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REVIEW

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Applications of the luminol chemiluminescent reaction in analytical chemistry

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Abstract This critical review discusses the results published between 2000 and 2005 on the development of analytical systems based on the luminol chemiluminescent and electrochemiluminescent reactions. An increasing number of non-specific detection systems based on the enhancing, inhibiting or catalysing effect of a large range of compounds have been published. Possible detected compounds and their concomitant presence in samples are discussed. Chemiluminescent and electrochemiluminescent reactions were also found to merge in biochip and microarray development as a possible substitute to the well-established but hardly quantitative fluorescent detections.

Keywords Biochip · Chemiluminescence · FIA · Luminol · Microarray

Introduction

Luminescent transitions of excited molecules or atoms to a state of lower energy are characterized by electromagnetic radiation dissipated as photons in the ultraviolet, visible or near-infrared region. These luminescent reactions are classified according to the energy source involved during the excitation step: thus, most classical light emission reactions are referred to as bioluminescence (from in vivo systems), chemiluminescence (from a chemical reaction), electroluminescence (from an electrochemical reaction) and photoluminescence (from UV, visible or near-IR radiations). More trivial reactions are also described as pyroluminescence (from flame-excited metal atoms), radioluminescence (from irradiation by X-rays or γ-rays), sonoluminescence (from ultrasonication of dissolved substance) and thermoluminescence (from solids subjected to mild heating).

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Chemiluminescence reactions are generally oxido-reduction processes and the excited compound, that is the reaction product, has a different chemical structure from the initial reactant. Several hundreds of organic and inorganic compounds are at the origin of chemiluminescence reactions which can occur in liquid or solid phases, or at solid–liquid or solid–gas interfaces [1–3].

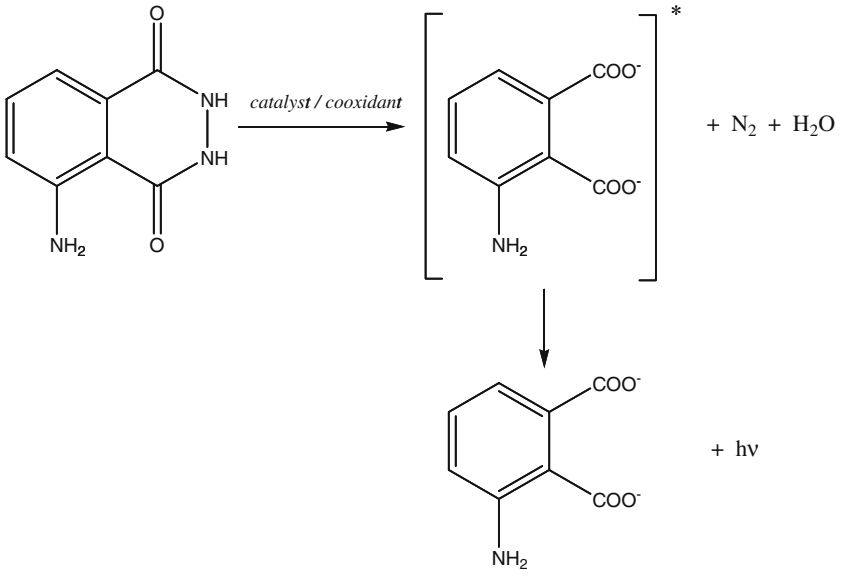
This critical review will mainly focus on liquid-phase light-emitting reactions based on the oxidation of 5-amino-2,3-dihydrophthalazine-1,4-dione (luminol). Scheme 1 shows the overall reactions in aqueous medium. Luminol oxidation leads to the formation of an aminophthalate ion in an excited state, which emits light when returning to the ground state. The quantum yield of the reaction is low (≈0.01) and the emission spectrum shows a maximum at 425 nm [4].

As suggested above, this reaction can be triggered through a wide range of catalysts, more or less specific for a particular oxidizing species and with varying efficiencies. Peroxidase enzymes, and particularly the one extracted from horseradish, are usually considered as the most effective catalyst, working at pH relatively close to neutral (8–10) and having a quite high specificity for a particular co-oxidant, the hydrogen peroxide. This chemiluminescence production system is the basis of biochemistry-based analytical systems such as biosensors, immunoassays, immunosensors and microarrays. The reaction is then used to reveal any interaction with a peroxidase-labelled entity, or to detect hydrogen peroxide first produced by a specific reaction.

The electrochemical oxidation of luminol is usually considered as the second most efficient way of triggering the reaction. The scheme is similar to the peroxidase-catalysed one with hydrogen peroxide as co-oxidant and a large range of working pH.

