Chapter 8

CHEMILUMINESCENCE-BASED SENSORS

Loïc J. Blum and Christophe A. Marquette
Laboratoire de Génie Enzymatique et Biomoléculaire
Enzymes Membranes Biologiques et Biomimétiques (EMB2)
UMR 5013 CNRS-Université Claude Bernard Lyon 1
Bâtiment CPE - 43, Bd du 11 novembre 1918
69622 Villeurbanne Cedex, France

1. INTRODUCTION

Luminescence is the emission of light from an electronically excited compound returning to the ground state. The source of excitation energy serves as a basis for a classification of the various types of luminescence. Chemiluminescence occurs in the course of some chemical reactions when an electronically excited state is generated. Bioluminescence is a special case of chemiluminescence occurring in some living organisms and involves a protein, generally an enzyme.

Bio- or chemiluminescence measurements consist in monitoring the rate of production of photons and thus, the light intensity depends on the rate of the luminescent reaction. Consequently, light intensity is directly proportional to the concentration of a limiting reactant involved in a luminescence reaction. With modern instrumentation, light can be measured at a very low level, and this allows the development of very sensitive analytical methods based on these light-emitting reactions. Bioluminescence-based and chemiluminescence-based sensors have been then developed with the aim of combining the sensitivity of light-emitting reactions with the convenience of sensors. Optical fibres associated with a sensitive light detector appeared to be convenient transducers for designing biosensors involving these kind of luminescent reactions. In addition to these optical fibre-based sensors several luminescence analytical systems including immobilized reagents but not optical fibres have been described. More recently, chemi- and electrochemiluminescence detections have been also used instead of fluorescence for the development of biochips and microarrays.

F. Baldini et al. (eds.), Optical Chemical Sensors, 157–178.

2. LIGHT-EMITTING REACTIONS

2.1 Chemiluminescence

Chemiluminescence reactions are currently exploited mainly either for analyte concentration measurements or for immunoanalysis and nucleic acid detection. In the latter case, a compound involved in the light emitting reaction is used as a label for immunoassays or for nucleic acid probes. In the former case, the analyte of interest directly participates in a chemiluminescence reaction or undergoes a chemical or an enzymatic transformation in such a way that one of the reaction products is a coreactant of a chemiluminescence reaction. In this respect, chemiluminescent systems that require H_2O_2 for the light emission are of particular interest in biochemical analysis. Hydrogen peroxide is in fact a product of several enzymatic reactions, which can be then coupled to a chemiluminescent detection.

Among the different synthetic compounds used for hydrogen peroxide determination, only luminol and oxalate esters have found widespread use and were really evaluated for H_2O_2 detection.

2.1.1 Luminol Chemiluminescence Reaction

The chemiluminescent properties of luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) were first reported in 1928 by Albrecht[1]. Since that time, the chemiluminescence of luminol and related hydrazides has been studied extensively.

In aprotic media, the chemiluminescent oxidation of luminol requires only oxygen and a strong base[2-4].

In protic solvents (*e.g.* water), an oxidation system and an oxidative catalyst are required in addition to alkaline conditions[1] (pH 10-13). Hydrogen peroxide is the most frequently used oxidizing agent. Transition metal cations (Cr^{3+}, Mn^{4+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}), either free or complexed to organic or inorganic ligands, catalyze the luminol chemiluminescence oxidation. This is why heme-containing proteins, particularly horseradish peroxidase (EC 1.11.1.7) are able to catalyze the chemiluminescence of luminol in the presence of hydrogen peroxide. The use of this enzyme has the advantage over other catalysts such as ferricyanide in that the chemiluminescent reaction can proceed at near neutral pH values[5] (8-8.5).

Another way to produce light from luminol is electrogenerated chemiluminescence[6, 7]. Luminol is oxidized using a positively biased electrode and in the presence of hydrogen peroxide the light emission occurs.

The overall reaction scheme of the luminol chemiluminescence in an aqueous medium is shown in Figure 1. The luminol oxidation leads to the formation of an aminophthalate ion in an excited state, which then emits light on return to the ground state. The quantum yield of the reaction is low (≈ 0.01) compared with bioluminescence reactions and the emission spectrum shows a maximum[1] at 425 nm.

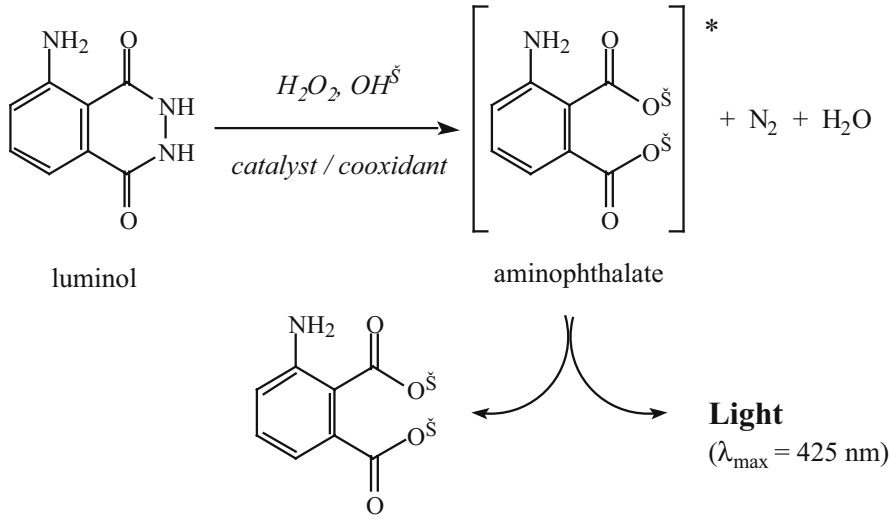

Figure 1. Overall luminol chemiluminescence reaction in aqueous medium.

2.1.2 Oxalate Ester Chemiluminescence Reaction

Some esters of oxalic acid, mainly aryl-oxalates, react with the oxidant H_2O_2 in the presence of suitable fluorescers to give rise to light emission. The chemical system is generally known as the peroxyoxalate chemiluminescence system and is an energy transfer system. By reacting with hydrogen peroxide, the oxalic acid ester gives an unstable intermediate of high energy, 1,2-dioxetanedione, which in turn excites the fluorescer. The excited fluorescer returns to the ground state emitting light in a typical fluorescence process[8, 9]. The overall reaction of aryl-oxalate chemiluminescence is shown in Figure 2. In fact, the production of 1,2-dioxetanedione proceeds in two steps with first, the production of one phenol molecule and peroxyoxalic acid which decomposes into a second phenol molecule and 1,2-dioxetanedione intermediate. Then, the intermediate forms a charge-transfer-type complex with the fluorescer. This unstable intermediate releases excited fluorescer and two carbon dioxide molecules.

The best known ester of oxalic acid and probably the more efficient is TCPO (bis-(2,4,6-trichlorophenyl)oxalate). With suitable fluorescers such as

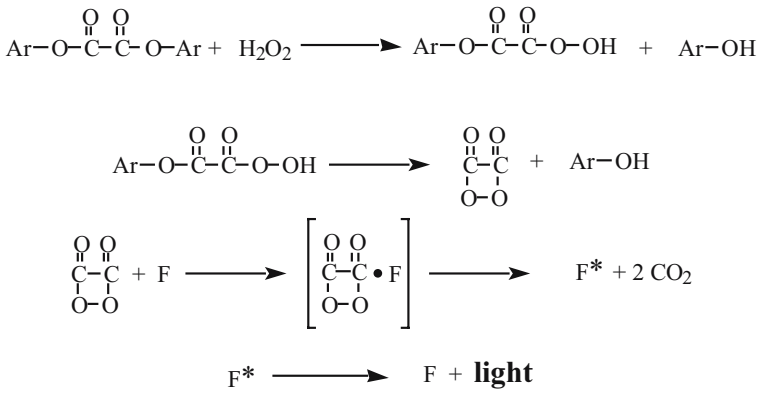

Figure 2. Aryl-oxalate chemiluminescence reaction. F is a fluorescer.

perylene, rubrene or 9,10-diphenylanthracene the quantum yield of the chemiluminescence reaction is of the order of 0.22 - 0.27 and the colour of the emitted light depends on the fluorescer used.

