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Fiberoptic Biosensors Based on Chemiluminescent Reactions

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Abstract

The chemiluminescence of luminol in the presence of H_2O_2 has been exploited to develop fiberoptic biosensors associated with flow injection analysis systems. A chlorophenol sensor was developed based on the ability of certain halophenols to enhance the peroxidase-catalyzed luminol chemiluminescence. Horseradish peroxidase immobilized on a collagen membrane was used. Ten chlorophenols have been tested with this chemiluminescent-based sensor. The lower detection limit was obtained with 4-chloro-3-methylphenol and was equal to 0.01 μ*M*. Electrochemiluminescent-based fiberoptic biosensors for glucose and lactate were also developed using glucose oxidase or lactate oxidase immobilized on polyamide membranes. In the presence of oxidase-generated H_2O_2, the light emission was triggered electrochemically by means of a glassy carbon electrode polarized at +425 mV vs a platinum pseudo-reference electrode. The detection limits for glucose and lactate were 150 and 60 pmol, respectively, and the dynamic ranges were linear from 150 pmol to 600 nmol and from 60 pmol to 60 nmol, respectively.

Index Entries: Biosensors; chemiluminescence; chlorophenols; electrochemiluminescence; fiberoptic sensor; flow injection analysis; glucose; lactate; luminol; peroxidase.

Introduction

Bio- and chemiluminescent sensors are devices that make use of light emitted during the course of particular reactions *(1)*. Light is taken as the analytical signal, and its intensity is correlated to the concentration of an analyte involved in the light-emitting reaction. Because of the particular nature of one of the reaction products, i.e., light, which can be detected at

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a very low level, bioluminescent and chemiluminescent reactions associated with an optical transduction can be used to design highly sensitive biosensors. Such sensors, involving fiberoptic probes, have been developed for several years in our laboratory for the direct measurements of adenosine triphosphate, NAD(P)H, and H_2O_2 concentrations using the firefly luciferase, the bacterial oxidoreductase/luciferase system, and the luminol peroxidase system, respectively.

The enzymes catalyzing these luminescent reactions were then covalently immobilized on membranes placed at the distal end of a glass fiber bundle, with the proximal end connected to the photomultiplier tube of a luminometer. Batchwise and flow injection analysis (FIA) systems were as previously described *(2–9)*.

To extend the potentialities of these fiberoptic biosensors to other analytes, multienzymatic systems with auxiliary enzymes were used. For that purpose, two approaches have been developed. The classic one consists in coimmobilizing all the necessary enzymes on the same support *(10,11)*. In a more original approach, the sensing layer of the biosensor is compartmentalized *(12–16)*. This is realized by immobilizing the auxiliary enzyme on one membrane and the light-emitting system on another membrane, while both enzymatic supports are stacked and placed at the tip of the fiberoptic bundle. In such a configuration, the sensor response is enhanced compared with a sensing layer consisting of coimmobilized enzymes. Depending on the enzymatic system involved, the amplification factor varies between 5 and 20.

More recently, we focused on the development of chemiluminescent-based fiberoptic biosensors. A new method for the chemiluminescent detection of chlorophenols was studied. Because of their toxicity, phenolic compounds and especially chlorophenols are classified among the priority pollutants to be detected. The proposed luminescent method was based on the ability of certain substituted phenols to enhance the chemiluminescent reaction of luminol catalyzed by horseradish peroxidase (Eq. 1).

$$\text{luminol} + 2\ H_2O_2 \longrightarrow \text{3-aminophthalate} + N_2 + 3\ H_2O + \text{light}\ (\lambda_{max} = 425\ \text{nm}) \quad (1)$$

The magnitude of enhancement depends on the particular enhancer employed as well as on the enhancer concentration. By taking advantage of the properties of these enhancers, an FIA method for chlorophenols involving a fiberoptic sensor and immobilized peroxidase has been developed *(17)*. The electrochemiluminescence of luminol was also exploited in order to develop lactate and glucose fiberoptic biosensors incorporated in FIA systems as well as an immunosensing system for the detection of 2,4-dichlorophenoxyacetic acid *(18)*. We present the results recently obtained in the development of these luminol chemiluminescence-based sensors for chlorophenols, glucose, and lactate.