Finally, transition metal cations (Co^{2+}, Cu^{2+}, Cr^{2+}, Fe^{2+}+, Fe^{3+}, Hg^{2+}, Mn^{4+}, Ni^{2+}) and their complexed forms (e.g. ferrocene, ferricyanide) can be used as catalyst with mitigated performances which are linked to the relatively low signal to noise ratio obtained and the required elevated pH of the reaction.

Scheme 1



Applications of these three types of chemiluminescent reactions during the last five years will be presented and discussed. Particular attention will be given to: (i) the detection of compounds through their effect on the chemiluminescence reaction rate, (ii) the use of the chemiluminescent reaction as a label in separation systems, (iii) the coupling of this reaction to other enzymatic reactions and (iv) the on-chip uses on these reactions.

Reaction triggering

Bio-catalysis and chemi-catalysis

As suggested above, the bio-catalysis of the luminol chemiluminescence is considered as the most powerful way of triggering the light emission reaction. The heme-containing proteins, particularly horseradish peroxidases (EC 1.11.1.7), are able to catalyse the chemiluminescent reaction of luminol in the presence of hydrogen peroxide. The use of this enzyme has the advantage over other catalysts that the chemiluminescent reaction can proceed at near-neutral pH values (8–8.5).

In spite of numerous works [5–9], the complex mechanism of the peroxidase-catalysed reaction and the stoichiometry remain hypothetical. Roughly speaking, the reaction sequence leading to light generation can be divided into two main processes (Fig. 1a). First, in the course of a series of enzymatic steps and in the presence of luminol (LH^-) and hydrogen peroxide, horseradish peroxidase (HRP) is successively converted into intermediary complexes (complex I and complex II) before being regenerated to free peroxidase. These enzymatic steps produce luminol radicals ($L^{-\cdot}$, $LH^{\cdot}$) which then enter a complex chemical pathway to finally generate luminol hydroperoxide (LO_2H^-), the precursor of the light emitter (excited 3-aminophthalate ion). This second part of the mechanism can be slightly modified, in terms of the species

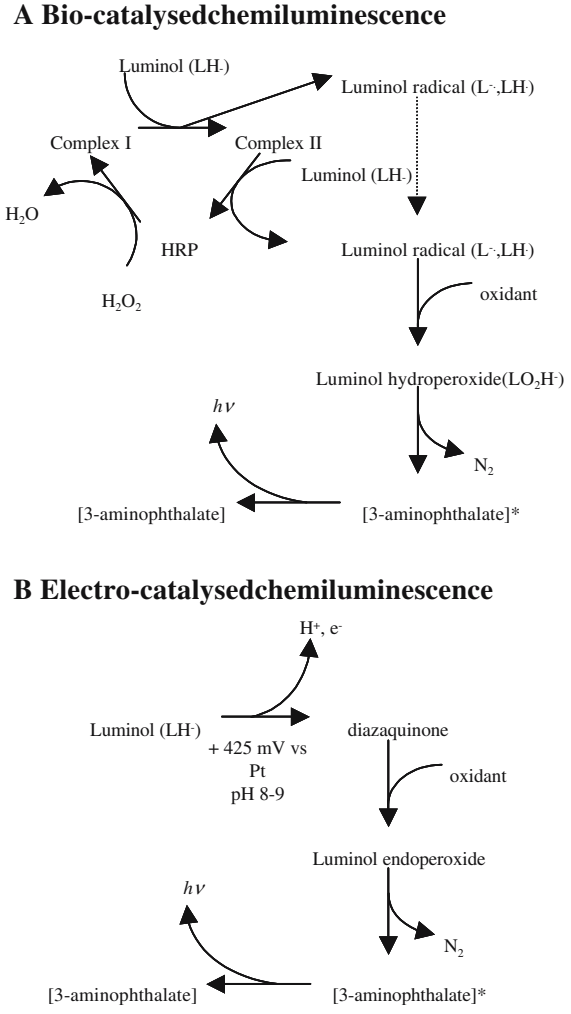


Fig. 1 Schematic representation of the **a** horseradish peroxidase catalysed reaction and **b** electro-catalysed chemiluminescent reaction

involved and their relative concentrations, when the enzyme to hydrogen peroxide molar ratio changes: when this ratio is high, a superoxide pathway is involved; for low ratios the predominant pathway is the diazaquinone route.

This reaction can be used for detecting peroxidase-labelled molecules, hydrogen peroxide generating labelled enzymes and hydrogen peroxide enzymatic precursors, i.e. substrates for oxidase enzymes generating hydrogen peroxide.