The chemiluminescence reaction of esters of oxalic acid can proceed within a wider pH range than for luminol. However, the most efficient oxalate derivatives are only soluble in organic solvents such as ethyl acetate, acetonitrile, dioxane or dimethoxyethane and dissolution problems of these solvents in aqueous media are encountered. This can limit the use of this chemiluminescence reaction for a direct coupling to an H_2O_2-generating enzymatic reaction.

2.2 Bioluminescence

Although numerous luminous organisms are known, only a few of them has been studied and really exploited. Analytical applications of bioluminescence concern mainly the detection of ATP with the firefly luciferase and of NADH with some marine bacteria systems. Luciferase from the North American firefly, i.e., *Photinus pyralis*, has been extensively studied[10–12] and afterwards, attention has been paid to the luciferase from *Luciola mingrelica*, i.e., the North Caucasus firefly[13-15].

In some bioluminescent organisms, light is produced without the intervention of a luciferase, directly from a protein-luciferin complex, called a photoprotein, where the luciferin is tightly or covalently bound to the protein. These systems are able to release energy in the form of light emission, independently of a chemical or enzymatic reaction. This energy "discharge" occurs in the presence of a triggering compound, generally H^+ or Ca^{2+} ions depending on the bioluminescent systems.

For example, the jellyfish *Aequorea* contains a photoprotein called aequorin of molecular weight about 20,000 and with a heterocyclic compound called coelenterazine covalently linked to it. The protein contains bound oxygen and three calcium binding sites and upon addition of calcium ions, a blue light is produced[16]. This bioluminescence system can be used for imaging the Ca^{2+} content in living cells. However, it has not been exploited for sensor development since the protein does not turnover and consequently is efficient only once for the production of light.

2.2.1 Firefly Bioluminescence

The firefly luciferase (EC 1.13.12.7) catalyzes the emission of light in the presence of ATP, Mg^{2+}, molecular oxygen and firefly luciferin a specific natural substrate (Figure 3).

The colour of the light emission is yellow-green with a maximum at 560 nm. The quantum yield of the firefly luciferase bioluminescence reaction is close to 1 under optimum conditions of temperature and pH and in the presence of saturating luciferin concentration[17].

Synthetic luciferin as well as purified preparations of native and recombinant firefly luciferases are now commercially available allowing the bioluminescent determination of ATP to be used as a routine analysis technique in some laboratories.

a

$$\text{ATP + luciferin + } O_2 \xrightarrow[\textit{luciferase}]{\textit{firefly}} \text{AMP + PPi + oxyluciferin + } CO_2 + \textbf{light}$$

(λ_{max} = 560 nm)

b

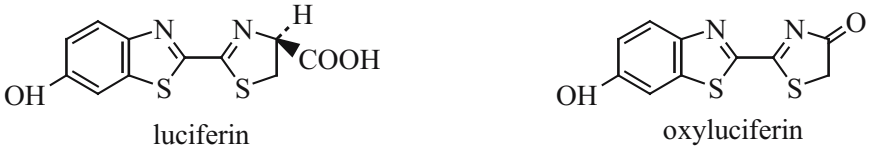

Figure 3. (a) The firefly luciferase bioluminescence reaction. (b) Structure of the specific substrate luciferin and the corresponding reaction product oxyluciferin.

2.2.2 Bacterial Bioluminescence

The bacterial bioluminescent reaction is also catalyzed by a luciferase (EC 1.14.14.3) isolated from marine bacteria. The four most studied types are *Vibrio harveyi*, *Vibrio fischeri*, *Photobacterium phosphoreum* and *Photobacterium leiognathi*[18, 19]. In these different luminescent bacteria the

same light emission mechanism is involved and the luciferases are similar[20]. The substrates of the bacterial luciferase reaction include reduced flavin mononucleotide ($FMNH_2$), molecular oxygen and a long-chain aldehyde (R-CHO). In vitro, decanal is generally used as the aliphatic aldehyde and light emission occurs with a peak at 490 nm. $FMNH_2$ is only a transient intermediate and is produced in the course of an oxido-reduction reaction, catalyzed by an oxidoreductase, which involves the oxidation of NADH or NADPH concomitantly with the reduction of FMN.

This reductase was isolated from various strains of bioluminescent bacteria as well as in several species of non-luminous aerobic and anaerobic bacteria[21]. The two most useful light-emitting systems were isolated from *V. harveyi* and *V. fischeri*. Distinct oxidoreductases for NADH and NADPH were identified in extracts of *V. harveyi*[22] whereas *V. fischeri* appears to have only one oxidoreductase acting on both NADH and NADPH. Thus, the bacterial bienzymatic system allows NAD(P)H to be assayed, providing it is the limiting substrate (Figure 4). In addition, the use of coupled reactions makes it easy to determine various substrates and enzymes involved in NAD(P)H producing or consuming reactions.

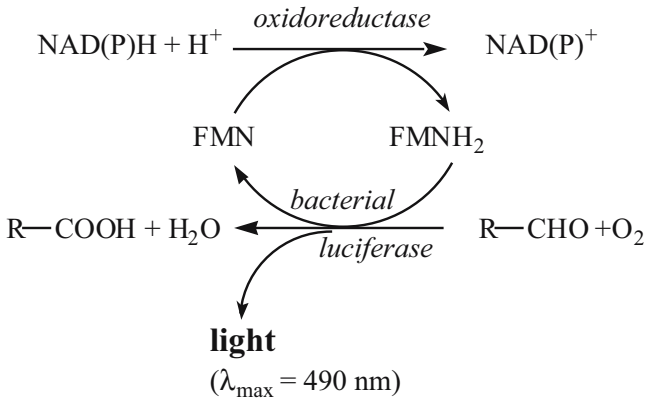

Figure 4. Coupled bacterial bioluminescent reaction allowing the detection of NADH or NADPH.

3. COUPLED ENZYMATIC REACTIONS

Bioluminescence and chemiluminescence are very powerful analytical tools, since in addition to the direct measurement of ATP, NAD(P)H or hydrogen peroxide, any compound or enzyme involved in a reaction that generates or consumes these metabolites can be theoretically assayed by one of the appropriate light-emitting reactions. Some of these possibilities have been exploited for the development of optical fibre sensors, mainly with bacterial bioluminescence and with luminol chemiluminescence.

3.1 NADH-Dependent Reactions

The bioluminescent determinations of ethanol, sorbitol, L-lactate and oxaloacetate have been performed with coupled enzymatic systems involving the specific suitable enzymes (Figure 5). The ethanol, sorbitol and lactate assays involved the enzymatic oxidation of these substrates with the concomitant reduction of NAD^+ in NADH, which is in turn reoxidized by the bioluminescence bacterial system. Thus, the assay of these compounds could be performed in a one-step procedure, in the presence of NAD^+ in excess. Conversely, the oxaloacetate measurement involved the simultaneous consumption of NADH by malate dehydrogenase and bacterial oxidoreductase and was therefore conducted in two steps.