Materials and Methods

Reagents

Glucose oxidase (GOD) (grade I, EC 1.1.3.4, from *Aspergillus niger*) and peroxidase (grade I, EC 1.11.1.7, from horseradish; 250 U/mg) were supplied by Boehringer Mannheim. Luminol (3-aminophthalhydrazide), L-lactic acid (lithium salt), and lactate oxidase (LOD) (EC number not available, from *Pedioccocus*) were purchased from Sigma (St. Louis, MO). The phenolic compounds were obtained from Aldrich. Ethanol was from Merck. Normal (Lyotrol N) and pathological (Lyotrol P) human serum were supplied by BioMérieux (France). All other reagents were of analytical reagent grade. All buffers and aqueous solutions were made with distilled deionized water. Fresh aqueous H_2O_2 solutions were prepared daily. A stock solution of 5.5 m*M* luminol was prepared in 0.01 *M* KOH. Stock solutions of phenolic compounds (10 m*M*) were prepared in absolute ethanol, and sequential dilutions were made as appropriate with reaction medium solution.

Immobilization of LOD and GOD on Polyamide Membranes

Preactivated polyamide membranes, Immunodyne ABC type, supplied by Pall Gelman Sciences were cut out as 7-mm-diameter disks. Seven microliters of a 10 mg/mL enzyme solution (GOD or LOD) in phosphate-buffered saline (PBS) (10 m*M* phosphate buffer, 0.14 *M* NaCl, 1 m*M* KCl, pH 7.4) was applied dropwise on each side of the disk. The coupling procedure was performed at ambient temperature and was complete after 5 min. Membranes were then washed first for 10 min in PBS, then for 20 min in 0.1 *M* phosphate buffer (pH 7.0) containing 1 *M* KCl, and finally for 10 min in PBS.

GOD membranes were stored in PBS at 4°C and LOD membranes were stored at –20°C in PBS also containing 20% glycerol.

Immobilization of Peroxidase on Collagen Membranes

Peroxidase was immobilized on Cellagen™ membrane supplied by ICN. For the enzyme immobilization, 11-mm-diameter disks were cut out of a collagen membrane. The collagen activation process and the enzyme coupling were performed according to the method previously described *(19)*. The activation process implies the transformation of surface-available carboxyl groups into acyl-azide according to the following steps: (1) esterification by acidic methanol, (2) hydrazine treatment, and (3) action of nitrous acid. The enzyme coupling was performed at 4°C by simply dipping the activated collagen disk into 250 µL of a peroxidase solution (5 mg/mL) for 2 h. The coupling buffer was 0.5 *M* glycine NaOH, pH 7.7. After coupling, the disks were washed first in 0.1 *M* phosphate buffer (pH 7.0) containing 1 *M* KCl and then in the same buffer but without KCl, and were stored at 4°C in 0.1 *M* phosphate buffer, pH 7.0.

