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Optically-based chemical and biochemical sensors for the detection of some drugs and biological compounds*

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Abstract: The development of new methods for determining at a very low level a large spectrum of substances affecting the behaviour of living organisms is still a challenging goal. For such a purpose, chemical sensors which can be defined as the intimate combination of a sensitive and specific layer with a transducer, are undoubtedly among the more promising devices.

In this field, optical sensors are expanding rapidly, mainly based on absorption, fluorescence, chemi- and bioluminescence. Beside pH and gases, drugs (anticonvulsant, antitumour, anaesthetic . . .) and other compounds of biological interest can be determined with specifically designed optical sensors, for instance immunosensors.

Special attention will be given to optical biosensors with emphasis on chemi- and bioluminescence-based devices which are highly selective and ultrasensitive. When co-immobilizing various auxiliary enzymes in the sensing layer, the potentialities of such devices can be greatly extended as demonstrated by promising results recently obtained in our group.

Keywords: *Chemical sensors; optical sensing; fibre optic; biosensors; immobilized reagents; chemi- and bioluminescence.*

I. Introduction

A chemical sensor is a device resulting from the intimate combination of a sensing element and a transducer able to monitor a physico-chemical signal.

The chemical selectivity is provided by the sensitive element which includes either a chemical (selective ionophore, antibiotic . . .) or a biological receptor (enzymes, antigens/antibodies, bacteria or whole cells), generally immobilized on a support or in a matrix.

The transducer monitors physico-chemical changes occurring in the microenvironment of the sensing area when in contact with a target-molecule. It can be based on electrochemical, mass, thermal or optical detection and provides an electrical signal. For

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that purpose, the devices which are used, are amperometric or potentiometric electrodes, field effect transistors (FET), piezoelectric crystals, thermistors and optoelectronic systems.

The signal processing electronic system, more or less sophisticated, includes generally a digital result display. It may be miniaturized and can be connected to a printer and/or a recorder.

Well documented reviews dealing with both theorical and analytical aspects of chemical sensors have recently been published [1–7] including immunosensors based on an optical transduction allowing the detection at a very low level of either antigens, antibodies or haptens.

When thinking of the detection of drugs or related compounds of biological interest, the design of the sensor will mainly depend on its specific use in three main areas: biomedical analysis, food science and environment. However, the requirements to be met for a practical use can be different depending on the site where the system has to be utilized. For example, for biomedical applications different locations are possible: either in the hospital for routine clinical analysis or in intensive care service or at patient's bedside with possible connection to feedback mechanisms or anywhere with a portable self-monitoring system (pen-sized type sensor). *In vivo* sensing [6] or *ex vivo* body-fluids monitoring are sectors under development where improvements appear highly desirable concerning biocompatibility, lifetime of the sensor, removal of interferences, reduced drift and sensitivity changes, recalibration and sterilization. When designing new types of operational sensors, it must be also stressed that performances of the system are strongly dependent on detection limit, dynamic range, sensitivity and response-time.

Furthermore, with biosensors, stability of the bioactive layer remains the weakest point and special attention must be paid both to operational stability (number of assays with the same sensing layer) and to storage stability (in buffer or dry state).

II. Colorimetry and Fluorimetry-based Optical Sensors

A growing interest in the design of sensors based on fibre optics and optoelectronic devices appeared in recent years. Seitz [4], Place *et al.* [7] and Arnold and Meyerhoff [8] have reviewed the general characteristics of optically-based chemical sensors. Most of them involve colorimetric or fluorimetric reactions and it can be noted that up to now only a few of them deal with bio- or chemiluminescence.

Optical sensors for biomedical applications including partial pressure of blood gases (pO_2, pCO_2) and pH have been developed by some groups and thoroughly reviewed by Peterson and Vurek [9], Wolfbeis [10, 11] and Opitz and Lübbers [12]. They are not included in this paper which has been purposely restricted to other compounds of pharmaceutical and biomedical interest (Table 1).

(a) *Constitutive parts of optical sensors*

The sensing layer. Numerous methods for immobilizing a reagent onto the surface of a transducer have been described [51]. Different approaches can be used in associating the sensing element with the transducer, depending on the stage of integration which is considered [52].