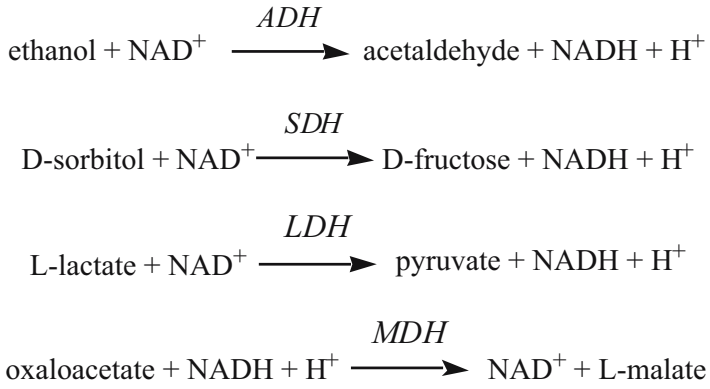

Figure 5. Example of dehydrogenase reactions which can be coupled with the bienzymatic bacterial bioluminescent system. ADH = alcohol dehydrogenase (EC 1.1.1.1), SDH = sorbitol dehydrogenase (EC 1.1.1.14), LDH = lactate dehydrogenase (EC 1.1.1.27), MDH = malate dehydrogenase (EC 1.1.1.37).

3.2 H_2O_2-Generating Enzymatic Systems

The main interest of luminol chemiluminescence in biochemical and clinical analysis is the possibility of coupling this light-emitting reaction with enzyme-catalyzed reactions generating hydrogen peroxide. Simple auxiliary H_2O_2-generating reactions as well as multi-enzymatic systems leading to the production of hydrogen peroxide can be used for the specific chemiluminescent detection of different metabolites. Some of these systems, used for the design of chemiluminescence-based biosensors, are listed in Table 1.

Table 1. H_2O_2-generating enzymatic systems for chemiluminescence-based optical fibre biosensors (Abbreviations: OX = oxidase, PNPase = purine nucleoside phosphorylase).

Metabolite	*Enzyme*	*Enzymatic reaction*
Choline	choline OX (EC 1.1.3.17)	choline + 2 O_2 = betaine + 2 H_2O_2
Ethanol	alcohol OX (EC 1.1.3.13)	primary alcohol + O_2 = aldehyde + H_2O_2
D-Glucose	glucose OX (EC 1.1.3.4)	β-D-glucose + O_2 = H_2O_2 + D-glucono-1,5-lactone
L-Glutamate	L-glutamate OX (EC 1.4.3.11)	2 L-glutamate + O_2 + H_2O = 2 NH_3 + 2 α-ketoglutarate + H_2O_2
L-Glutamine	Glutaminase (EC 3.5.1.2)	L-glutamine + H_2O = NH_3 + L-glutamate
	L-glutamate OX (EC 1.4.3.11)	2 L-glutamate + O_2 + H_2O = 2 NH_3 + 2 α-ketoglutarate + H_2O_2
Hypoxanthine	xanthine OX (EC 1.1.3.22)	hypoxanthine + O_2 + H_2O = xanthine + H_2O_2
	xanthine OX (EC 1.1.3.22)	xanthine + O_2 + H_2O = urate + H_2O_2
L-Lactate	lactate OX	L-lactate + O_2 = pyruvate + H_2O_2
L-Lysine	L-lysine OX (EC1.4.3.14)	L-lysine + O_2 = NH_3 + H_2O_2 + 6-amino-2-oxo-hexanoate
Phosphate	PNPase (EC 2.4.2.1)	Pi + inosine = ribose-1-phosphate + hypoxanthine
	xanthine OX (EC 1.1.3.22)	hypoxanthine + O_2 + H_2O = xanthine + H_2O_2
	xanthine OX (EC 1.1.3.22)	xanthine + O_2 + H_2O = urate + H_2O_2
Sulphite	sulphite OX (EC 1.8.3.1)	SO_3^{2-} + O_2 + H_2O = SO_4^{2-} + H_2O_2
Xanthine	xanthine OX (EC 1.1.3.22)	xanthine + O_2 + H_2O = urate + H_2O_2

4. DESIGN OF FIBEROPTIC BIOSENSORS

The first biosensor combining an optical fibre and an enzyme-catalyzed light-emitting process was described about twenty-five years ago by Freeman and Seitz[23]. It consisted of a 2 feet-long optical fibre with a 1/8 inch diameter at the tip of which was placed horseradish peroxidase immobilized in a thin layer of polyacrylamide. The other end of the optical fibre was placed in front of a photomultiplier tube. The sensing part of the sensor was immersed in a 10-ml stirred solution of buffer containing luminol. When hydrogen peroxide was injected in this reaction medium, light was emitted at the enzymatic phase and transmitted through the optical fibre to the detector. The first optical fibre biosensor based on bioluminescence reactions was

described in 1988 and consisted of a 1-meter glass fibre bundle of 8 mm in diameter[24]. The bioluminescence enzymes were immobilized on a white polyamide membrane maintained in close contact with the surface of one end of the fibre bundle, the other end being connected to the photomultiplier tube of a luminometer. The sensor was immersed in a 4.5 ml stirred and thermostated reaction medium protected from the ambient light by a polyvinyl chloride jacket.

Finally, all the bio- and chemi-luminescence-based optical fibre biosensors described were designed according to nearly the same principle, that is an optical fibre or a fibre bundle with one end connected to a light detector, generally a photomultiplier tube, and with the other end bearing the immobilized light-emitting enzymes and protected from ambient light to avoid interferences. This basic configuration was used either in batch systems or included in flow injection analysis (FIA) systems. As an example, schematic representations of the devices developed in our laboratory for batch analysis and FIA with luminescence-based optical fibre biosensors are shown in Figures 6 and 7, respectively.

4.1 Sensing Layer Design

Except the physical entrapment of horseradish peroxidase in a polyacrylamide gel used by Freeman and Seitz[23], immobilization of the

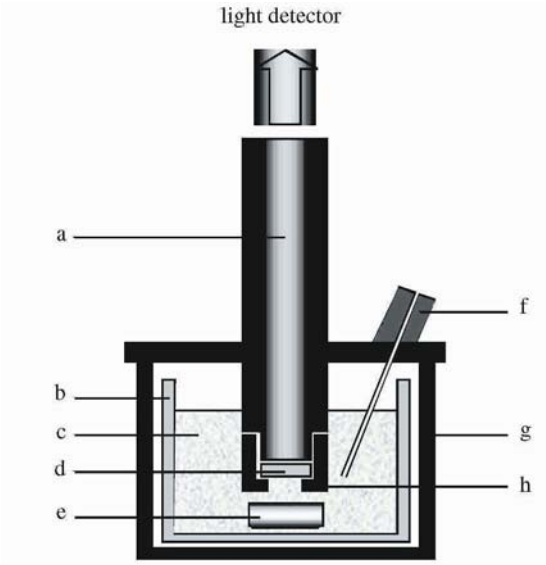

Figure 6. Optical fibre biosensor setup. (a) Optical fibre bundle; (b) thermostated reaction vessel; (c) reaction medium; (d) sensing layer; (e) stirring bar; (f) septum and needle guide for sample injection; (g) PVC jacket; (h) screw-cap for securing the sensing layer.

luminescence enzymes was mainly performed *via* a covalent coupling on a synthetic membrane. Commercially available membranes, supplied in a preactivated form, were used by several authors: Immunodyne from Pall, Immobilon-AV from Millipore or UltraBind from Gelman Sciences.

