JOURNAL OF MATERIALS SCIENCE: MATERIALS IN MEDICINE 2 (1991) 202–204

Fibre optic biosensors with immobilized bioluminescence enzymes

L. J. BLUM, S. M. GAUTIER, P. R. COULET
Laboratoire de Génie Enzymatique, UMR 106, CNRS, Université Lyon 1, 43 Bd du 11 Novembre 1918, F-69622 Villeurbanne Cedex, France

A fibre optic biosensor involving immobilized bioluminescence enzymes associated with a glass fibre bundle has been designed. The firefly luciferase from *Photinus pyralis*, as well as the bacterial luciferase–oxidoreductase system from either *Vibrio fischeri* or *Vibrio harveyi*, have been immobilized on preactivated polyamide membranes. First, the validity of the approach is demonstrated by performing the microdetermination of adenosine triphosphate (ATP) and sodium dehydrogenase (NADH) in a batch system. The detection limits are equal to 0.1 nM for ATP and 0.3 nM for NADH. With the NADH-based system, the extension of the biosensor potential to other analytes (ethanol, sorbitol and oxaloacetate) has been achieved using suitable dehydrogenases co-immobilized with the bacterial system. Second, the fibre optic biosensor is associated with a specially designed flow cell for the continuous-flow bioluminescent assay of NADH over the range 2 pmol–1 nmol with an r.s.d. of 3.4% at 0.1 nmol. Finally, a multifunction biosensor for the determination of either ATP or NADH using a single bioluminescence-based fibre optic probe is described. This was made possible by co-immobilizing the firefly luciferase with the bacterial system on the same preactivated polyamide membrane.

1. Introduction

Fibre optic biosensors are devices involving an immobilized biological active material on the end of a single optical fibre or a fibre bundle. Immobilized enzyme membranes have been utilized in our group for several years for the design of enzyme electrodes. With the requirement of new highly selective and ultrasensitive biosensors, we considered the use of luminescent enzyme systems associated with optical transducers. The monitoring of light emission occurring during bioluminescence reactions offers strong advantages when considering its analytical potential in terms of sensitivity, dynamic range and detection limit. Although the potential of immobilized bioluminescence reagents has been widely explored in the past decade [1], little attention has been paid to their use in convenient devices. In the novel type of fibre optic sensor we developed, the membrane-bound biocatalysts were placed in close contact with the tip of a fibre bundle. The light emitted at the membrane level during the bioluminescent process was transmitted to a photomultiplier tube allowing the quantification of adenosine triphosphate (ATP) or reduced nicotinamide adenine dinucleotide (NADH). The main developments concerning the use of our bioluminescence-based fibre optic sensor with either a batch or a continuous-flow system are presented.

2. Bioluminescence reactions

The firefly luciferase catalyses the production of light in the presence of ATP, Mg^{2+}, molecular oxygen and a specific substrate, luciferin (Reaction 1)

$$\text{ATP} + \text{luciferin} + O_2 \xrightarrow[Mg^{2+}]{\text{firefly luciferase}} \text{AMP} + \text{oxyluciferin} + \text{PPi} + CO_2 + \text{light} \quad (1)$$

($\lambda_{max} = 560$ nm)

Under appropriate reaction conditions, the light intensity is proportional to the ATP concentration.

In the marine bacteria system used for analytical purposes, the light is produced by two consecutive enzymatic reactions

$$\text{NAD(P)H} + H^+ + \text{FMN} \xrightarrow{\text{oxidoreductase}} \text{NAD(P)}^+ + FMNH_2 \quad (2)$$

$$FMNH_2 + O_2 + \text{R–CHO} \xrightarrow{\text{bacterial luciferase}} \text{FMN} + \text{R–COOH} + H_2O + \text{light} \quad (3)$$

($\lambda_{max} = 490$ nm)

In Reaction 2, catalysed by the NAD(P)H:FMN oxidoreductase, $FMNH_2$ is produced and then oxidized in the second reaction (Reaction 3) catalysed by a luciferase to produce light in the presence of molecular oxygen and of a long-chain aldehyde (R–CHO). When NAD(P)H is the limiting substrate of this bi-enzymatic system, the light intensity is proportional to NAD(P)H concentration. The two most useful light-emitting enzyme systems were isolated from *Vibrio harveyi* and *Vibrio fischeri*.

0022–2461/91 $03.00 + .12

3. Enzyme immobilization on synthetic membranes

Immobilized bioluminescence enzymes from both the firefly and bacteria have been obtained by several groups during the last decade and used with existing or modified analytical devices. Characteristics and performances of such systems have been reviewed in detail [1, 2] and it appears that most of the supports used are gel matrices or beads. In our laboratory, membranes have been chosen because of the handiness of such a support. First, we explored the potential of immobilized luminescence systems on collagen and polyamide membranes [3–5]. Then we developed fibre optic biosensors more suitable for using immobilized enzymatic light-emitting systems. The immobilization procedures for the covalent binding of enzymes on these synthetic membranes are briefly presented.