In optical sensor design, the two most common methods utilized involve either the direct attachment of the reagent phase to the optical device or the binding on/in a membrane layer closely associated to it. The first approach involves direct physical

Table 1
Main characteristics of some selected optical sensors for biomedical analysis

Analyte	Transmitter*/ transducer	Detection mode	Immobilized reagent	Detection limit	Reference
H_2O_2	Fibre optic	Chemiluminescence	Peroxidase on polyacrylamide gel	1×10^{-6}M	[20]
H_2O_2	Photodiode	Chemiluminescence	Peroxidase on polyacrylamide gel	1×10^{-3}M	[21]
H_2O_2	Fibre optic	Chemiluminescence	Glucose oxidase in a polyamide membrane	2.5×10^{-9}M	[22]
H_2O_2	Fibre optic	Chemiluminescence	Peroxidase in polyamide membrane	2×10^{-8}M	[23]
H_2O_2	CCD	Chemiluminescence	None	17.6 fmol	[14]
H_2O_2	O_2 fibre optic sensor	Quenching of fluorescence	(1) Catalase and ruthenium — co-adsorbed onto silica gel covered with a silicone membrane — adsorbed on different silica gel covered with a silicone membrane (2) Ruthenium adsorbed on silica gel and covered with a membrane containing silver powder	1×10^{-4}M	[24]
H_2O_2	Fibre optic spectroelectrochemical probe	Luminol mediated chemiluminescence	None	1×10^{-6}M	[25]
Glucose	Fibre optic/electrode	Fluorescence (fluorescein–dextran)	Concanavalin A on hollow dialysis fibre	2×10^{-3}M	[26]
Glucose	Photodiode	Chemiluminescence	Peroxidase + glucose oxidase on polyacrylamide gel	1×10^{-1}M	[21]
Glucose	O_2 fibre optic sensor	Fluorescence	Glucose oxidase in a BSA matrix	—	[27]
Glucose	Fibre optic	Chemiluminescence	Glucose oxidase in a polyamide membrane	6×10^{-7}M	[22]

Table 1
Continued

Analyte	Transmitter*/ transducer	Detection mode	Immobilized reagent	Detection limit	Reference
Glucose	Fibre optic	Absorbance	Glucose oxidase on polyamide membrane	$\approx 3 \times 10^{-6}$M	[28]
Glucose	pH fibre optic sensor	Fluorescence	Glucose oxidase + HPTS in hydrogel layer	1×10^{-4}M	[29]
Glucose	O_2 fibre optic sensor	Quenching of fluorescence	Glucose oxidase in polyamide membrane plus decacyclene in a silicone membrane	1×10^{-4}M	[30]
Glucose	Fibre optic	Fluorescence	Glucose dehydrogenase in a nylon mesh cartridge	6×10^{-4}M	[31]
Ammonia	pH fibre optic sensor	Fluorescence	pH indicator in silicone rubber	$\approx 10^{-5}$M	[32]
Ammonia	pH fibre optic sensor	Absorbance	Indicator solution retained at the tip of fibres by a gas permeable microporous Teflon membrane	1×10^{-5}M	[33]
Ammonia Creatinine (FIA)	pH fibre optic sensor	Reflectance	Creatinine iminohydrolase and glutamate dehydrogenase on controlled-pore glass	$\approx 1 \times 10^{-5}$M $\approx 1 \times 10^{-5}$M	[34]
Urea/urease	Fibre optic	Refractometry	Urease on glass beads (column)	—	[35]
Carboxylesterase	Fibre optic	Fluorescence	Fatty acid esters of HPTS on anion exchange membrane	$\approx 2 \times 10^{-2}$ g l^{-1} protein	[36]
IgG	Fibre optic	Fluorescence	IgG (ELISA)	10 pg	[37]
p-Nitrophenyl phosphate	Fibre optic	Absorbance	Alkaline phosphatase on nylon	$\approx 1 \times 10^{-5}$M	[38]
ATP	Fibre optic	Bioluminescence	Firefly luciferase in polyamide membrane	2.8×10^{-10}M	[23]
Ethanol	O_2 fibre optic sensor	Quenching of fluorescence	Alcohol oxidase + catalase plus ruthenium in silicon film	1×10^{-2}M	[39]

NADH Lactate Pyruvate	Fibre optic	Fluorescence	Lactate dehydrogenase on polyamide membrane	— 2×10^{-6}M 2×10^{-4}M	[40]
Ethanol	Fibre optic	Fluorescence	Alcohol dehydrogenase + NAD^+ + buffer retained at the tip of fibres by a gas permeable microporous Teflon membrane	9×10^{-4}M	[41]
NADH	Fibre optic	Bioluminescence	Bacterial luciferase in a polyamide membrane	3×10^{-10}M	[42]
Ethanol Sorbitol Oxaloacetate	Fibre optic	Bioluminescence	Bacterial luciferase with alcohol dehydrogenase or sorbitol dehydrogenase or malate dehydrogenase in a polyamide membrane	4×10^{-7}M 2×10^{-8}M 1×10^{-9}M	[43]
Doxorubicin	Fibre optic	Fluorescence	None	$\approx 10^{-7}$M	[44]
Phenytoin	Fibre optic	Fluorescence	B-phycoerythrin-labelled phenytoin + Texas Red-labelled antibody sealed inside a cellulose dialysis tubing cemented to the tip of a fibre	5×10^{-6}M	[45]
Halothane	O_2 fibre optic sensor	Quenching of fluorescence	Indicator in silicone membrane	0.1 M	[46]
Penicillin G sensor	pH fibre optic	Fluorescence	Penicillinase + pH-sensitive fluorescent dye in a polyacrylamide gel	2.5×10^{-4}M	[47]
Penicillin	pH fibre optic sensor	Reflectance	Penicillinase cross-linked with BSA into a cellulose pad or covalently bound on cellulose	1×10^{-5}M	[48]
Penicillin	pH fibre optic sensor	Fluorescence	Pencillinase cross-linked with BSA in a thin glutaraldehyde membrane on a FITC immobilized porous glass bead	1×10^{-4}M	[49]
Ascorbate	Fibre optic/electrode	Back-scattered reflectance	None	1×10^{-4}M	[50]