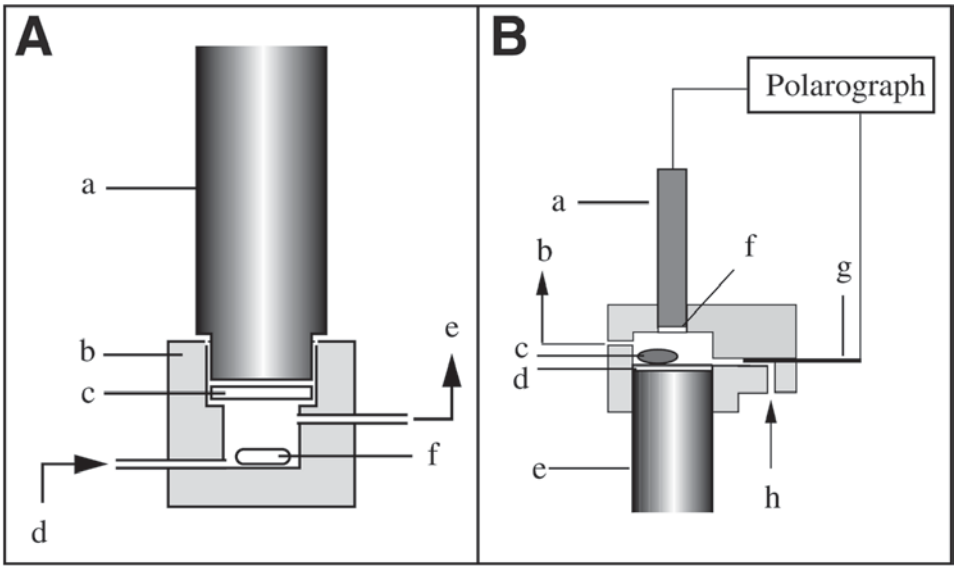

Fig. 1. **(A)** Flow cell for chemiluminescent determination of chlorophenols: (a) glass fiber bundle; (b) light tight flow cell; (c) peroxidase collagen membrane; (d) reagent solution inlet; (e) reagent solution outlet; (f) stirring bar. **(B)** Flow cell for electrochemiluminescent measurements: (a) glassy carbon electrode; (b) reagent solution outlet; (c) stirring bar; (d) Plexiglas window; (e) liquid core single optical fiber; (f) oxidase polyamide membrane; (g) platinum electrode; (h) reagent solution inlet.

Instrumentation

For chemiluminescent measurements with immobilized peroxidase, the FIA system used consisted of a one-channel peristaltic pump (Model P-1; Pharmacia), an injection valve (Model 5020; Rheodyne) on which a 50-μL sample loop was fitted, and a specially designed flow cell (130 μL inner volume) made of black polyvinyl chloride adapted to one end of a glass fiber bundle (1 m long, 8 mm diameter) in such a way that the enzymatic membrane was in close contact with the bundle and faced the reaction chamber (Fig. 1A). Stirring was effected in the flow cell with a small magnetic bar (3 × 6 mm). Teflon tubing (0.7 mm id) was used throughout the FIA system. The other end of the bundle was connected to the photomultiplier tube (R268; Hamamatsu) within a luminometer (Biocounter M2500; Lumac). The reagent/carrier stream (0.95 mL/min) was temperature controlled (30°C) by placing the flask reagent (0.1 *M* Tris-HCl buffer, pH 8.5, containing luminol at a final concentration of 55 μ*M*) in a thermostated water bath. The light intensity was monitored on a chart recorder (PE; Sefram) and expressed in arbitrary units (a.u.).

For electrochemiluminescent measurements, the flow system was similar to that we have already described. However, a liquid core single optical fiber from L.O.T. (Oriel, France) (5 mm core diameter, 7 mm overall diameter) was used instead of a glass fiber bundle. The design of the flow

cell was modified in order to adapt a glassy carbon electrode (BAS) 3 mm in diameter (Fig. 1B). The inner volume was then 250 μL and a 3 × 7 mm magnetic bar was used. The sample loop was 30 μL. The glassy carbon electrode was polarized against a pseudo-reference platinum electrode by a PRG-5 polarograph (Tacussel-Radiometer). The GOD or LOD membranes were placed in close contact with the glassy carbon electrode. Unless otherwise indicated, the reagent/carrier stream (0.375 mL/min) was a diethyl barbiturate (Veronal) buffer at pH 8.5 containing 50 μ*M* luminol, 30 m*M* KCl, and 1 m*M* $MgCl_2$.

Results and Discussion

Chemiluminescent-Based Fiberoptics for Determination of Chlorophenols

Because chlorophenols act as enhancers of the chemiluminescent reaction of luminol catalyzed by peroxidase and not as substrates of the enzyme, the chemiluminescence of a mixture of luminol and H_2O_2 must be measured first and thereafter the luminescence after the addition of a chlorophenol sample. Initially, a two-step procedure was used in which a standard H_2O_2 solution was first injected, and after the peak light intensity was measured, a mixed solution of chlorophenol and H_2O_2 was injected. The difference between the two light signals was then taken as the analytical response for the injected chlorophenol. However, injecting the chlorophenol sample in the reagent/carrier stream containing H_2O_2 gave rise to a peak light emission that could be correlated with the chlorophenol concentration. This simpler one-step procedure was then chosen.

