SESSION 4: ENVIRONMENTAL APPLICATIONS

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BIOLUMINESCENT WHOLE–CELL BIOSENSORS

Current legislation in the EU requires that water quality and the degree of contamination are assessed using chemical methods (European Groundwater Directive). Such methods do not consider the synergistic or antagonistic interactions that may affect the bioavailability and toxicity of pollutants in the environment (Dewhurst et al., 2002). Bioassays are methods for assessing the toxic impact of whole samples on the environment and are also methods of choice for screening environmental samples before going onto detailed chemical analyses that can be time consuming, expensive and do not allow monitoring (Forget et al., 2000). The utilization of organisms possessing lux – genes gained significant importance during the last decade once the toxicity bioassays have been recognized as essential tests along chemical analyses (Farre et al., 2003). The widely used marine photobacterium Vibrio fischeri is a self – maintained luminescent unit. The level of in vivo luminescence reflects the metabolic rate of luminous bacteria and the integrity of the bacterial cells (El–Alawi et al., 2002).

In resent years, the development of whole–cell biosensors has found increasing interest on the one hand due to the possibility of whole cells to convert complex substrates using specific metabolic pathways (D'Souza, 2001) and on the other hand due to potential applications of whole–cell biosensors for the monitoring of typical sum parameters, that cannot be monitored using enzyme–based sensors, such as toxicity (Farre et al., 2003), biological oxygen demand (Jia et al., 2003) xenobiotic compounds (Beyersdorf–Radec et al., 1998) or heavy metals (Petanen et al., 2002). The opportunity to module metabolic activities of specific cells can additionally be used for drug screening (Wu et al., 2001a) and combinatorial approaches for drug discovery (Durick et al., 2001). Additionally, microbial biosensors have been successfully applied for the specific determination of single components such as e.g. glucose, fructose, xylose and alcohols (Baeumner, 2003). A general advantage of microbial biosensors is that living cells are continuously repairing their integrated enzyme activities and enzyme cascades. This is a clear advantage in comparison to biosensors based on labile biological recognition elements (e.g. enzymes). On the other hand, the development of such biosensors allows the use of immobilized organisms that maintain their physiological status and thus the results obtained will represent the natural responses.

In comparison to the general field of biosensors where more than 11,000 papers have been published up to date, it is obvious that microbial biosensors are an emerging field in analytical chemistry and biotechnology. More than 90% of the č300 published articles appeared during the period 1995–2003. Most of the papers published up to date deal with schemes where the sample is incubated in a medium that contains microbial cells and measurement is achieved through classical analytical chemistry techniques. Only a small part of these papers exploit cell immobilization to facilitate the biological recognition element (microbes) and the detection technique in a single device. Although cell immobilization techniques were widely investigated over the last decades, immobilization of bioluminescent bacteria for application in biosensors is a relatively new research area (Belkin, 2003). Bioluminescent bacteria have been immobilized in alginate, agar, PVA, latex copolymer and sol–gel matrices. It is interesting to note that for Vibrio fisheri and P. leiognathi used for toxicity assessment there is no published application utilizing them in immobilized form. A further search on the literature shows that few articles describe the implementation of these devises in automated analysers for the construction of integrated analytical instrumentation for real sample analysis and monitoring (Han et al., 2002).

Flow injection is the only widely accepted technique for laboratory automation with more than 10,000 published articles. Variants of this mature technology introduced by Ruzicka's team in Seattle include Sequential injection and Lab–on Valve technologies (Wu et al., 2001b).

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Paper presented as a lecture.

This article describes the development of a fully automated flow injection analyser based on a bioluminescent whole cell biosensor unit.

MATERIALS AND METHODS

The HC135–01 photomultiplier (PMT) from Hammamatsu and cell immobilization techniques previously developed (Bugarski et al., 1993) are used for the development of the biosensor unit. An Ismatec IPC–8 peristaltic pump, a VCI valco C22Z–3186EH injection valve, a personal computer and the Lab–View object oriented programming language were used for the development of the flow injection analyser.

RESULTS AND DISCUSSION

Biosensor unit development

Light detection device: PMT's are the most sensitive devices for the detection of low level optical signals. An integrated PMT tube, incorporating the high voltage source and divider circuit along with an RS 232 signal output was selected resulting in a compact biosensor device.

Flow cell: Due to the use of a PMT detector, a wall–jet flow cell configuration was implemented. Fig. 1 depicts the design of the prototype that is based on previous studies (Divritsioti et al., 2003). The assemply was clamped between two stainles steel plates featuring appropriate openings for light detection and passage of the In and W PTFE tubes of 0.8 mm internal diameter. Cell species that exhibit bioluminescence and are sensitive to toxic stimuli are immobilized on the P surface. Although increased stability of immobilized bacteria is expected, biosensor unit design facilitates easy change of the low cost, disposable flow cell in order to renew the sensing cells.