*When fibre optics are used as a transmitter, they are associated with a photomultiplier tube to form the transducer.

adsorption or covalent binding through surface activated groups. When a membrane is used, several immobilization techniques can be chosen, i.e. gel entrapment, cross-linking with bifunctional agents, adsorption or covalent binding to activated groups.

With enzymes, the reusability of the same active layer for hundreds or even thousands of times can be achieved. Generally, an improvement in apparent stability is observed which strongly depends on the enzyme. Occasionally, molecules are not insolubilized but only confined in a minute compartment by a permselective membrane at the tip of the sensor [41].

Various types of support have been used including cellulose, polyvinylchloride matrices, polyamide, silicone, polyacrylamide or cross-linked glutaraldehyde–BSA membranes, glass beads and more recently Langmuir–Blodgett films.

Transducers and detection mode. The transducers which are utilized in such systems are fibre optics associated to a light detector, photodiodes or charge-coupled devices (CCD). Fibre optics are mostly used for the design of either intrinsic sensors involving a change in the transmission properties of the fibre or extrinsic sensors in which an external sensing element is associated to the fibre. Photomultiplier tubes (PMT) are routinely used as detectors. The most typical configurations of fibre-optic-based sensors are shown in Fig. 1.

Fluorescence-based optical sensors require a light source, monochromators and bifurcated optical fibres for conveying light in both ways from the source and to the detector. Measurements can be based on quenching or not.

Absorbance-based optical sensors involve basically remote UV-vis spectrophotometry and also bifurcated fibres. Alternatively, reflectance measurements can be performed.

Chemi- and bioluminescence optical sensors are based on light emission and thus do not require a light source. They involve either a fibre optic coupled to a PMT for a one way light transmission, a photodiode or a CCD capable of direct transduction of the light emission.

(b) *Optical sensing of drugs and related compounds*

Antitumor drug (doxorubicin). Sepaniak *et al.* [44] have developed single fibre optic fluoroprobes for the *in vivo* determination of the antitumor drug doxorubicin. These sensors were characterized by their extremely small internal volume and by the optically well-defined sample chamber in order to keep the sampled volume constant and unaffected by changes of the biological matrix. The transport of the body-fluids into the probe was performed by either an aspiration or a capillary action. The analyte concentration was measured by using two modes of detection: conventional fluorescence (CF) or sequentially excited fluorescence (SEF) techniques, the light excitation being provided by an argon ion laser source. In both cases the detection limit for doxorubicin *ex vivo* was about 10^{-7} M. In blood, the detection limit was 1×10^{-6} M by using CF detection mode whereas about 1×10^{-5} M doxorubicin could be detected with the SEF technique.

Anticonvulsant drug (phenytoin). Anderson and Miller [45] have recently described a fibre optic sensor based on homogeneous fluorescence energy transfer immunoassay for the continuous measurement of the anticonvulsant drug phenytoin (Fig. 2). The principle is as follows: B-Phycoerythrin-phenytoin and Texas Red-labelled antibody to phenytoin were sealed inside a piece of dialysis tubing cemented to the end of a fibre optic. When