When transition metal cations are used to catalyse the reaction, these are involved in the production of luminol radicals, in the same way as HRP is, but under harsher pH conditions. The analytical applications using such catalysts will therefore focus more on the detection of molecules affecting the rate or efficiency of the reaction and the detection of hydrogen peroxide enzymatic precursors.

Electro-catalysis

An original and unusual way to obtain highly sensitive hydrogen peroxide detection is the electrogenerated chemiluminescence or electrochemiluminescence of luminol (ECL). In a mechanistic study of this ECL reaction, Sakura [10] had proposed that luminol was first oxidised at the electrode surface and then reacted, mole to mole, with hydrogen peroxide (Fig. 1b). The theoretical ratio (photon produced)/(H_2O_2 consumed) is then 1, whereas it is only 0.5 for the peroxidase-catalysed reaction. Moreover, avoiding the use of fragile enzymes for the catalysis of the chemiluminescent reaction could lead to more stable and reproducible sensors. Consequently, regarding the sensitivity of hydrogen peroxide detection, the electrogenerated chemiluminescence of luminol will be more efficient than the peroxidase-catalysed reaction [11]. Because of this high sensitivity for hydrogen peroxide most of the applications of ECL are dedicated to the detection of hydrogen peroxide generating label enzymes and hydrogen peroxide enzymatic precursors. Less often, this electrogenerated reaction is used for the detection of luminol-labelled molecules.

The main competitors to luminol label in the field of analytical chemistry are the ruthenium complexes [12]. Such molecules, which can be regenerated after having emitted their photon(s), appeared to be more appealing electroluminescent labels.

Analytical applications

Direct chemiluminescent detection of compounds in flow injection analysis (FIA)

The last five years have witnessed a drastic increase of the number of publications focussed on the use of chemiluminescence for the flow injection analysis of compounds [13] as diverse as metal ions, pesticides, amino acids and more generally pollutants and toxicants. This trend, mainly owing to the simplicity of development and use of chemiluminescent FIA systems, has led to the description of numerous case studies whose shear volume reveals their lack of specificity [14–32].

Indeed, a large number and type of compounds were tested, according either to their inhibiting, enhancing or catalytic effect on the chemiluminescent reaction. An overall look on this literature evidences quite interesting detection limits, with absolute detected quantities down to the sub-femtomolar level, but also obvious potential cross reactivity problems (Table 1) [33]. The presence of different toxicants for example in human urine will lead to the measurement of a mean signal corresponding to both inhibited and enhanced chemiluminescent reaction. The same remark could be made from water samples that are, as the human fluids, quite complex media obviously contain-

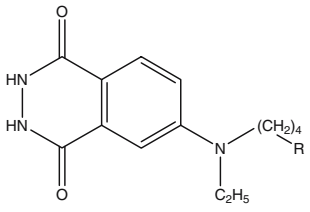
Table 1 Characteristics of chemiluminescent non-specific detection systems in FIA

Compound	CL effect (CL system)	Detection limit (quantity)	Sample	Reference
p-Aminophenol	Inhibition (DMSO/EDTA)	1.74 nM (0.17 pmol)	Water	[15]
Cobalt	Catalyst	5 pM (0.5 fmol)	Human sera, water	[16]
Carbofuran	Enhancement ($KMnO_4/OH^-$)	90 nM (6.3 pmol)	Water	[14]
Ascorbic acid, *L*-cysteine	Enhancement (Fe^{2+}/O_2)	0.3 μM (45 pmol) ascorbic acid 3 μM (0.45 nmol) cycteine 7.5 nM (1.1 pmol) cysteine	Human urine	[18, 19]
Rutin	Inhibition (ferricyanide)	0.6 nM (0.12 pmole 50 pM (2.5 fmol)	Human urine	[20, 21]
Phentolamine	Enhancement (ferricyanide)	9.5 nM (0.95 pmol)	Human samples	[22]
Uric acid	Enhancement (ferricyanide)	23 μM (3.45 nmol)	Human samples	[44]
Nitric oxide	Enhancement (OH^-)	1pM (constant)	–	[23–25]
Parathion	Enhancement (H_2O_2)	27 nM (1 pmol)	Rice	[26]
Isoniazid	Enhancement ($NaIO_4$)	4.2 nM (0.12 pmol)	Pharmaceutical preparations	[27]
Berberine	Inhibition (ferricyanide)	52 pM (10 fmol)	Pharmaceutical preparations	[28]
Dobutamine hydrochloride	Enhancement (ferricyanide)	77 pM (5.7 fmol)	Pharmaceutical preparations	[29]
Riboflavin	Enhancement (*N*-bromosuccinimide)	9.2 nM (5.5 pmol)	Pharmaceutical preparations	[30]
Iodine	Catalyst	0.4 μM (0.1 nmol)	Gas samples	[31]
Arsenic	Enhancement (ferricyanide)	4 nM (0.3 pmol)	–	[32]

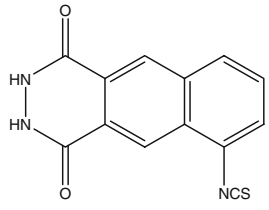
ing more than one chemiluminescently active component. Nevertheless, in particular situations—e.g. in process analytics—one and only one particular analyte is expected and needs to be quantified. In these cases, such sensitive analytical systems could then be really helpful.