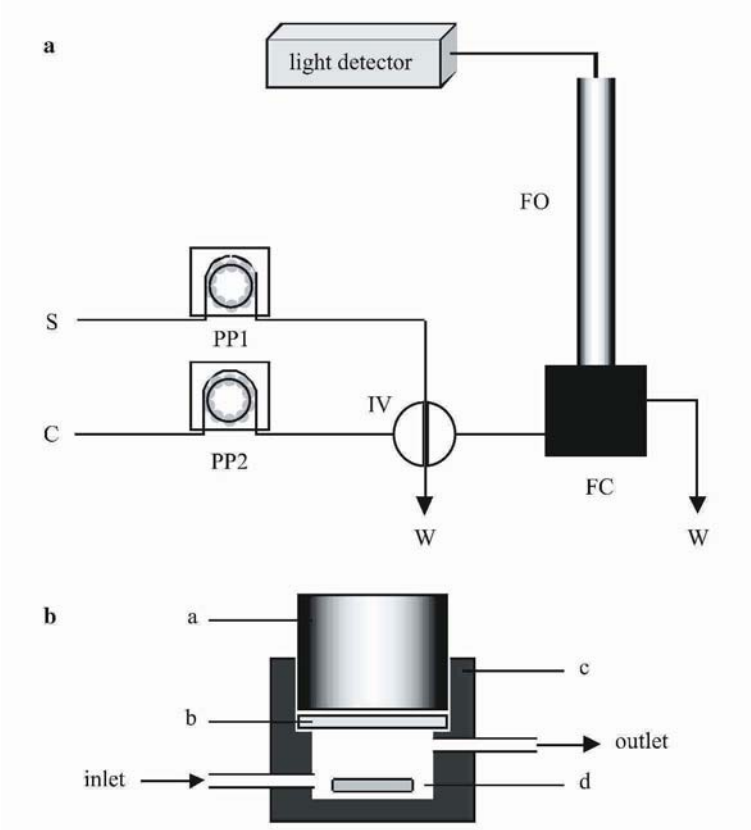

Figure 7. (a) Flow diagram of the optical fibre continuous-flow system for bioluminescence and chemiluminescence measurements: S, sample; C, carrier stream; PP, peristaltic pump; IV, injection valve; W, waste; FO, optical fibre; FC, flow-cell. (b) Details of the optical fibre biosensor/flow-cell interface: a, optical fibre; b, sensing layer; c, light-tight flow-cell; d, stirring bar.

4.1.1 Co-Immobilized Auxiliary Enzymes

As mentioned above, in order to extend the potentialities of the luminescence-based optical fibre biosensors to other analytes, auxiliary enzymes can be used. The classical approaches consist either of the co-immobilization of all the necessary enzymes on the same membrane or of the use of microreactors including immobilized auxiliary enzymes and

placed in a FIA system, upstream from the luminescence-based optical fibre sensor.

4.1.2 Compartmentalized Sensing Layer

Another approach, developed in our laboratory, consists of the compartmentalization of the sensing layer[25-27]. This concept, only applicable for multi-enzyme based sensors, consist in immobilizing the luminescence enzymes and the auxiliary enzymes on different membranes and then in stacking these membranes at the sensing tip of the optical fibre sensor. This configuration results in an enhancement of the sensor response, compared with the case where all the enzymes are co-immobilized on the same membrane. This was due to an hyperconcentration of the common intermediate, i.e. the final product of the auxiliary enzymatic system, which is also the substrate of the luminescence reaction, in the microcompartment existing between the two stacked membranes.

4.1.3 Entrapped Coreactants

Since ideally, a biosensor should be reagentless, that is, should be able to specifically measure the concentration of an analyte without a supply of reactants, attempts to develop such bioluminescence-based optical fibre biosensors were made for the measurements of NADH[28-30]. For this purpose, the coreactants, FMN and decanal, were entrapped either separately or together in a polymeric matrix placed between the optical fibre surface and the bacterial oxidoreductase-luciferase membrane. In the best configuration, the period of autonomy was 1.5 h during which about twenty reliable assays could be performed.

For luciferin, a firefly luciferase cosubstrate, another method of retention has been evaluated which consisted of incorporating the substrate in acrylic microspheres during their formation, these last being then confined in a polymeric matrix[31]. Using the suitable co-immobilized enzymes (adenylate kinase and creatine kinase), the three adenylic nucleotides (ATP, ADP and AMP) could be assayed continuously and reproducibly with a self-containment working time of 3 h.

4.2 Characteristics and Performances of Bioluminescence and Chemiluminescence-Based Fiberoptic Sensors

The main performances of batchwise and flow luminescence-based optical fibre sensors are summarized in Tables 2 and 3. As it can be shown

in these tables, the sensitivity achieved is generally better with bioluminescence-based sensors than with chemiluminescence-based sensors. This could be explained by considering the quantum yield of these light-emitting reactions. For the firefly luciferase reaction the quantum yield is closed to 1 and for the bacterial bioluminescence reaction it is about 0.3 whereas it is only 0.01 for the luminol chemiluminescence reaction.

Table 2. Performances of batchwise and flow injection analysis (FIA) bioluminescence-based optical fibre sensors.

Analyte	*Linearity or detection limit*	*Precision*	*System*	*Reference*
ADP[(a)]	1 x 10^{-11} mol	4.3%	FIA	31
AMP[(a)]	25 x 10^{-11} mol	6%	FIA	31
ATP	2.8 x 10^{-10}- 1.4 x 10^{-6} M	///	batch	24
ATP[(b)]	1 x 10^{-10}- 1 x 10^{-6} M	6% at 9 x 10^{-9} M	batch	32
ATP[(b)]	0.25 x 10^{-12} mol	4-4.5%	FIA	33
ATP[(a)]	2.5 x 10^{-12} mol	4%	FIA	31
Ethanol	4 x 10^{-7} - 7 x 10^{-5} M	5.4% at 5 x 10^{-6} M	batch	34
Lactate[(c)]	2 x 10^{-7} M	5.1%	batch	26
LDH	5 - 250 IU l^{-1}		FIA	35
NADH	3 x 10^{-9} - 3 x 10^{-6} M	///	batch	24
NADH	1 x 10^{-9} - 3 x 10^{-6} M	5% at 4 x 10^{-8} M	batch	36
NADH	2 x 10^{-9} M[(d)]	4.2% at 4 10^{-8} M	batch	37
NADH	0.3 x 10^{-9} M[(e)]	4.8% at 4 x 10^{-8} M	batch	37
NADH	2 x 10^{-12} - 1 x 10^{-9} mol	3.4% at 1 x 10^{-10} mol	FIA	38
NADH[(a)]	1 x 10^{-9} - 1 x 10^{-6} M	6% at 4 x 10^{-8} M	batch	32
NADH[(a)]	5 x 10^{-12} mol	4-4.5%	FIA	33
NADH[(f)]	5 x 10^{-12} - 5 x 10^{-10} mol	< 3%	FIA	30
oxaloacetate	3 x 10^{-9} - 2 x 10^{-6} M	5.1% at 5.5 x 10^{-8} M	batch	34
sorbitol	2 x 10^{-8} - 2 x 10^{-5} M	6% at 4.4 x 10^{-7} M	batch	34

(LDH) lactate dehydrogenase.
[(a)] reagentless biosensor with luciferin immobilized in microspheres included in a polymeric matrix.
[(b)] firefly luciferase co-immobilized with the bacterial oxidoreductase-luciferase system.
[(c)] compartmentalized system.
[(d)] bacterial oxidoreductase-luciferase system from *V. harveyi.*
[(e)] bacterial oxidoreductase-luciferase system from *V. fischeri.*
[(f)] reagentless biosensor, i.e. with FMN and decanal entrapped in a polymeric matrix.