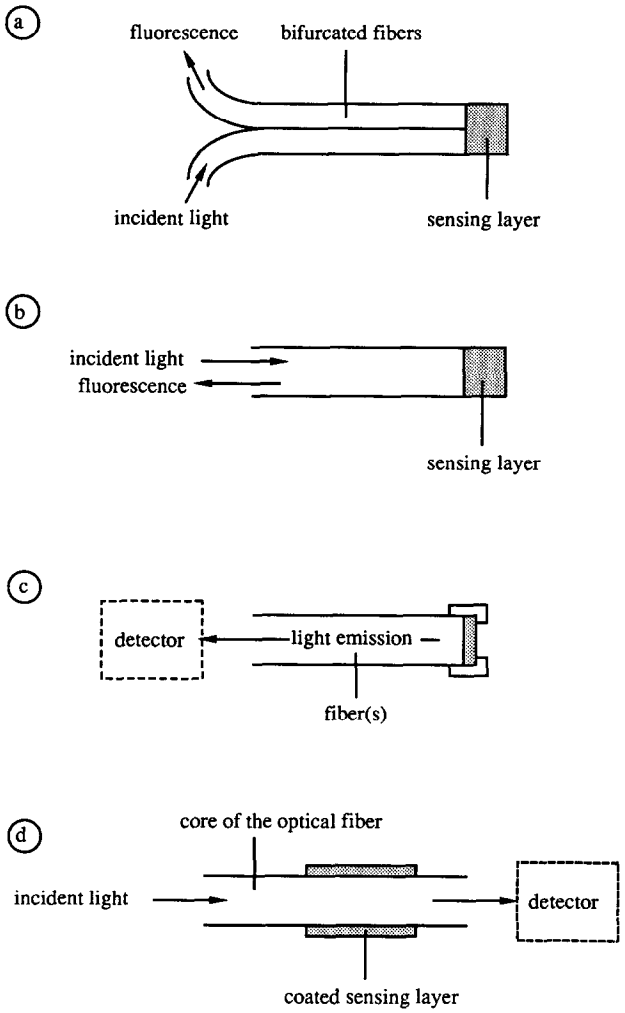

Figure 1
Some typical configurations of fibre-optic-based chemical sensors. Types (a), (b) and (c) are extrinsic sensors, i.e. the fibre(s) serves merely as a light transmitter, whereas in the intrinsic sensor (d), the sensing layer modifies the transmission properties of the fibre(s).

present in a sample where the sensitive part of the fibre is immersed, free phenytoin crossed the dialysis membrane and displaced a fraction of the B-Phycoerythrin–phenytoin from the antibody and as a result, an increase in B-Phycoerythrin fluorescence occurred which can be monitored. The reagents are not consumed and no physical separation is necessary. The detection limit was 5×10^{-6} M phenytoin and the dynamic range was 5×10^{-6}–5×10^{-4} M. Equilibrium response-time ranged from 5 to 30 min. According to the authors, any small molecule for which an antibody is available, could be detected; *in vivo* applications are still hampered by problems of biocompatibility, stability and calibration.

Anaesthetic (halothane). A fibre optic fluorescence sensor for measuring concentrations of halothane in the presence of oxygen has been reported by Wolfbeis *et al.* [46].

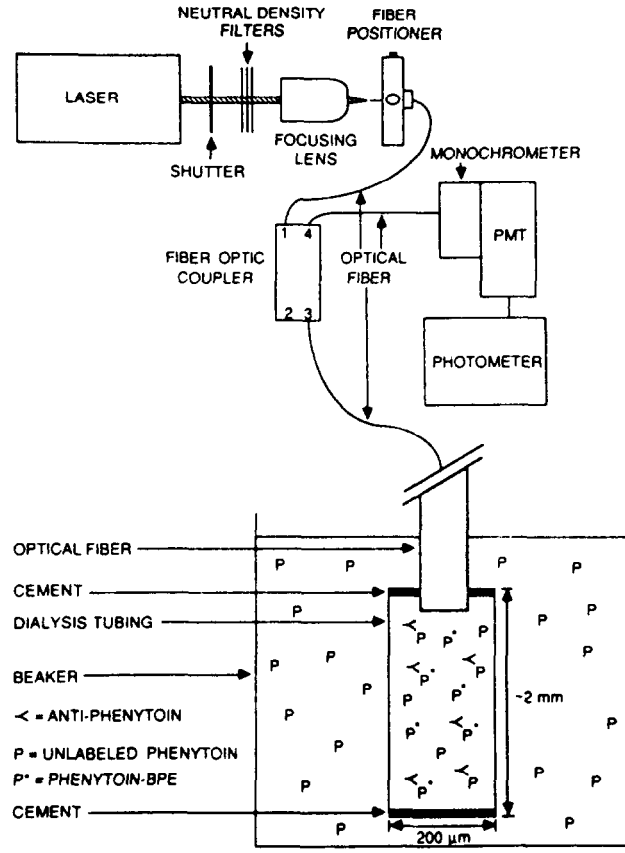

Figure 2
Schematic representation of the fibre optic fluorometer and sensor system for the detection of phenytoin. From Anderson and Miller [45]. See text for explanations. Reprinted with permission from Fig. 1 in *Clin. Chem.* **34**, 1417–1421 (1988). Copyright American Association for Clinical Chemistry.

It involves a highly halothane-sensitive indicator layer and is based on dynamic fluorescence quenching using a two-sensor technique: interferences by molecular oxygen being taken into account by a second indicator layer highly sensitive to oxygen. The technique presented is practically specific for both halothane and oxygen and allowed their determination with a good precision. Among potential applications, continuous *in vivo* monitoring of halothane in blood, measurement of oxygen in the presence of halothane and continuous monitoring of anaesthetic breath gases can be considered as particularly attractive.