Temperature control of the flow cell: To maintain immobilized bacteria at their optimal temperature, the flow cell is enclosed in a thermostated aluminium frame.

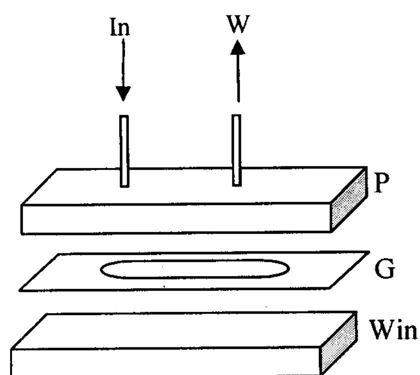

Figure 1. Flow cell design. In: carrier solution feed; W: waste; P: plexiglas plate with immobilized cells; G: Gasket; Win: optical window from Plexiglas or Quartz (PMT detector is opposite). Dimensions after assembly: 2 cm x 1 cm x 1 cm.

Beyond a water circulator, a peletier based electrothermostating system is been evaluated in order to develop a portable instrument.

Flow injection analyser development and optimisation

Implementation of the biosensor unit was achieved through a laboratory made flow injection analyser (Fig. 2). The analyser design is based on the continuous flow of a carrier solution that contains the appropriate nutrient solution for bioluminescent cells immobilized in the biosensor unit's flow cell. The sample is automatically injected in the carrier solution by a four way valve and transported to the biosensor unit where the flow is stopped for the assessment of the sample's toxic effect to the immobilized cells. The analytical protocol is fully automated and controlled by the computer that is also used for data acquisition from the biosensor unit (Georgiou et al., 1991). Parameters for optimisation are: a) flow rate (typically in the range of 0.2 to 3 mL/min); b) volume of the injected toxic sample (typically in the range of 0.020 mL to 0.2 mL); c) carrier solution (nutrient solution suitable for each individual cell species) composition for optimal bioluminescence exhibition; d) monitoring time to asses the toxicity to the immobilized cells (typically in the range of 5 – 15 min). To increase sample through put, the possibility of using shorter contact times through appropriate changes of the other parameters will be assessed. It should be pointed out that serial dilutions required for the determination of EC50 are automatically achieved by a single injection using concentration gradients formed along the flow injection peaks (Georgiou et al., 1999).

Data acquisition & control interface and software: Appropriate subroutines were developed in Lab–View for a) data acquisition through the RS–232 port of the PMT, b) control of the pump and the injection valve through a relay interface card utilizing the computers digital output, c) the graphic user interface facilitating user input and presentation of results, d) storage to data files and d) data treatment.

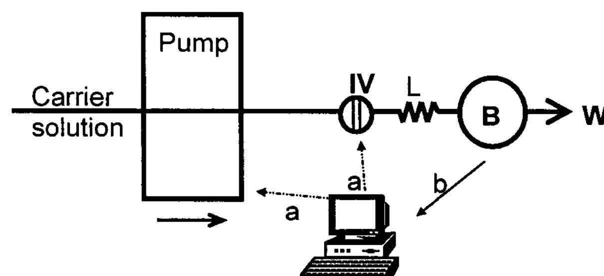

Figure 2. Automated flow injection analyser for biosensor implementation. B: biosensor unit; IV: injection valve; L: mixing coil; W: waste; a: digital control signals; and b: data acquisition line.

Analyser operation: The carrier solution is propelled through the biosensor unit yielding a signal that represents the cells' healthy state. When a toxic stimulus (pollutant) is injected through the injection valve, cells are affected exhibiting lower luminescence levels. The drop in the detected signal represents the response to the pollutant.

CONCLUSIONS

The fully automated analyser developed facilitates kinetic monitoring along single point (fixed time) measurements. It is anticipated that interesting results concerning toxicity assessment will come through monitoring the "rate" of toxic effects of individual samples to cells.

The biosensor based analyser will be evaluated by analysis of synthetic/model aqueous samples of known toxicity containing different dilutions of:

1. Pesticides/insecticides: carbamates (manozeb, zineb, carbendazim etc); organophosphates (methyl–parathion, carbaryl, fipronil, bifenthrin, indoxacarb etc) and triazines (atrazine, simetryn, irgarol etc).

2. Phenolic compounds: Olive mill waste water phenolics (tyrosol, hydroxytyrosol, caffeic acid, oleuropein etc) and chlorophenols.

3. Heavy metals, alcohols, chlorobenzenes, and aromatic hydrocarbons.

Beyond single compounds, binary mixtures of compatible compounds will also be assessed in order to detect any synergistic or antagonistic interactions.

The analyzer will be further evaluated through analysis of waters, municipal waste waters, landfill leachates, and agricultural waste effluents from the olive oil (alpechin), wine and milk industries.

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