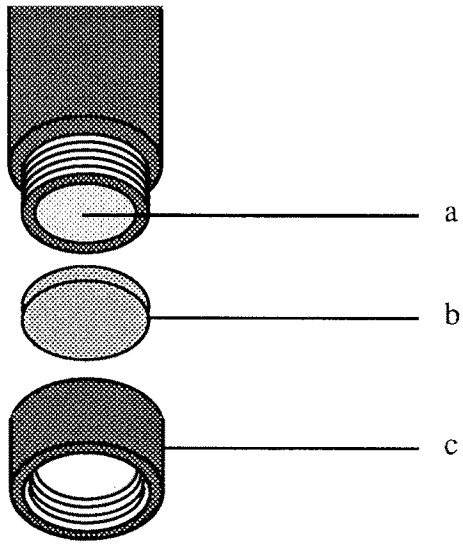

Figure 1 Sensing tip of the fibre optic biosensor. a, fibre bundle; b, enzymatic membrane; c, screw-cap.

3.1. Covalent binding on collagen membranes

Collagen films were supplied in the form of flat sheets of 15 cm × 18 cm. Strips of 1 cm × 4.5 cm cut from a collagen film were used for the immobilization of bioluminescence enzymes. Activation and coupling were performed according to the general procedure previously described [6]. The collagen activation process implies the transformation of surface-available carboxyl groups from lateral chains of aspartate and glutamate residues into acyl-azide. The method consists of three consecutive steps: esterification for 3–4 days at 20–22 °C, in a hydrochloric acid/methanol mixture, treatment overnight in a 1% hydrazine solution followed by soaking in a nitrous acid solution prepared extemporaneously. Then, the membrane is ready for enzyme coupling which must be done immediately.

3.2. Covalent binding on preactivated polyamide membranes

White polyamide membranes (immunodyne type) supplied in a preactivated form for immunodiagnostic use have been selected and adapted in our group for enzyme immobilization [7]. Covalent binding of enzymes was performed by simply applying 10 µl of an enzymatic solution on each side of a disc (10 mm diameter) cut out of the membrane. The reaction was complete after 1 min at room temperature.

4. Bioluminescence-based fibre optic sensors

4.1. Design of the fibre optic sensor

The fibre optic sensor consisted of a 1 m long glass fibre bundle, 8 mm diameter. One end of the bundle was connected to the photomultiplier tube of a Berthold Biolumat LB 9500 luminometer [8]. At the other end, an enzymatic membrane was maintained in close contact with the surface of the bundle by a screw-cap (Fig. 1). For batch analysis, the sensing tip of the sensor was immersed in a stirred and thermostatted reaction medium of 4.5 ml. To avoid ambient light interference, the reaction vessel was surrounded by a light-tight polyvinyl chloride jacket and the samples were injected through a septum. For continuous-flow measurements, the screw-cap was removed and a specially designed flow-cell, constructed in such a way that ambient light did not interfere, was fixed at the sensing tip [9]. Stirring was performed in the flow-cell and samples were injected in a reagent stream using a syringe through a light-proof septum (Fig. 2).

4.2. Performances

When used in a batch system, the fibre optic sensor equipped with the suitable enzymatic membrane allowed ATP measurements in a linear dynamic range from 0.1 nM–1.4 µM. The response-time of this biosensor varied from 1 min to 6–7 min at 3 µM [10].

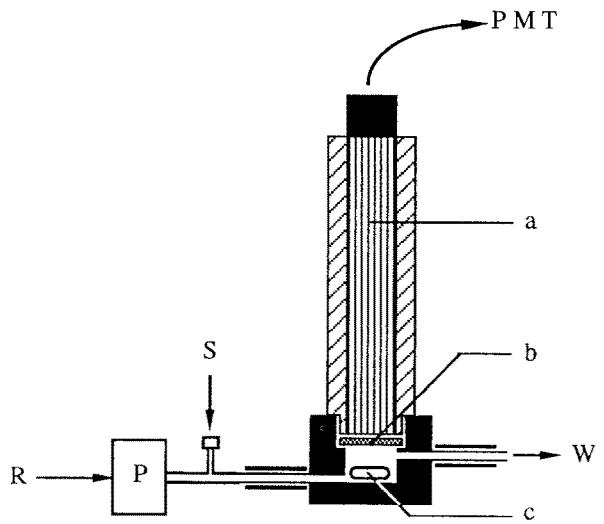

Figure 2 Schematic representation of the fibre optic continuous-flow system. P M, photomultiplier tube; R, reagent/carrier stream; P, peristaltic pump; S, sample injection, W, waste; a, fibre bundle; b, enzymatic membrane; c, stirring bar.

Much attention has been paid to the NADH fibre optic sensor because of the interest of microdetermination of this analyte in clinical analysis and special emphasis was put on stability which is one of the essential key-points in designing biosensors. For measurements performed with the bioluminescence system from *V. fischeri*, the calibration graph was linear from 1 nM–3 μM with a detection limit of 0.3 nM [11]. The response-time was 1–3 min depending on the NADH concentration. However, using this bacterial bioluminescence system, a recalibration had to be done every ten assays. On the contrary, although the immobilized system from *V. harveyi* exhibited a lower activity, its excellent operational stability allowed 100 assays to be performed within a day without loss in activity [12].