Ascorbic acid (in the presence of dopamine). Fibre optic probes intended for *in situ* measurements in hard-to-reach environments and based on both spectroscopy and electrochemistry have been recently described by Van Dyke and Cheng [50]. As shown in Fig. 3, they consist of fused silica optical fibres embedded in electrically conductive graphite/epoxy material with the optical end face and the working electrode active surface in a coplanar arrangement. Spectral measurements in the UV-vis region were carried out using a bifurcated fibre configuration, one arm transmitting incident light to

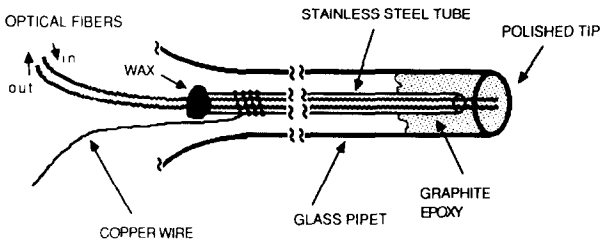

Figure 3
Schematic representation of the spectroelectrochemical probe. From Van Dyke and Cheng [50]. See text for explanations. Copyright 1988 by courtesy of the American Chemical Society.

the sample and the other carrying light back to the detector. The concept of electrochemical modulation of spectral signals for fibre-optic-assisted spectroscopy was demonstrated by using an ascorbate/dopamine gel model with which back-scattered reflection/absorbance measurements were performed. Although the calibration curve was non-linear, ascorbate could be determined in the concentration range 1×10^{-4}–1×10^{-3} M with no significant interference induced by the presence of dopamine added to simulate the extracellular chemical environment of brain tissue. The response-time ranged from 5 to 10 min. Experiments were also performed on an isolated dog brain to demonstrate the validity of the approach for spectroelectrochemical measurements in brain tissue.

Antibiotics (penicillin). Among antibiotics, the determination of β-lactamins has been the most commonly reported using biosensors. A penicillin–β-lactamase (penicillinase) commercially available, allowed the design of some penicillin fibre optic biosensors operating in a fluorometric or a colorimetric mode.

According to the reaction:

$$\text{penicillin} + H_2O \xrightarrow{\text{penicillinase}} \text{penicilloic acid}, \qquad (1)$$

an increase in the amount of H^+ issued from the penicilloic acid liberated from pencillin can be monitored using a pH-sensitive dye. Either the decrease in the fluorescent intensity or the change in colour of the dye could be directly related to the decrease in pH and thus to penicillin concentration.

Two penicillin fibre optic biosensors have been described by Kulp *et al.* [47] which differ essentially by the matrix in which penicillinase and the pH-sensitive fluorescent dye were co-immobilized and by the type of dye used. The first biological sensing layer consisted of a polyacrylamide gel containing both the entrapped enzyme and a covalently bound fluorescein derivative, whereas the other one was obtained by cross-linking BSA, penicillinase and hydroxypyrenetrisulphonate (HPTS) with glutaraldehyde. In both cases, the bioactive membrane was covalently attached to the tip of a glass fibre optic. For each sensor a linear response was obtained in the range 2.5×10^{-4}–1×10^{-2} M penicillin G with short response times (40–60 s) by measuring the decrease in fluorescence resulting from the decrease in pH. Under conditions of constant use, both fibre optic probes retained their full activity for an average of 2–3 weeks.

In another work, Fuh *et al.* [49] described an optical biosensor based on fluorescence, for the determination of penicillin (Fig. 4). The probe itself was a single fibre optic pH sensor. The fluorescent dye (fluorescein isothiocyanate, FITC) was bound to a porous glass bead attached to the end of the optical fibre. The glass bead was coated with a membrane containing penicillinase cross-linked with BSA by glutaraldehyde. The fluorescence excitation was obtained by using an argon ion laser source. When using this probe, a concentration of penicillin as low as 1×10^{-4} M could be detected in approximately 30 s in a small sensing volume by measuring the decrease in the fluorescence intensity of the dye concomitant to the decrease in pH. The dynamic range was 1×10^{-4}–1×10^{-2} M. Concerning the stability, 95% of the original activity of the biosensor was retained over a 5-day period.

Yerian *et al.* [48] have developed a penicillin sensor for flow injection analysis (FIA). The sensor consisted of penicillinase cross-linked with BSA into a cellulose pad or covalently bound to the cellulose. The pH-sensitive indicator dye (Merck indicator 9582) was covalently immobilized on the surface of the cellulose. An integrated microconduit was designed to contain the solid support with the immobilized enzyme and dye within the flow cell. The flow-through cell interfaces with a bundle of plastic optical fibres in order to transmit the light illumination to the flow cell and the reflected signal from the flow cell to the detector. Measurement could be performed in <1 min with a detection limit of 1×10^{-5} M for both penicillin G and penicillin V by monitoring the change in colour of the indicator concomitant to the change in pH. According to the authors, although it is difficult to get an exact prediction of enzyme lifetime, the stability of the sensing element was excellent since responses were obtained 10 months after immobilization with activity similar to that initially measured.