Table 3. Performances of batchwise and flow injection analysis chemiluminescence-based optical fibre sensors.

Analyte	*Linearity or detection limit*	*Precision*	*System*	*Reference*
Choline	1×10^{-6} - 1×10^{-3} M	///	FIA	39
	0.5×10^{-12} mol	2.6%	FIA	40
Ethanol	3×10^{-6} - 7.5×10^{-4} M	2.4% at 3×10^{-4} M	FIA	41
D-Glucose	3×10^{-7} - 3×10^{-4} M	3.8% at.5×10^{-5} M	batch	42
	2×10^{-3} - 1.8×10^{-2} M	///	batch	43
	2.5×10^{-10} - 2.5×10^{-7} mol	3.8% at 1×10^{-9} mol	FIA	44
L-Glutamate	1×10^{-7} - 6×10^{-5} M	///	FIA	45
L-Glutamine	1×10^{-6} - 2.5×10^{-3} M	///	FIA	45
H_2O_2	1×10^{-6} - 1×10^{-4} M	5% at 1×10^{-4} M	batch	23
	2×10^{-8} - 2×10^{-5} M	///	batch	24
	2.4×10^{-8} - 1.2×10^{-4} M	2.6% at 2.5×10^{-5} M	batch	42
	2.5×10^{-12} - 2.5×10^{-8} mol	2.5% at 1×10^{-10} mol	FIA	44
	1×10^{-8} - 1×10^{-3} M	3%	FIA	46
	6.25×10^{-12} - 2.5×10^{-8} mol	3% at 2.5×10^{-10} mol	FIA	47
Hypoxanthine	1×10^{-6} - 3.1×10^{-4} M	///	batch	48
L-Lactate	2.5×10^{-10} - 1.25×10^{-6} mol	1.7% at 6×10^{-9} mol	FIA	49
L-Lysine	1×10^{-5} - 1×10^{-3} M	///	FIA	50
	1×10^{-6} - 1×10^{-3} M	///	FIA	51
Phosphate	1×10^{-7} - 1×10^{-4} M	///	FIA	51
Sulphite	1×10^{-6} - 1×10^{-4} M	///	batch	52
Xanthine	3.1×10^{-6} - 3.1×10^{-4} M	///	batch	48

4.3 Electrochemiluminescence-Based Fiberoptic Biosensors

As mentioned above, an original and unusual way to obtain a high sensitive hydrogen peroxide detection is the electrogenerated chemiluminescence of luminol (ECL). Based on this electro-optical process, a flow injection analysis optical fibre H_2O_2 sensor has been developed[53,54]. The electrochemiluminescence was generated using glassy carbon electrode polarized *vs* a platinum pseudo-reference electrode and integrated in a flow injection analysis system (Figure 8).

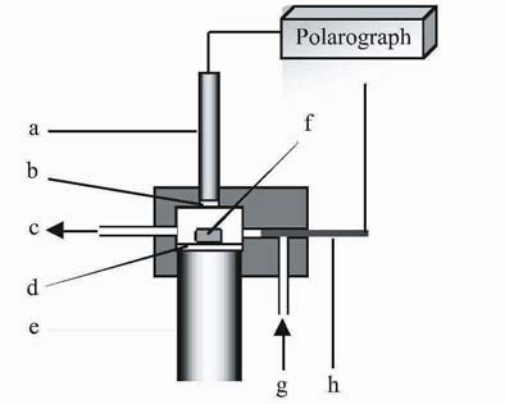

Figure 8. Flow cell for electrochemiluminescence measurements: (a) glassy carbon electrode; (b) sensing layer; (c) reagent solution outlet; (d) Plexiglas window; (e) liquid core single optical fiber; (f) stirring bar; (g) reagent solution inlet; (h) platinum electrode.

The optimization of the reaction conditions showed that an applied potential of + 425 mV *vs* a platinum pseudo-reference electrode enabled the realization of a sensitive H_2O_2 sensor while avoiding passivation of the working electrode. An optimum pH measurement of 9 was found and moreover, the pH dependence of the ECL sensor appeared less pronounced than when using immobilized HRP as the sensing layer. Under optimum conditions, hydrogen peroxide measurements could be performed in the range 1.5 pmol - 30 nmol. This ECL H_2O_2 sensor then exhibited slightly higher performances than membrane-based horseradish peroxidase chemiluminescent FIA biosensors (see [44], Table 3).

4.3.1 Glucose and Lactate Electrochemiluminescent Biosensor

For the development of glucose and lactate ECL FIA biosensors[53], the hydrogen peroxide ECL sensor was associated with the catalytic action of glucose oxidase and lactate oxidase. The oxidases were immobilized on synthetic preactivated membranes brought into contact with the glassy carbon electrode. The glucose or lactate electro-optical biosensor was then able to detect the target analyte with detection limits of 150 pmol and 60 pmol, respectively. In each case, glucose and lactate measurements could be performed over 4 decades.

These biosensors were tested for glucose and lactate measurements in sera, and for lactate measurements in whey solutions. Good agreements were obtained between the present method and reference methods. For glucose analysis in serum, the coefficient of variation for 53 repeated measurements performed over a 10 h period was 4.8% while for lactate analysis, 80 assays performed over a 15 h period gave a coefficient of variation of 6.7%. Thus,

the ECL-based biosensors gave the possibility to sensitively detect glucose and lactate in complex matrices without pre-treatment of the samples.

4.3.2 Choline Electrochemiluminescent Biosensor

A flow injection optical fibre biosensor for choline was also developed[55]. Choline oxidase (ChOX) was immobilized by physical entrapment in a photo-cross-linkable poly(vinyl alcohol) polymer (PVA-SbQ) after adsorption on weak anion-exchanger beads (DEAE-Sepharose). In this way, the sensing layer was directly created at the surface of the working glassy carbon electrode. The optimization of the reaction conditions and of the physicochemical parameters influencing the FIA biosensor response allows the measurement of choline concentration with a detection limit of 10 pmol. The DEAE-based system also exhibited a good operational stability since 160 repeated measurements of 3 nmol of choline could be performed with a variation coefficient of 4.5%.

4.3.3 Cholesterol Electrochemiluminescent Biosensor

A new cholesterol flow injection analysis biosensor has also been described as an application of the H_2O_2 ECL sensor[56]. In that work, the luminol electrochemiluminescence, previously studied in aqueous media, was implemented in Veronal buffer added of 0.3% triton X-100 (v/v), 0.3% PEG and 0.4% cholate to enable the solubilisation of the cholesterol and then its efficient oxidation catalyzed by the immobilized cholesterol oxidase. The ECL reaction occurred thus in a micellar medium and the performances of the H_2O_2 ECL sensor were investigated.

The calibration curve obtained for hydrogen peroxide exhibited a detection limit of 30 pmol and ranged over three decades at least. These performances compared well with those previously obtained in non-micellar media[54]. The presence of surfactant compounds in the ECL measurement buffer appeared thus to have little effect on the H_2O_2 ECL sensor performances. In optimized conditions, the determination of free cholesterol could be performed with a detection limit of 0.6 nmol and a calibration curve ranging over two decades at least.

5. BIOCHIPS AND MICROARRAYS

The chemiluminescence and electrochemiluminescence of luminol have been also exploited for the development of enzyme, DNA and immuno-biochips. Different approaches were studied in our laboratory, in which

bioactive molecules were anchored on microbeads subsequently entrapped in a suitable polymeric structure. Whatever immobilization method of the biological probe and the type of biochip (DNA, enzyme or protein) a unique detection system consisting of a CCD camera was used and both formats, low density arrays and microfluidic biochips were studied.