Different values of the H_2O_2 concentration in the reagent/carrier stream were tested in the range of 10–250 μ*M*. A 100 μ*M* concentration was chosen because for each chlorophenol tested, the detection limit at a signal:noise (S:N) ratio of 3 was better at this value.

Of the 10 chlorophenols that have been assayed with the chemiluminescent-based fiberoptic sensor, 7 could be detected. Table 1 gives the detection limits determined for an S:N ratio of 3. The lower detection limit was obtained with 4-chloro-3-methylphenol and was equal to 0.01 μ*M* (concentration in the sample). For the other chlorinated phenols, the values of the detection limits increased from 0.05 to 10 μ*M* according to the following order: 4-chlorophenol > 2,4-dichlorophenol > 2-chlorophenol = 3-chlorophenol > 2,4,5-trichlorophenol > 2,4,6-trichlorophenol. Pentachlorophenol, 2,6-dichlorophenol, and 2-amino-4-chlorophenol could not be detected with this chemiluminescent method. Calibration curves established for 4-chlorophenol, 2,4-dichlorophenol, and 4-chloro-3-methylphenol were linear up to at least 100 μ*M* chlorophenol. The reproducibility of the present method was evaluated by performing 10 repeated injections of 5 μ*M* 2,4-dichlorophenol. With a mean value of 10 955 a.u. and a standard deviation of 438 a.u., the coefficient of variation was 4.0%.

Table 1
Detection Limits (S:N = 3)
of Chlorophenols Determined
with Luminol Chemiluminescent-Based Fiberoptic
Biosensor Including Peroxidase Immobilized
on a Collagen Membrane

Chlorophenol	Detection limit (μ*M*)
2-Chlorophenol	0.25
3-Chlorophenol	0.25
4-Chlorophenol	0.05
2,4-Dichlorophenol	0.1
2,6-Dichlorophenol	Not detectable
2,4,5-Trichlorophenol	2.5
2,4,6-Trichlorophenol	10
Pentachlorophenol	Not detectable
4-Chloro-3-methylphenol	0.01
2-Amino-4-chlorophenol	Not detectable

Optimization of Electrochemiluminescent Detection of H_2O_2

A preliminary study was necessary to determine the optimum reaction conditions for the electrochemiluminescent detection of enzyme-generated H_2O_2, i.e., the nature of the buffer and its pH, the concentration of the coreactants, and the value of the applied potential. After testing carbonate, Tris, and Veronal buffers at different concentrations and in the presence of varying concentrations of KCl, a 30 m*M* Veronal buffer containing 30 m*M* KCl was chosen because it gave the higher S:N ratio. Moreover, it was observed that the presence of $MgCl_2$ in the Veronal buffer at the optimum concentration of 1 m*M* increased the S:N ratio of the electrochemiluminescent response.

To fix the value of the optimum potential applied at the glassy carbon electrode vs the platinum pseudo-reference electrode, the electrochemiluminescent response was measured on the injection of 0.1 nmol of H_2O_2 into the 30 m*M* Veronal buffer, pH 10.0, containing 100 μ*M* luminol, for different values of the applied potential from +250 to +650 mV. The highest S:N ratio was obtained at +425 mV, and this value was used for all subsequent experiments.

The optimum values of the luminol concentration and pH were determined by measuring the sensor response for different luminol concentrations (10 to 300 μ*M*) and at different pH values (6.5 to 10.0). For each condition tested, a calibration curve for H_2O_2 was established and the characteristics of the curves then obtained (slope of the log-log plot, detection limit, and dynamic range) were compared. Considering the S:N ratio, the optimum value was observed at pH 9.0 and in the presence of a luminol concentration equal to 50 or 100 μ*M*, indifferently. However, keeping in