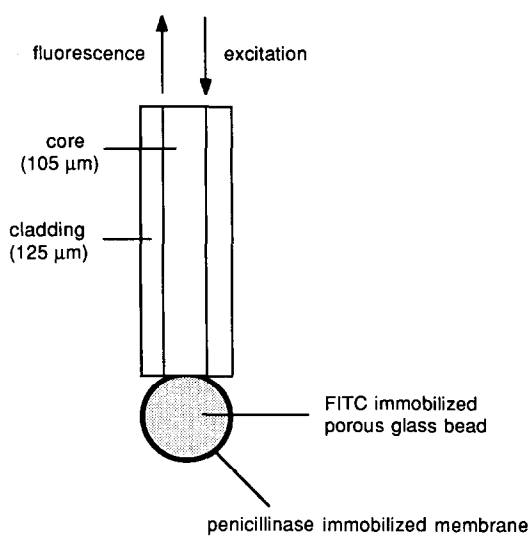

Figure 4
Configuration of a fluorescence-based enzyme probe for the determination of penicillin. Fluorescein isothiocyanate was bound to a glass bead coated with penicillinase cross-linked with BSA by glutaraldehyde and attached to the optical fibre. From Fuh *et al.* [49]. Copyright 1988 by courtesy of the American Chemical Society.

(d) *Sensing of other species*

The principle of the detection of ions is in most cases based on the measurement of the fluorescence intensity of a sensitive dye [15, 16, 19] or of a complex formed between a receptor (for instance a natural carboxylic polyether antibiotic) and the target ion [13, 17]. Glucose with glucose oxidase in the sensing layer is obviously the most popular analyte monitored with all kinds of biosensors. For optical devices, several principles of detection have been utilized involving fluorescence-based pO_2 or pH sensors [27, 29, 30]. Another approach using glucose dehydrogenase has been reported in which the intrinsic fluorescence of NADH was measured [31]. This approach has also been used with lactate dehydrogenase for pyruvate and lactate determination [40]. The detection of ammonia is based on either fluorimetry or UV-vis spectrophotometry by using a pH indicator immobilized or not at the tip of a sensor [32, 33]. The extension to creatinine and urea has also been reported [34, 35].

III. Chemi- and Bioluminescence-based Optical Sensors

Luminescence is for some types of molecules the possibility, when in an excited state, to return to the ground state with energy released as a light emission. According to the energy source involved in the excitation process, different types of luminescence can be characterized: when the energy needed to reach the excitated state is provided only by a chemical source, the term chemiluminescence is used. Bioluminescence can be considered as a special form of chemiluminescence in which the reaction of the light emission is enzymatically catalysed. The fast and reliable microdetermination of biologically important analytes still remains a very attractive aim in pharmaceutical and biomedical analysis and chemi- and bioluminescence are very appealing in this way, due to their very high sensitivity and their extendable potentialities with auxiliary reactions.

Chemiluminescence-based sensors

Based on the light emision phenomenon, detection at a very low level of hydrogen peroxide is possible with the following reaction (2):

$$2\ H_2O_2 + \text{luminol} \xrightarrow{\text{catalyst}} \text{3-aminophthalate} + N_2 + 3\ H_2O + h\nu \quad (\lambda_{max} = 430\ \text{nm}) \qquad (2)$$

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) is the mostly used chemiluminescent synthetic molecule leading to light emission in the presence of hydrogen peroxide with a catalyst such as ferricyanide or a biocatalyst like peroxidase.

Auxiliary enzyme systems producing hydrogen peroxide as for instance glucose oxidase [reaction (3)] can be coupled to this chemiluminescence reaction providing an interesting alternative to colorimetric or electrochemical measurements with a lowered detection limit:

$$\text{glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2. \qquad (3)$$

A decade ago, the approach developed by Freeman and Seitz [20] who have immobilized peroxidase in a polyacrylamide gel on the end of a fibre optic paved the way

for designing optical biosensors. The enzymatically catalysed luminol–hydrogen peroxide reaction emitted light at the enzyme level which was transmitted through the fibre optic to a photomultiplier tube. A response-time of a few seconds and a detection limit close to 1×10^{-6} M peroxide was obtained with such a system.

Since then, a biophotodiode for the photovoltaic determination of hydrogen peroxide and glucose has been described by Aizawa and his group [21]. In this device, horseradish peroxidase was entrapped in a polyacrylamide gel attached to a silicon photodiode, and luminol was used as chemiluminescent reagent. When operating the device in a suitable medium, the photocurrent was linearly related to the hydrogen peroxide concentration in the range 1×10^{-3}–1×10^{-2} M. When adding co-immobilized glucose oxidase, the use of the system could be extended to the measurement of glucose in the range 0.1–1.5 M.

