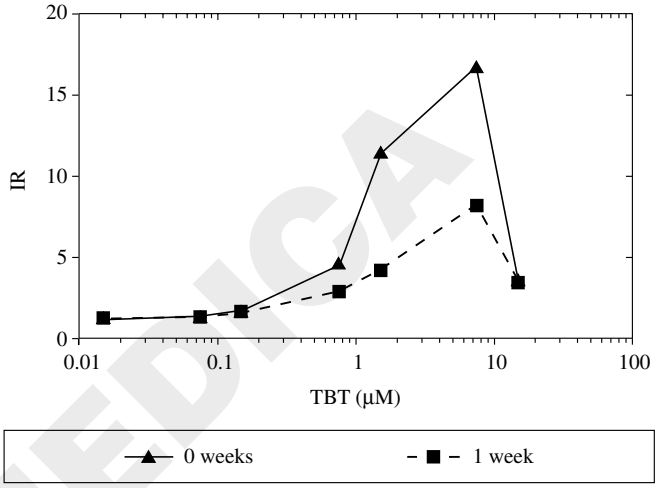

Figure 5.—Induction profile of bacteria immobilized in alginate, after 0, 1 and 2 weeks storage.

in refrigerator for several weeks (they can be stored only after being freeze-dried, which is not suitable to a biosensor). The evaluation of stability properties therefore allows pointing out the specificity and the advantages of immobilization. Two tests were carried out: re-use tests, to investigate the possibility of performing repetitive measurements with the same membrane containing bacteria, storage tests, to evaluate the shelf-life of membranes containing bacteria. As a matter of fact, during storage bacteria should be inactive and non-growing, but they must be able to quickly recover their activity and to be ready for measurements.

STORAGE TEST

The viability loss of bacteria immobilized in alginate stored at 4 °C was insignificant after 1 week, of 5% after 2 weeks and of 78% after 3 weeks. The latter dramatic loss may be avoided in the future with further optimization of both storage process (sealing and progressive cooling) and preconditioning of immobilization bacteria (reacclimation in glucose medium and progressive warming). However, up to 2 weeks storage, the agarose-immobilized bacteria kept similar induction profile and detection limit, while the slope and the values of induction ratios decreased (Figure 4). To take into account the changes in IR values, the biosensor should always be calibrated before any measurement of an unknown

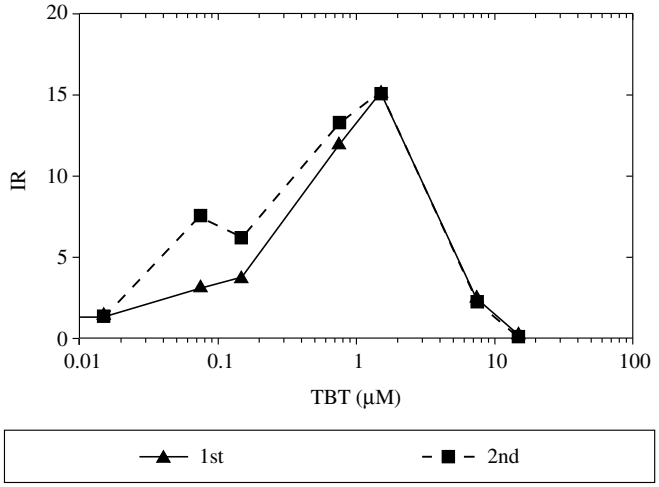

Figure 6.—Reuse test: induction profiles of bacteria immobilized in agarose, due to the first measurement (1st) and the second measurement (2nd), after washing.

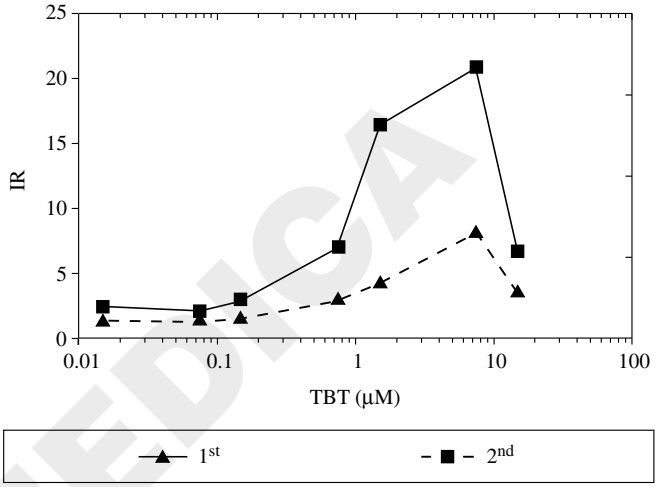

Figure7.—Reuse test: induction profiles of bacteria immobilized in alginate, due to the first management (1st) and to the second measurement (2nd), after washing.

sample. Bacteria immobilized in alginate showed no loss in viability after 1 week, nevertheless the induction profile resulted very different from the original one and detection limit was significantly reduced (Figure 5). Finally, bacteria in PVA showed a viability loss of 53% after one-week storage, which was unacceptable.

Reuse tests

Immobilized bacteria after 1-week storage were employed for reuse tests. As it can be observed in Figure 6, bacteria in agarose during the second measurement after regeneration gave a detection limit and an induction profile close to the first measurement. This means that these membranes can be employed for at least two consecutive measurements. Alginate gave negative results, since the induction profile and the detection limit of the first and the second measurements were completely different (Figure 7). This may be due to the adsorption of TBT on the alginate matrix, due to its free carboxyl groups; nevertheless, this hypothesis should be validated with further investigation and analysis.

Discussion and conclusions

In order to develop an environmental biosensor for the detection of Tributyltin in water, bioluminescent bacteria were immobilized in agarose, alginate and freeze-thawed PVA. Acclimation of immobilized bacteria with glucose medium enhanced luminescence activity, so that immobilized bacteria showed a good inducibility and an induction ratio, comparable with suspended bacteria.

Agarose showed the best results in terms of biocompatibility and signal emission. While suspended bacteria cannot be neither regenerated nor stored for longtime, it was proved that immobilization allows the reuse and the storage: the same membrane containing bacteria could be used for repetitive measurements and stored in refrigerator, with evident cost reduction, compared to a disposable kit. Finally, through immobilization, reproducibility may be enhanced, since a stock of membranes may be easily prepared from the same microbial culture, guaranteeing constant physiological state and activity. However, further studies are in progress to test the performances of a real biosensor in continuous mode, with real samples of seawater.

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