A solution to overcome these interferences and cross reactivity problems should be found in the use of a separation step such as liquid chromatography [34], high-performance liquid chromatography [35–37] or capillary electrophoresis [34, 38–40] before the detection. The

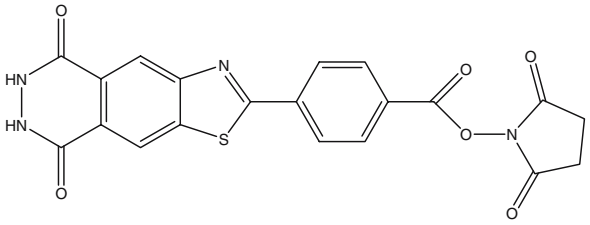
a



	R
N-(4-Aminobutyl)-N-ethylisoluminol (ABEI)	-NH2
N-(4-Aminobutyl)-N-ethylisoluminol isothiocyanate(ABEI-ITC)	-NCS

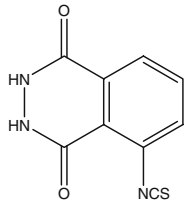


6-isothiocyanatobenzo[g]phthalazine-1,4(2H,3H)-dione (IPO)

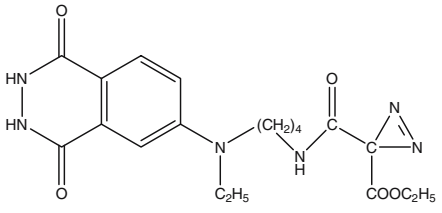


4-(6,7-dihydro-5,8-dioxothiazolo[4,5-g]phthalazin-2-yl)benzoic acid N-hydroxysuccinimide ester (TPB-Suc)

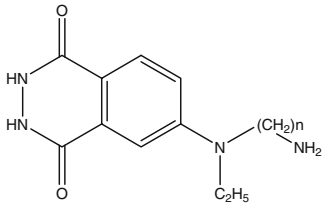
b



Isoluminol isothiocyanate (ILITC)

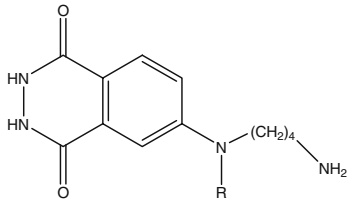


4-[(N-α-ethoxycarbonyldiazoacetyl)aminobutyl]-N-ethylisoluminol (EDA-ABEI)

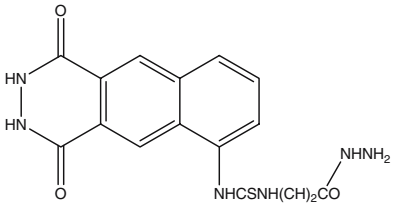


	n
ABEI	4
N-(4-Aminohexyl)-N-ethylisoluminol (AHEI)	6

c



	-R
ABEI	$-C_2H_5$
N-(4-Aminobutyl)-N-methylisoluminol (ABMI)	$-CH_3$



6-[N-(3-propionohydrazino)thioureido]benzo-[g]phtalazine-1,4(2H,3H)-dione (PROB)

Fig. 2 Chemiluminescent luminol reagents for labelling of **a** amines, **b** amino acids and peptides and **c** carboxylic acids

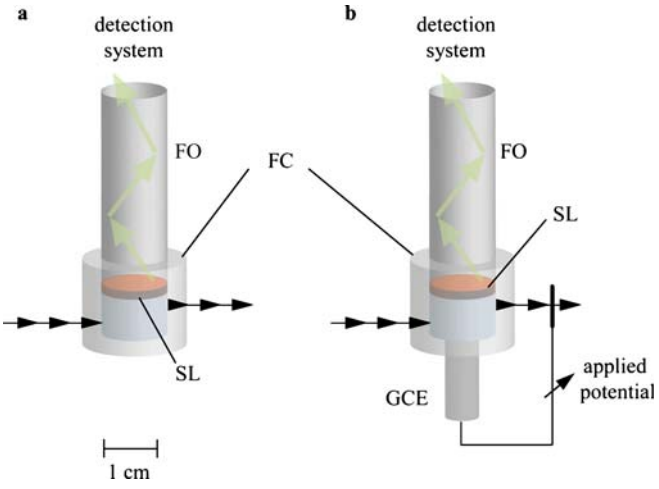


Fig. 3 Flow injection analysis systems for enzyme-based chemiluminescent (**a**) and electrochemiluminescent (**b**) specific systems. *FO* optical fibre, *FC* flow cell, *GCE* glassy carbon electrode, *SL* sensing layer