More recently, Abdel-Latif and Guilbault [22] have described a fibre optic sensor for the peroxyoxalate-mediated chemiluminescence detection of hydrogen peroxide and glucose. The use of a surfactant such as cetyltrimethylammonium bromide to enhance the chemiluminescence generated from the reaction of bis(2,4,6-trichlorophenyl)oxalate (TCPO) with hydrogen peroxide in the presence of perylene allows solution of the problems of mixing and reproducibility. The light emitted was transmitted through fibre optics to the photomultiplier tube of a luminometer. For the determination of glucose, glucose oxidase was immobilized in a polyamide membrane and the enzymatic layer was mounted around a 2 cm long glass spacer which in turn was fixed to the end of the fibre cable. The peak light intensity, reached in 4–10 s, was linearly related to the hydrogen peroxide concentration in the range 8×10^{-9}–8×10^{-4} M with a detection limit of 2.5×10^{-9} M. For glucose, the dynamic linear range was 3×10^{-6}–3×10^{-2} M and the detection limit was 6×10^{-7} M.

A different approach was used by Jalkian and Bonner Denton [14] for the luminol-mediated chemiluminescence microdetermination of hydrogen peroxide. The authors utilized a solid-state two-dimensional CCD to perform their light measurements. As stressed by the authors, this detector system has an excellent sensitivity, linearity and dynamic range since with a detection limit of 17.6×10^{-15} mol, hydrogen peroxide could be determined in a linear range up to 2×10^{-9} mol. Although the device described is not a sensor, the detection system used has very interesting performances which justify a mention of their work being made here.

Bioluminescence-based sensor

In our laboratory, after having explored the potentialities of immobilized luminescence systems on collagen and in polyamide membranes for both theoretical and analytical purposes [53–55], and taking into account the laboratory's know-how in enzyme electrode design [56], focus in recent years has been on luminescence biosensors.

Among the various reactions of bioluminescence, the two best known and most utilized are those catalysed by the firefly luciferase or by bacterial luciferases which allow the ultra-sensitive determination of ATP and NAD(P)H, respectively; these two important compounds being involved in a number of metabolic pathways.

In the presence of luciferin (LH_2), ATP, Mg^{2+} and molecular oxygen, the firefly luciferase (EC 1.13.12.7) catalyses a light emission according to the overall reaction:

$$\text{ATP} + \text{LH}_2 + \text{O}_2 \xrightarrow{\text{luciferase}} \text{AMP} + \text{PPi} + \text{oxyluciferin} + \text{CO}_2 + h\nu \quad (\lambda_{max} = 560 \text{ nm}) \qquad (4)$$

The intensity of light is proportional to the ATP concentration under appropriate conditions.

In the luminescent marine bacteria system, the production of light results from two consecutive reactions. The first, catalysed by the NAD(P)H–FMN oxidoreductase (EC 1.6.8.1), produces $FMNH_2$ [reaction (5)] acting as a substrate for the second reaction catalysed by a luciferase (EC 1.14.14.3) to generate light [reaction (6)] in the presence of an aliphatic aldehyde (R-CHO) and of molecular oxygen:

$$NAD(P)H + FMN \xrightarrow{\text{oxidoreductase}} NAD(P)^+ + FMNH_2, \quad (5)$$

$$FMNH_2 + R\text{-}CHO + O_2 \xrightarrow{\text{luciferase}} FMN + R\text{-}COOH + H_2O + h\nu. \quad (6)$$
$$(\lambda_{max} = 490 \text{ nm})$$

When operating with NAD(P)H as the limiting substrate of the overall system, light intensity is proportional to NAD(P)H concentration. *Vibrio fischeri* and *Vibrio harveyi* are the two bacteria from which the most utilized light-emitting enzyme systems were isolated.

In addition to the direct determination of either ATP or NAD(P)H, by this approach, the analysis of some ATP or NAD(P)H dependent enzymes and of their substrates can also be performed via the bacterial or the firefly luminescent systems.

Design of the extrinsic fibre optic biosensor. The photobiosensor designed is shown in Fig. 5 and consists of a 1-m long glass fibre bundle (8 mm dia.). One end of the bundle can be connected to the photomultiplier tube of a luminometer. The sensing tip is composed of a bioactive disk (8 mm dia.) maintained in close contact with the surface of the fibre bundle by a screw-cap. When in use, the biosensor is immersed in 4.5 ml of a stirred and thermostatted reaction medium and samples are injected through a septum. Ambient light interferences must be avoided and the reaction vessel is surrounded by a black PVC jacket. A single channel chart recorder is coupled to the luminometer for an easier monitoring of the light signal [23].