5.1 Composite Sensing Layer on Glassy Carbon Electrode

Beads bearing bioactive molecules have been used to develop generic biochips based on chemi- and electro-chemiluminescent detection. The biochips were composed of arrayed biosensors, including enzyme-charged beads, antigen-charged beads or oligonucleotide-charged beads, entrapped in polyvinyl alcohol (PVA-SbQ) photopolymer. In each case the sensing layers were spotted at the surface of a glassy carbon electrode (25 mm^2) as 0.3 µl drops, generating 500-800 µm spots. The luminescent reactions were either catalyzed by the horseradish peroxidase or triggered by the application of a +850 mV potential between the glassy carbon electrode and a platinum pseudo-reference.

Enzyme biochips were designed for the concomitant detection of choline, glucose, glutamate, lactate, lysine and urate, based on the corresponding oxidase-charged beads and the ECL reaction of the produced hydrogen peroxide with luminol-immobilized beads[57, 58]. Limits of detection of 1 µM for glutamate, lysine and uric acid, 20 µM for glucose and 2 µM for choline and lactate were found with detection ranging over three decades at least.

A tri-enzymatic sensing layer based on kinase-oxidase activities for the detection of acetate was also described. A reaction sequence using acetate kinase, pyruvate kinase and pyruvate oxidase enabled the production of H_2O_2 in response to acetate injection in the range 10 µM – 100 mM[59].

Based on IgG-bearing beads, a chemiluminescent immuno-biochip has been also realized for the model detection of human IgG. Biotin-labeled anti-human IgG were used in a competitive assay, in conjunction with peroxidase labelled streptavidin[59]. In that case, the planar glassy carbon electrode served only as a support for the sensing layer since the light signal came from the biocatalytic activity of horseradish peroxidase. Free antigen could then be detected with a detection limit of 25 pg (10^8 molecules) and up to 15 ng.

In a similar way, the use of oligonucleotide-immobilized beads enabled the realization of DNA sensitive biochips that could be used to detect biotin labelled sequence as 5.10^8 molecules[59].

5.2 Direct Entrapment of Micrometric Beads in PDMS

Poly(dimethylsiloxane) (PDMS) as an immobilization matrix has been successfully used to design multi-purpose biochips i.e., for either nucleic

acids, proteins or enzymes[60]. A new arraying method based on the properties of PDMS polymer to entrap beads bearing biologically active compounds was described. Such beads could be spotted and dried at the surface of a PVC master and subsequently transferred at the PDMS interface by direct moulding of the polymer on the mask (Figure 9). The use of the PDMS-assisted-immobilization enables the development of either a low density array (100 spots) or a micro-channel biochip with a direct incorporation of the sensing element in a fluidic system for the quantitative detection of enzyme substrates, antigens and oligonucleotides, depending on the immobilized sensing element. As a result, arrays of beads bearing active enzymes, antibodies and oligonucleotides were successfully obtained and enabled the achievement of biochips for the chemiluminescent detection of enzyme substrates, protein antigens and oligonucleotides sequence with detection limit of 1 μM, 1.5 x 10^7 molecules and 10^8 molecules, respectively.

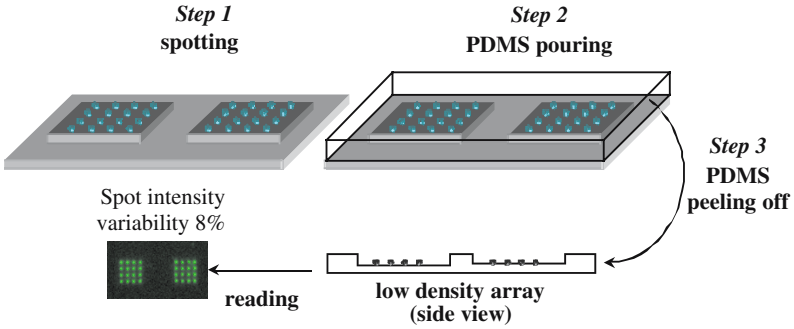

Figure 9. Direct immobilization in PDMS of beads bearing biologically active compounds. Step1: Sepharose beads bearing bioactive compounds are arrayed as 0.3 μl drop onto a flat PVC master; Step 2: a PDMS solution is poured onto the spotted array; Step 3: the PDMS-based array is peeled off from the master and ready to use.

5.3 Conducting Elastomer Surface Texturing

A new active support for biochip preparation has been developed based on graphite modified PDMS[61]. The addressed inclusion of Sepharose beads at the surface of the obtained elastomeric electrode generated local high specific surfaces. This electrode structure was characterized by electrochemical and imaging methods and an increase factor of the surface area equal to 50 was found. This was due to the texturing of the surface generated by the presence of the Sepharose beads (Figure 10).

This new material was used to design biochips based on the electro-chemiluminescence reaction of luminol in the presence of enzymatically

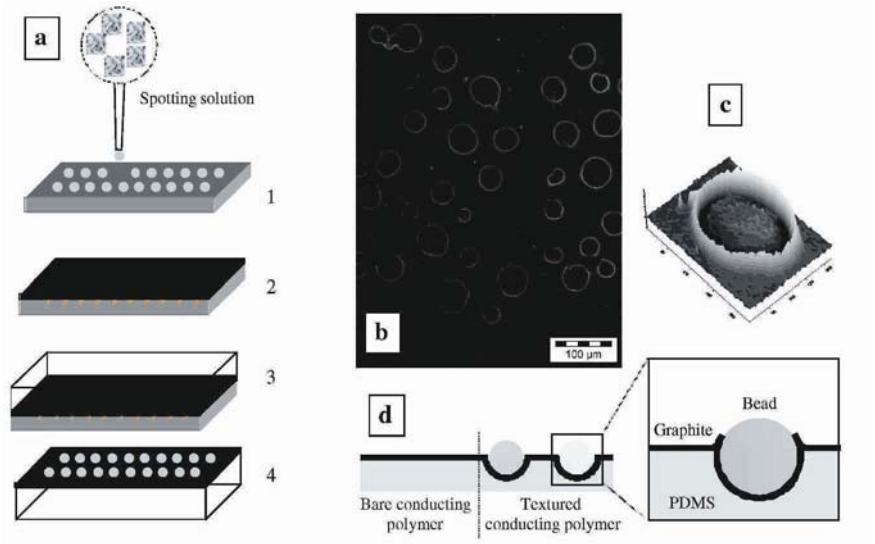

Figure 10. (a) Steps of the preparation of the addressed texturing of the PDMS-graphite conducting elastomer: (1) bead spotting; (2) graphite coating; (3) PDMS molding; (4) PDMS removal. (b) Electron microscopy (15 kV) image of Sepharose bead spots at the surface of the PDMS-graphite elastomer. (c) 3D representation of the scanning electron microscopy image of a Sepharose bead inserted in the the PDMS-graphite elastomer. (d) Schematic representation of the bead-containing layer.

produced H_2O_2. Using beads bearing biomolecules such as oligonucleotides or IgG, in conjunction with glucose oxidase-labelled DNA or antibody, sensitive biochips could be obtained with detection limits of 10^{11} and 10^{10} molecules respectively (Figure 11).

Multi-parameter enzyme-based biochips could also be obtained by locally adsorbing, at the PDMS-graphite surface, several oxidase enzymes.