characterization of the retention time for each species present in the samples then leads to more accurate results. Two different uses of these coupled systems are (i) separation and detection through the effect of each separated compound on the CL reaction [36, 37, 39, 40], (ii) labelling of the sample components with luminol before the separation and detection through their emitted chemiluminescence [34, 35]. For the latter purpose, a large range of luminol derivatives (Fig. 2) can be used for the labelling of aminoacids and peptides, ibuprofen, amphetamine and methamphetamine, histamine and much more. The detection limits obtained with these chemiluminescent separative systems are usually in the fmol–pmol range.

An extension of such separation systems is the use of molecularly imprinted polymer recognition elements jointly with chemiluminescent detection [41, 42]. Polymers are synthesised by using a process of template-induced formation of specific molecular recognition sites (binding or catalytic) in a material where the template directs the positioning and orientation of the material's structural components by a self-assembling mechanism. The binding by the polymer of the target molecule, which has chemiluminescent activity (usually catalytic), will then help to increase the specificity of the analytical systems.

Bio-specific chemiluminescent detection of compounds

Interesting approaches to the specific detection of particular compounds in a complex mixture are based on the sequential use of highly specific enzymatic reactions and chemiluminescent detection. Chemiluminescent systems requiring hydrogen peroxide for the light emission (Fig. 1) are of particular interest because in addition to the determination of H_2O_2, several other compounds can be analysed by coupling with H_2O_2-generating enzymatic reactions [43–49]. This makes it possible to detect specific compounds, including e.g. glucose, lactate, urate, cholesterol, xanthine, choline, in complex media, taking advantage of the specificity of the oxidase enzyme used.

For this type of biosensor, FIA fibre optic sensors associated with membrane- or polymer-based immobilized enzymes (Fig. 3a) have been extensively used; however, these appear to be declining in popularity (according to the number of publications) since the introduction of miniaturisation. Nevertheless, the immobilization of both the oxidase enzyme and the peroxidase in millimetre-sized sensing layers, in contact with a fibre optic, has led to the achievement of sensitive, specific and stable analytical systems [3, 50, 51]. Interesting results were also obtained based on ferricyanide or electrochemically catalysed chemiluminescence (Fig. 3b). The typical detection performances of such macrosystems are presented in Table 2. They exhibit satisfactory detection limits and ranges and are in most cases validated in complex samples such as human sera. These systems benefit from the high sensitivity of the light measurement systems such as photomultipler tubes. More applications of these coupled reactions are detailed in the following section, which presents the up-to-date miniaturized chemiluminescent microarrays and biochips systems.

Table 2 Analytical characteristics of enzyme-based chemiluminescent sensors for bio-specific detection in FIA systems

Compound	CL system	Detection limit	Range	Reference
L-Glutamate	Luminol/peroxidase	40 nM	40–1,000 nM	[48]
	Luminol/peroxidase	10 nM	20 nM–5 μM	[49]
L-Lysine	Luminol/peroxidase	50 nM	50–1,200 nM	[48]
Lactate	Luminol/ferricyanide	0.2 μM	1–1,000 μM	[43]
	Luminol/electrochemical	1 μM	1 μM–3 mM	[11]
Uric acid	Luminol/ferricyanide	4 nM	10 nM–30 μM	[44]
Choline	Luminol/electrochemical	0.3 μM	0.3–1,000 μM	[73]
	Luminol/peroxidase	15 nM	15 nM–300 μM	[45]
Glucose	Luminol/OH^-	4 μM	10 μM–1 mM	[46]
	Luminol/peroxidase	0.12 mM	0.2–2 mM	[47]
	Luminol/electrochemical	2 μM	2 μM–30 mM	[11]
Cholesterol	Luminol/electrochemical	20 μM	20 μM–2.5 mM	[74, 75]

Table 3 Analytical characteristics of antibody-based chemiluminescent sensors for bio-specific detection