In order to facilitate automation, we have developed a continuous-flow method for the bioluminescent determination of NADH using the fibre optic sensor [9]. The assay was linear from 2 pmol–1 nmol NADH with a precision of 3.4% for 10 pmol and 25 samples/h could be measured with no carryover. Owing to the very good operational stability of the immobilized enzymes from *V. harveyi*, no loss of activity was observed after 150 assays performed in a 3 day period.

4.3. Extension of the fibre optic biosensor use

4.3.1. Co-immobilization of the bacterial system with various dehydrogenases

By co-immobilizing the bioluminescent bacterial system with the suitable dehydrogenase, the determination of sorbitol, ethanol and oxaloacetate has been performed at the nanomolar level [13]. For alcohol and sorbitol determinations, the dehydrogenase reactions, catalysed by sorbitol dehydrogenase (SDH) and alcohol dehydrogrenase (ADH), respectively (Reactions 4 and 5), have been directly coupled to the luminescent system in the presence of excess NAD^+. Then the rate of NADH production was measured by the peak light intensity.

$$\text{D-sorbitol} + NAD^+ \xrightarrow{\text{sorbitol dehydrogenase}} \text{D-fructose} + NADH + H^+ \quad (4)$$

$$\text{alcohol} + NAD^+ \xrightarrow{\text{alcohol dehydrogenase}} \text{aldehyde} + NADH + H^+ \quad (5)$$

The assay of oxaloacetate involves the concomitant consumption of NADH by malate dehydrogenase (MDH) (Reaction 6) and oxidoreductase and must be conducted in a sequential manner.

$$\text{oxaloacetate} + NADH + H^+ \xrightarrow{\text{malate dehydrogenase}} NAD^+ + \text{L-malate} \quad (6)$$

First, the injection of NADH leads to a constant light emission, then the oxaloacetate-containing sample is injected resulting in a light decrease. Either the rate of light decrease or the final variation of light intensity could be linearly related to the oxaloacetate concentration.

4.3.2. Multifunctional fibre optic sensor

The possibility of specifically determining different compounds with a single biosensor without changing its sensing part, i.e. the bioactive membrane, appears particularly attractive. In this way, we have developed a multifunctional fibre optic biosensor allowing the alternate determination of either ATP or NADH [14]. For that purpose, the firefly luciferase has been co-immobilized with the bacterial oxidoreductase–luciferase system on the same membrane. Compatible analytical conditions with regard to the activity and stability of each bioluminescent system were selected, enabling them to attain their highest performances. It was possible to perform continuous-flow measurements of ATP and NADH over a wide linear calibration range with detection limits of 0.25 pmol ATP and 5 pmol NADH.

5. Conclusion

Among optical sensors described in the literature, the device based on bioluminescence developed in our group appears very promising due to its high sensitivity associated with the practicality of the sensor design. Unlike fluorescence or absorbance/reflectance-based sensors, our bioluminescence-based sensor produces its own light and does not require a light source and monochromators. Such a device could be adapted in extracorporeal loops for monitoring analytes of clinical importance.

References

1. P. R. COULET and L. J. BLUM, in "Analytical Uses of Immobilized Biological Compounds for Detection, Medical and Industrial Uses", edited by G. G. Guilbault and M. Mascini (Reidel, Dordrecht, Netherlands, 1988) p. 237.
2. *Idem.*, in "Practical Fluorescence", 2nd Edn, edited by G. G. Guilbault (Marcel Dekker, New York, 1990) p. 543.
3. L. J. BLUM and P. R. COULET, *Anal. Chim. Acta* **161** (1984) 355.
4. *Idem., Biotechnol. Bioengng* **28** (1986) 1154.
5. L. J. BLUM, J. M. PLAZA and P. R. COULET, *Anal. Lett.* **20** (1987) 317.
6. P. R. COULET, J. H. JULLIARD and D. C. GAUTHERON, *Biotechnol. Bioengng* **16** (1986) 1055.
7. C. H. ASSOLANT-VINET and P. R. COULET, *Anal. Lett.* **19** (1986) 875.
8. L. J. BLUM, S. M. GAUTIER and P. R. COULET, *ibid.* **21** (1988) 717.
9. *Idem., Anal. Chim. Acta* **226** (1989) 331.
10. S. M. GAUTIER, L. J. BLUM and P. R. COULET, *Sensors Actuators* **B1** (1990) 580.
11. *Idem., Biosensors* **4** (1989) 181.
12. L. J. BLUM, S. M. GAUTIER and P. R. COULET, *Anal. Lett.* **22** (1989) 2211.
13. S. M. GAUTIER, L. J. BLUM and P. R. COULET, *J. Biolum. Chemilum.* **5** (1990) 57.
14. *Idem., Anal. Chim. Acta* **235** (1990) 243.

Received 21 December 1990
and accepted 19 April 1991