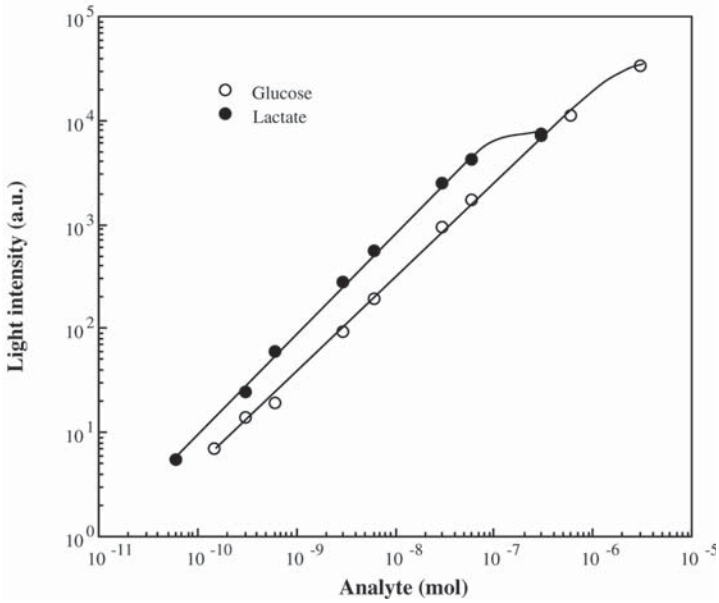

Fig. 2. Calibration curves for glucose and lactate obtained with the luminol electrochemiluminescent-based fiberoptic biosensor including GOD or LOD immobilized on polyamide membranes.

mind that enzyme-generated H_2O_2 had to be detected, we chose a pH value of 8.5 because of the pH requirement of the associated oxidase enzymes, which is generally close to the neutrality. Moreover, at this pH value, the sensor response still represented 80% of the value obtained at pH 9.0. Regarding the luminol concentration, the best performances for the electrochemiluminescent detection of H_2O_2 were obtained with a luminol concentration between 50 and 100 μ*M* with which the detection limit was 1.5 pmol and the dynamic range was 1.5 pmol to 30 nmol. However, long-term use of the fiberoptic sensor showed that luminol concentrations higher than 50 μ*M* induced the passivation of the glassy carbon electrode, and, consequently, reagent/carrier streams containing only 50 μ*M* luminol were used subsequently.

Glucose and Lactate Electrochemiluminescent-Based Fiberoptic Biosensors

For the electrochemiluminescent measurement of either glucose or lactate, the suitable polyamide enzymatic membrane was placed between the fiberoptic and the glassy carbon electrode. The optimum reaction conditions determined above were used, and calibration curves were obtained for glucose and lactate measurements (Fig. 2). The detection limits were 150 pmol for glucose and 60 pmol for lactate, and the dynamic ranges (log-log plots) were linear up to 600 nmol for glucose and 60 nmol for lactate.

FIA of glucose and lactate on real samples was performed to test the validity of this approach. For that purpose, normal and pathological recon-

Table 2
Measurements of Glucose and Lactate in Reconstituted Human Sera with Electrochemiluminescent-Based Fiberoptic Biosensors

	Normal serum		Pathological serum	
	ECLB[a]	Reference value	ECLB[a]	Reference value
Glucose (m*M*)	4.11 ± 0.10	4.13 ± 0.6	11.0 ± 0.40	10.9 ± 0.7
Lactate (m*M*)	1.87 ± 0.09	2.00 ± 0.7	4.4 ± 0.15	4.9 ± 0.7

[a]ECLB, electrochemiluminescence-based fiberoptic biosensors.

stituted human sera were used after a 1:10 dilution. As shown by the results presented in Table 2, the values obtained with the electrochemiluminescent-based fiberoptic biosensor compared well with the reference values given by the supplier.

Conclusion

The feasibility of the chemiluminescent FIA of chlorophenols with a fiberoptic sensor including immobilized peroxidase was demonstrated. The chemiluminescent method compared well in terms of sensitivity and selectivity with enzyme electrode-based methods involving immobilized tyrosinase, laccase, or peroxidase *(20–25)*. In addition, the electrochemiluminescent detection of immobilized oxidase-generated H_2O_2 was realized. The fiberoptic sensors developed for glucose and lactate, using immobilized GOD and LOD, respectively, were tested with real samples, and the results obtained were in good agreement with the reference values obtained with classic methods.

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