Compound	CL system	Detection limit	Range	Reference
IgG	Luminol/peroxidase	2 μg L^{-1}	2–60 μg L^{-1}	[57]
Salmonella typhimurium	Luminol/peroxidase	1.97×10^{3} CFU mL^{-1}	1.97×10^{3} to 1.97×10^{6} CFU mL^{-1}	[58]
2,4-D	Luminol/peroxidase	4 μg L^{-1}	4 μg L^{-1}–160 mg L^{-1}	[52]
	Luminol/electrochemical	0.2 μg L^{-1}	0.2 μg L^{-1}–200 mg L^{-1}	[59]
Okadaic acid	Luminol/peroxidase	0.1 μg L^{-1}	0.1 μg L^{-1}–100 μg L^{-1}	[53]
TNT	Luminol/peroxidase	0.1 μg L^{-1}	0.1–1000 μg L^{-1}	[54]
Serologic IgG	Luminol/peroxidase	1:800,000 titer	–	[56]
Alkylbenzene sulfonates	Luminol/peroxidase	50 ppb	–	[55]

Table 4 Analytical characteristics of chemiluminescent-based bio-specific detection on biochips and microarrays

Compound	CL system	Range of detection limit	Reference
Ricin, viscumin, staphylococcal enterotoxin B, tetanus and diphtheria toxins, lethal factor of anthrax	Luminol/peroxidase	0.1–50 μg L^{-1} depending on the compound	[62]
Penicillin G, cloxacillin, cephapirin, sulfadiazine, sulfamethazine, streptomycin, gentamicin, neomycin, erythromycin, and tylosin	Luminol/peroxidase	0.12–32 μg L^{-1} depending on the compound	[63]
Allergen-specific antibodies	Luminol/peroxidase	0.16–1.9 μg L^{-1} depending on the compound	[64]
Influenza A viruses (H1N1, H3N2, H1N2, and H5N1) and influenza B viruses genes	Luminol/peroxidase	–	[67]
Choline, glucose, glutamate, lactate, lysine, urate	Luminol/electrochemical	1–20 μM depending on the compound	[72]
Choline, glucose, lactate	Luminol/electrochemical	3–10 μM depending on the compound	[70]

When the compound of interest is not a substrate for oxidase enzymes or could not be converted into such a substrate, the chemiluminescence reaction can still be used in a biospecific system through the use of immunoreactions with peroxidase- or luminol-labelled antibodies. In such cases the specifically detected target can be pesticides [52], toxins [53], explosives [54], detergent [55], proteins [56, 57] or living cells [58] (Table 3).

Peroxidase-labelled antibodies are the most widely used chemiluminescent tools in the bio-analytical field. Indeed, these protein complexes are used for immunoassay on standard 96-well microplates, for immunosensor detection and for on-chip immunodetection (see below).

Antibodies labelled with luminol or luminol derivatives [59] have exclusively been used as a label for electrochemiluminescent systems. Only a few works with such a label have been [59, 60], mainly because of the difficulties encountered when attempting to achieve its attachment through standard chemical reactions compatible with retaining the protein integrity. Indeed, the luminol molecule has an aromatic amine as the only available functional group and this is not easily covalently linked to bio-

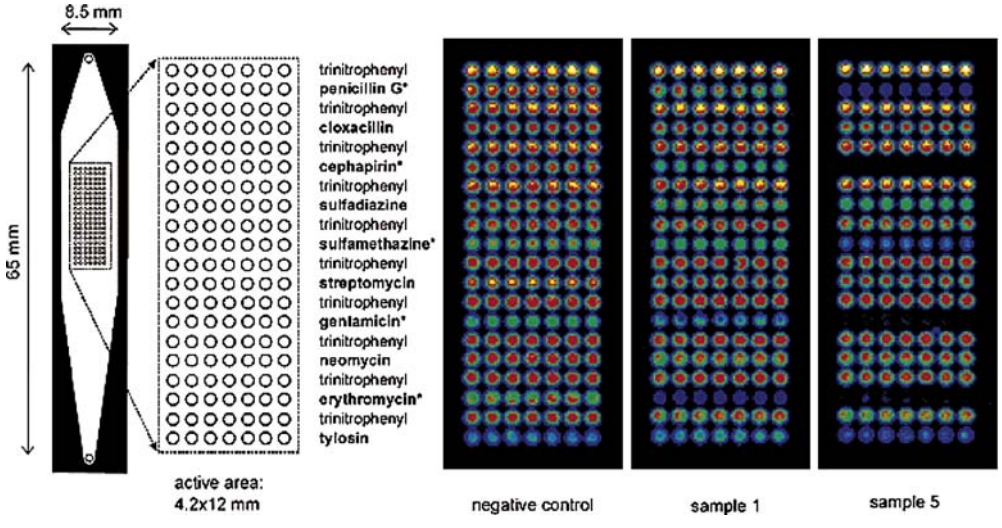


Fig. 4 Schematic representation and chemiluminescent images of a passive microarray for the multiplexed detection of antibiotics. Reproduced from ref. [63]