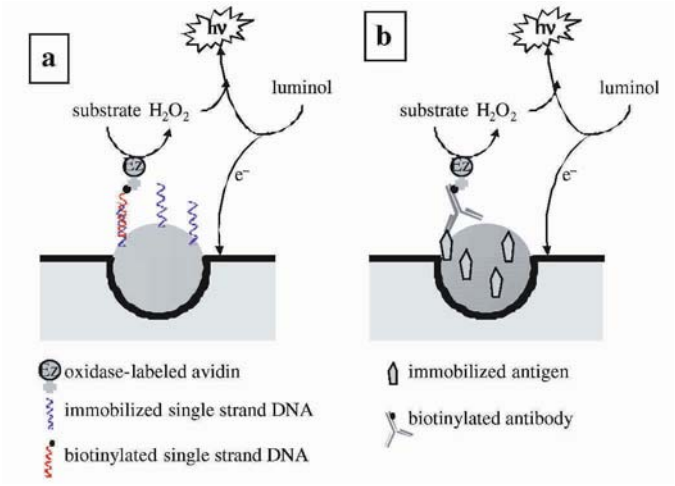

Figure 11. Electrochemiluminescent PDMS-graphite biochip formats: (a) nucleic acid-based biochip; (b) immunochip (competitive immunoassay).

With the specific suitable oxidases, lactate, choline and glucose could be assayed. Concentration measurements of these metabolites could be performed over at least two decades wit a detection limit of 10 μM for lactate and choline and 20 μM for glucose.

6. CONCLUSIONS

The field of biosensors is expanding continuously with a constant search for new transducing systems associated with stable biosensing elements. The ultra-sensitivity of bio- and chemiluminescence techniques together with the convenience of immobilized compounds in combination with optical fibres constitutes an attractive opportunity for designing biosensors. In addition to the advantages of optical fibre-based sensors, *i.e.* the possibilities of miniaturization and of remote sensing, bio- and chemiluminescence-based sensors require a simpler instrumentation than those based on other spectroscopic techniques. The coupling of auxiliary enzymes allows to extend the range of compounds that can be monitored at the trace level, including enzyme activities.

The current trends toward miniaturization and the need of massively parallel measurements led to the development of biochips. In this area, biocatalyzed and electrogenerated chemiluminescence reactions appear attractive and represent an alternative to fluorescence detection which is still widespread used despite the numerous problems of quantitative measurements and interference fluorescence emission.

Composite sensing layers, consisting of bioactive molecule-charged beads entrapped in a polymeric structure, have been successfully used to realize multi-purpose biochips for DNA, proteins or enzymes. For all these different biochips, the chemiluminescence and electro-chemiluminescence measurements required only a CCD camera and neither light sources nor optical filters are needed.

REFERENCES

1. Roswell D.F., White E.H., The chemiluminescence of luminol and related hydrazides, *Methods Enzymol.* 1978; 57: 409-423.
2. White E. H., Zafiriou O., Kägi H. H.,. Hill J.H.M., Chemiluminescence of luminol: the chemical reaction, *J. Am. Chem Soc.* 1964; 86: 940-941.
3. White E.H.,Bursey M.M., Chemiluminescence of luminol and related hydrazides. The light emission step, *J. Am. Chem. Soc.* 1964; 86: 941-942.
4. Yamazaki I., Yokota K.N., Oxidation states of peroxidase, *Mol.Cell. Biochem*. 1973; 2: 39-52.
5. Cormier M.J.,Prichard P.M., An investigation of the mechanism of the luminescent peroxidation of luminol by stopped flow techniques, *J. Biol. Chem.* 1968; 243: 4706-4714.

6. Kuwana T., Electro-oxidation followed by light emission, *J. Electroanal.Chem.* 1963; 6: 164-167.
7. Epstein B., Kuwa.na T., Electrooxidation of phthalhydrazides, *J. Electroanal. Chem.* 1967; 15: 389-397.
8. Thorpe G.H.G., Kricka L.J., Carter T.J.N., Chemiluminescence, in *Clinical and Biochemical Luminescence*, L. J. Kricka and T.J.N. Carter, eds. Marcel Dekker, New York, 1982, 21-42.
9. Mohan A.G., *Chemi- and Bioluminescence*, J.G. Burr, ed. Marcel Dekker, New York, 1985, 245-258.
10. De Luca M., Firefly luciferase, *Advances Enzymol.* 1976; 44: 37-68.
11. De Luca M., McElroy W. D., Purification and properties of firefly luciferase, *Methods Enzymol.* 1978; 57: 3-15.
12. McElroy W.D., De Luca M., Firefly bioluminescence, in *Chemi- and Bioluminescence*, JG Burr, ed., Marcel Dekker, New York, 1985, 387-399.
13. Ugarova N.N., Luciferase of *Luciola mingrelica* fireflies. Kinetics and regulation mechanism, *J. Biolumin. Chemilumin.* 1989; 4: 406-418.
14. Brovko L.Y., Gandelman O.A., Polenova T.E., Ugarova N.N. Kinetics of bioluminescence in the firefly luciferin-luciferase system. *Biochemistry (Russia)* 1994; 59(2): 195-201.
15. Gandelman O.A., Brovko L.Y., Chikishev A.Y., Shkurinov A.P., Ugarova N.N., Investigation of the interaction between firefly luciferase and oxyluciferin or its analogues by steady state and subnanosecond time-resolved fluorescence, *J. Photochem. Photobiol. B :Biol.* 1994; 22: 203-209.
16. Shimomura O., Johnson F.H., Saiga Y., Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan Aequorea, *J. Cell Comp. Physiol.* 1962; 59: 223-239.
17. Seliger H.H., McElroy W.D., Spectral emission and quantum yield of firefly bioluminescence, *Arch. Biochem. Biophys.* 1960; 88: 136-141.
18. Gonzalus-Miguel A., Meighen E.A., Ziegler M.M., Nicoli M., Nealson K.H., Hastings J.W., Purification and properties of bacterial luciferases, *J. Biol. Chem.* 1972; 247: 398-404.
19. Hastings J.W., Baldwin T.O., Nicoli M.Z., Bacterial luciferase: assay, purification and properties, *Methods Enzymol.* 1978; 57: 135-152.
20. Nealson K.H., Isolation, identification, and manipulation of luminous bacteria, *Methods Enzymol*. 1978; 57: 153-166.
21. Puget K., Michelson A.M., Studies in bioluminescence. VII. bacterial NADH: Flavin mononucleotide oxidoreductase, *Biochimie*, 1972; 54: 1197-1204.
22. Gerlo E., Charlier J., Identification of NADH-specific and NADPH-specific FMN reductase in *Beneckea harveyi*, *Eur. J. Biochem.* 1975; 57: 461-467.
23. Freeman T.M., Seitz W.R., Chemiluminescence fiber optic probe for hydrogen peroxide based on the luminol reaction, *Anal. Chem.* 1978; 50(9): 1242-1246.
24. Blum L.J., Gautier S.M., Coulet P.R., Luminescence fiber-optic biosensor, *Anal. Lett.* 1988; 21(5): 717-726.
25. Berger A., Blum L.J., Enhancement of the response of a lactate oxidase/peroxidase-based fiberoptic sensor by compartimentalization of the enzyme layer, *Enzyme Microb. Technol.*, 1994; 16: 979-984.
26. Michel P.E., Gautier S.M., Blum L.J., Effect of compartmentalization of the sensing layer on the sensitivity of a multienzyme-based bioluminescent sensor for L-lactate, *Anal. Lett.* 1996; 29 (7): 1139-1155.
27. Michel P.E., Gautier S.M., Blum L.J., A high-performance bioluminescent trienzymatic sensor for D-sorbitol based on a novel approach of the sensing layer design". *Enzyme Microb.Technol.* 1997; 21(2): 108-116.