Enzyme immobilization. Enzymes were covalently immobilized onto a commercially available polyamide membrane of white colour, Immunodyne type, supplied in a

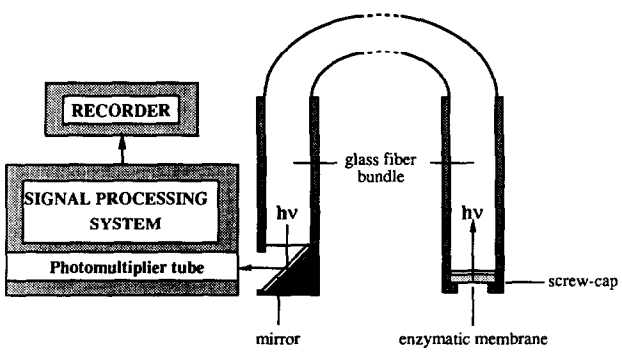

Figure 5
Schematic set-up of the bioluminescence-based fibre optic sensor. The light emitted at the enzyme level is conveyed through the fibre bundle to the photomultiplier tube of a luminometer.

Table 2
Performance of the chemi- and bioluminescence fibre optic sensors

Analyte	H_2O_2	ATP	NADH	Ethanol	D-Sorbitol	Oxaloacetate
Bioactive disk	Horseradish peroxidase	Firefly luciferase	BL + OR*	BL + OR* + ADH	BL + OR* + SDH	BL + OR* + MDH
Detection limit (M)	2×10^{-8}	2.8×10^{-10}	3×10^{-10}	4×10^{-7}	2×10^{-8}	1×10^{-9}
Linearity (M)	2×10^{-8}–2×10^{-5}	2.8×10^{-10}–1.4×10^{-6}	1×10^{-9}–3×10^{-6}	4×10^{-7}–7×10^{-5}	2×10^{-8}–2×10^{-5}	1×10^{-9}–3×10^{-6}
Precision (RSD)†	6.8% On the entire linear range	3.7% On the entire linear range	4.8% 10 replicates at 4×10^{-8}M	5.4% 10 replicates at 6×10^{-6}M	6.0% 10 replicates at 4×10^{-7}M	5.1% 10 replicates at 5×10^{-8}M
Storage stability‡	Not tested	70% At −20°C after 3 months	100% At −20°C after 5 months	100% At −20°C after 3 months	100% At −20°C after 3 months	85% At −20°C after 9 months

*BL, bacterial luciferase; OR, oxidoreductase; ADH, alcohol dehydrogenase; SDH, sorbitol dehydrogenase; MDH, malate dehydrogenase.
†RSD, relative standard deviation.
‡Residual activity of the enzymatic membrane.

preactivated form by Pall Industrie S.A. (France) [57]. This support sold for immunodiagnostic use, has been selected and adapted in our laboratory for enzyme immobilization by simply wetting the membrane with 10 μl of enzyme solution at the chosen concentration and applied on one or both sides of the membrane [58]. The reaction is normally completed within 1 min. Bioactive disks of a size matching the biosensor tip are cut out from the enzymatic membrane and can be used immediately or stored either in the suitable buffer or in a dry state.

Performances. The main results obtained by using the luminescence-based fibre optic sensor are summarized in Table 2. In a first step, the validity of the approach has been demonstrated by performing the microdetermination of either ATP, NADH or hydrogen peroxide [23]. For this purpose, the biosensor was equipped with the suitable solid-phase enzymatic layer and each analyte could be assayed at the nanomolar level with good precision. The optimization of the sensor has been performed by focusing on the bacterial bioluminescent system with special emphasis put on stability [42]. The next step consisting in the extension of the biosensor use to the detection of other analytes has been realized by co-immobilizing several NADH-dependent dehydrogenases; with these new sensing layers, ethanol, sorbitol and oxaloacetate have been successfully assayed [43].

Automation is of critical interest in biomedical analysis and recently a continuous flow method for the bioluminescent quantification of NADH has been described using the fibre optic sensor associated with a specially designed flow cell [59]. NADH could be determined in the linear range 2×10^{-12}–1×10^{-9} mol with a precision of 3.4% for 1×10^{-10} mol injected. Twenty five samples/h could be analysed with no carry-over. No loss of activity was observed after 150 assays performed within 3 days.

IV. Conclusion

Optical sensing as an alternative to electrochemical detection is a rapidly emerging field. The attractiveness of both sensor type design and advantages allied to light detection and monitoring make such an approach very promising for overcoming technological hurdles existing with other methods. Noteworthy results have been obtained on antitumor and anticonvulsant drugs as well as antibiotics.

Among optical sensors, biosensors appear very promising due to the high degree of selectivity provided by the biological layer. New strategies for the design of operational luminescence-based fibre optic sensors have been developed. Besides the possibilities of classical detection modes based on fluorescence or colorimetry, the very low detection limit attainable with chemi- and bioluminescence and the simpler instrumentation required appear as two key-points which undoubtedly will favour the extended development of such devices in the near future, now under investigation in our laboratory.

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