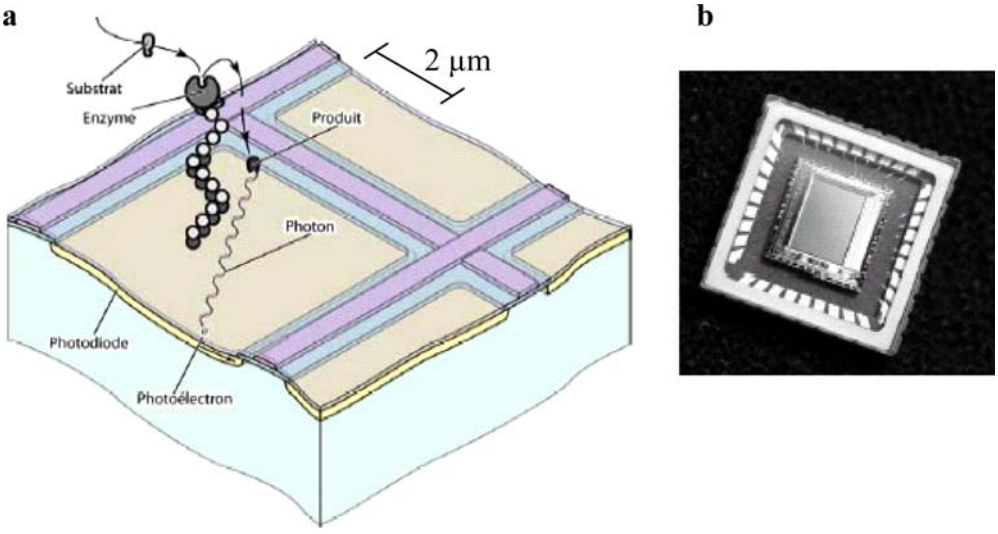


Fig. 5 **a** Schematic representation of a chemiluminescent active biochip based on a commercial CCD photodetector. **b** Photograph of the commercial photodetector used. Reproduced from ref. [68]

molecules. Luminol derivatives with more reactive functional groups, such as *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI), have also been tested as labels [61] but these have shown lower light emission properties when grafted directly to proteins.

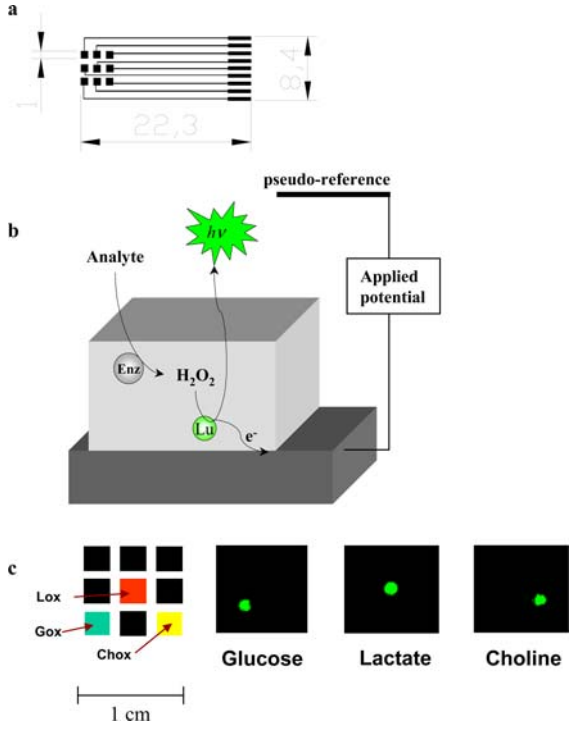


Fig. 6 **a** Schematic representation of an electrochemiluminescent active biochip based on screen-printed electrodes array. **b** Principle of the electrochemiluminescent enzyme-based biochip. **c** Distribution of the different sensing layers at the biochip surface and electrochemiluminescent images of the biochip in the presence of either glucose, lactate or choline. *Chox* choline oxidase, *Gox* glucose oxidase, *Lox* lactate oxidase, *Lu* luminol

Bio-specific on-chip chemiluminescent detection of compounds

One of the major advances in chemiluminescent analytical tools during the last five years has been the use of these light-emitting reactions in miniaturized systems such as biochips and microarrays. Thus, the sensing layers' multiplexing has led to the achievement of multi-parameter systems with interesting quantitative possibilities when compared to the equivalent fluorescent systems.