28. Gautier S.M., Blum L.J., Coulet P.R., Bioluminescence-based fiber-optic sensor with entrapped co-reactant: an approach for designing a self-contained biosensor, *Anal. Chim. Acta*, 1991; 243: 149-156.
29. Gautier S.M., Blum L.J., Coulet P.R., Cofactor-containing bioluminescent fiber-optic sensor: new developments with poly(vinyl) alcohol matrices, *Anal. Chim. Acta*, 1991; 255: 253-258.
30. Gautier S.M., Michel P.E., Blum L.J., Reagentless bioluminescent sensor for NADH, *Anal. Lett.*, 1994; 27(11): 2055-2069.
31. Michel P.E., Gautier S.M., Blum L.J. , Luciferin incorporation in the structure of acrylic microspheres with subsequent confinement in a polymeric film : a new method to develop a controlled release- based biosensor for ATP, ADP and AMP, *Talanta* 1998; 47: 167-181.
32. Gautier S.M., Blum L.J., Coulet P.R., Alternate determination of ATP and NADH with a single bioluminescence-based fiber-optic sensor, *Sens. Actuators* 1990; B1: 580-584.
33. Gautier S.M., Blum L.J., Coulet P.R., Multifunction fibre-optic sensor for the selected bioluminescent flow determination of ATP or NADH, *Anal. Chim. Acta* 1990; 235: 243-253.
34. Gautier S.M., Blum L.J., Coulet P.R., Fibre-optic biosensor based on luminescence and immobilized enzymes: microdetermination of sorbitol, éthanol and oxaloacetate, *J. Biolumin. Chemilumin.* 1990; 5: 57-63.
35. Gautier S.M., Blum L.J., Coulet P.R., Dehydrogenase activity monitoring by flow injection analysis combined with luminescence based fibre-optic sensors, *Anal. Chim. Acta* 1992; 266: 331-338.
36. Gautier S.M., Blum L.J., Coulet P. R., Fibre-optic sensor with co-immobilised bacterial bioluminescence enzymes, *Biosensors* 1989; 4: 181-194.
37. Blum L.J., Gautier S.M., Coulet P.R., Highly stable bioluminescence-based fiber-optic sensor using immobilized enzymes from *Vibrio harveyi*, *Anal. Lett.* 1989; 22(10): 2211-2222.
38. Blum L.J., Gautier S.M., Coulet P.R., Continuous flow bioluminescent assay of NADH using a fibre-optic sensor, *Anal. Chim. Acta* 1989; 226: 331-336.
39. Lapp H., Spohn U., Janasek D., An enzymatic chemiluminescence optrode for choline detection under flow injection conditions, *Anal. Lett.* 1996; 29: 1-17.
40. Tsafack V.C., Marquette C.A., Pizzolato F., Blum L.J., Chemiluminescent choline biosensor using histidine-modified peroxidase immobilized on metal-chelate substituted beads and choline oxidase immobilized on anion-exchanger beads co-entrapped in a photocrosslinkable polymer, *Biosens. Bioelectron*, 2000; 15: 125-133.
41. Xie X., Suleiman A.A., Guilbault G.G., Yang Z., Sun Z., Flow-injection determination of ethanol by fiber-optic chemiluminesecnce measurement, *Anal. Chim. Acta* 1992; 266: 325-329.
42. Abdel-Latif M.S., Guilbault G.G., Peroxide optrode based on micellar-mediated chemiluminescence reaction of luminol, *Anal. Chim. Acta* 1989; 221: 11-17.
43. Zhou X., Arnold M.A., Internal enzyme fiber-optic biosensors for hydrogen peroxide and glucose, *Anal. Chim. Acta* 1995; 304: 147-156.
44. Blum L.J., Chemiluminescent flow injection analysis of glucose in drinks with a bi-enzyme fiber optic biosensor, *Enzyme Microb.Technol.* 1993; 15: 407-411.
45. Blankenstein G., Preuschoff F., Spohn U., Mohr K.H., Kula M.R., Determination of L-glutamate and L-glutamine by flow-injection analysis and chemiluminescence detection: comparison of an enzyme column and enzyme membrane sensor, *Anal. Chim. Acta* 1993; 271: 231-237.
46. Preuschoff F., Spohn U., Blankenstein G., Mohr G., Kula M.R., Chemiluminometric hydrogen peroxide sensor for flow injection analysis, *Fresenius J. Anal. Chem.*, 1993; 346: 924-929.
47. Berger-Collaudin A., Blum L. J., Enhanced luminescent response of a fiberoptic sensor for H_2O_2 by a high-salt concentration medium, *Sens. Actuators B* 1997; 38-39: 189-194.

48. Hlavay J., Haemmerli S.D., Guilbault G.G., Fibre-optic biosensor for hypoxanthine and xanthine based on a chemiluminescence reaction, *Biosens. Bioelectron* 1994; 9: 189-195.
49. Berger A., Blum L. J., Enhancement of the response of a lactate oxidase/peroxidase-based fiberoptic sensor by compartmentalization of the enzyme layer, *Enzyme Microb. Technol.* 1994; 16: 979-984.
50. Preuschoff F., Spohn U., Weber E., Unverhau K., Mohr K.H., Chemiluminometric L-lysine determination with immobilized lysine oxidase by flow-injection analysis, *Anal. Chim. Acta* 1993; 280: 185-189.
51. Spohn U., Preuschoff F., Blankenstein G., Janasek D., Kula M.R., Hacker A., Chemiluminometric enzyme sensors for flow injection analysis, *Anal.Chim. Acta* 1995; 303: 109-120.
52. Hlavay J., Guilbault G.G., Determination of sulphite by use of a fiber-optic biosensor based on a chemiluminescent reaction, *Anal. Chim. Acta* 1994; 299: 91-96.
53. Marquette C.A., Blum L.J., Luminol electrochemiluminescence-based fibre optic biosensors for flow injection analysis of glucose and lactate in natural samples, *Anal. Chim. Acta* 1999; 381: 1-10.
54. Marquette C.A., Blum L.J., Electrochemiluminescence of luminol for 2,4-D optical immunosensing in a flow injection system, *Sens. Actuators B* 1998; 51: 100-106.
55. Tsafack V. C., Marquette C. A., Leca B., Blum L. J., An electrochemiluminescence-based fibre optic biosensor for choline flow injection analysis, *Analyst* 2000; 125: 151-155.
56. Marquette C. A., Raveau S., Blum L. J., Luminol electrochemiluminescence-based biosensor for total cholesterol determination in natural samples, *Anal. Lett.* 2000; 33(9): 1779-1796.
57. Marquette C.A., Blum L.J., Self-containing reactant Biochips for the electrochemiluminescent determination of glucose, lactate and choline, *Sens. Actuators B* 2003; 90: 112-117.
58. Marquette C.A., Degiuli A., Blum L.J., Electrochemiluminescent biosensors array for the concomitant detection of choline, glucose, glutamate, lactate, lysine and urate, *Biosens. Bioelectron.* 2003; 19: 433-439.
59. Marquette C.A., Thomas D., Blum L.J., Design of luminescent biochips based on enzyme, antibody or DNA composite layer, *Anal. Bioanal. Chem.* 2003; 377: 922-928
60. Marquette C.A., Blum L.J., Direct immobilisation in PDMS for DNA, protein and enzyme fluidic biochips, *Anal. Chim. Acta* 2004; 506: 127-132.
61. Marquette C.A., Blum L.J., Conducting elastomer surface texturing: a path to electrode spotting. Application to the biochip production, *Biosens. Bioelectron*, 2004, 20 (2): 197-203.