Passive systems for immunodetection (Table 4), i.e. where the solid phase only provides an immobilization support, have been described for the quantitative detection of multiple toxins [62], antibiotics [63] or allergen-specific antibodies [64]. An example of such systems is presented in Fig. 4. A homogeneous immobilization chemistry was performed on the entire surface of the support (microscope glass slide) and different proteins spotted to generate the

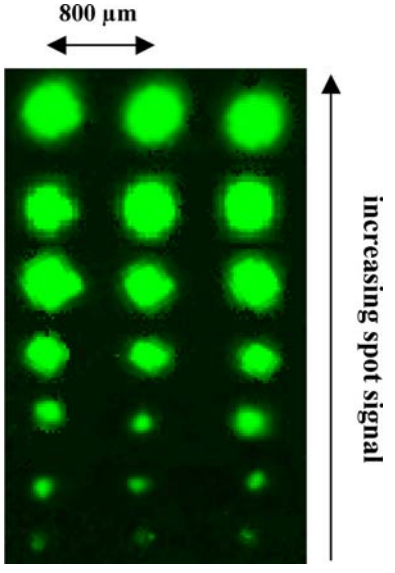


Fig. 7 Chemiluminescent image of a protein microarray incubated with increasing amounts of peroxidase-labelled antibodies

array [63]. The low light background of the charge coupled device (CCD) system and the bright light emission of the peroxidase labelling system (see Fig. 4 micrographs) enabled the quantitative detection of the different molecules (11 antibiotics) concomitantly.

More elaborate systems involving heterogeneous immobilization chemistry based on latex bead deposition [65, 66] have also been described. These systems use a chemiluminescent detection and exhibited interesting analytical performances for the detection of HIV-1-specific antibodies or p53 gene sequence mutations. In these cases, specific immobilization chemistry could be performed according to the molecule to be immobilized and furthermore the low background signal of the CCD imaging system led to sensitive quantification.

Similar work with passive supports but for the multiparametric detection of DNA sequences were also published recently [67]. Analysis of influenza A viruses (H1N1, H3N2, H1N2, and H5N1) and influenza B viruses showed that chemiluminescent microarray-based methods were capable of rapid and unambiguous identification of all types and subtypes of viruses by use of random PCR products. DNA sequences were then labelled with peroxidase through biotin–streptavidin adducts.

Interesting results were also obtained, for DNA sequence detection, by directly using the CCD sensor as an immobilization substrate [68]. Thus, the photons emitted by the peroxidase-labelled DNA were detected with high sensitivity through the CCD array (Fig. 5) and led to really interesting performances with low-pM detection limits for target sequences.

Micro-chemiluminescent biosensors and flow injection biosensors (μFIA) were also described based on the HRP-catalysed reaction [69] or electrochemically triggered reactions (Fig. 2b) [70–72]. Figure 6 presents one of these ECL micro-biosensors based on the use of screen-printed electrode arrays. This active support acts as immobilization substrate and ECL triggering material, and enabled the detection of different compounds (glucose, lactate and choline) concomitantly and with good sensitivities (Table 4). The micrographs in Fig. 6 present the typical electrochemiluminescent images obtained, which are characterised by good spatial resolution and no interference problem between the different sensing elements.

Conclusions

Luminol chemiluminescence reactions are very attractive, mainly owing to their ease of use, high sensitivity and simplicity of the instrumentation used for the technique. During the last five years, a large number of studies have been reported on the use of non-specific chemiluminescent reactions for the detection of an unexpectedly large number of compounds. As has been discussed, such a large number of potential activators, inhibitors and catalysers evidences obvious lacks of specificity of the developed direct detection systems. More specific results were obtained by coupling these non-specific reactions with separation steps such as chromatography or electrophoresis systems. Major advances were also obtained through the miniaturization and multiplexing of chemiluminescent analytical systems. Thus, micro-biosensors and arrayed immunoassays have achieved performances similar to those of the macro systems developed during the late 1990s.

Chemiluminescent reactions therefore appeared to be good candidates for biochip developments albeit with some potential restrictions. The chemiluminescent image presented in Fig. 7 evidences a possible problem of the chemiluminescent detection on microarrays. Indeed, for a constant spot diameter of 150 μm, increasing the density of peroxidase within the spot generates an increasing emission area. Such phenomenon, probably due to the lateral diffusion of luminol radical or excited 3-aminophthalate, will therefore impose a limit on the achievable spot density that could be detected through peroxidase-catalysed chemiluminescence [65]. A more complete understanding of such phenomenon is therefore required to fully explore the on-chip chemiluminescent detection possibilities.

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