Principles and recent analytical applications of chemiluminescence

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The potentials and possibilities offered by the rather unknown chemiluminescence-based analytical technique are discussed. Simplicity of instrumentation, low detection limits for various systems, and the inherent power of application to a vast number of natively fluorescing species or fluorophores formed after chemical derivatization broaden the scope of this relatively new detection technique. Drawbacks should be mentioned as well, amongst others limited selectivity of analysis (unless coupled to a powerful separational set-up), unexpected poor sensitivities for various analytes and the need to include an extra reaction step in the analytical procedures. Apart from the earlier gas-phase applications, the analysis in flowing streams (flow injection analysis, HPLC, and even capillary electrophoresis) has increased exponentially starting back in the early eighties. Various reagents have become available, including some originating from bioluminescent reactions (*e.g.* for ATP- and related analysis), not to forget the light emission as produced by diverse oxidation reactions, many of which the exact chemical pathways are not elucidated yet. On top of this, the power of chemiluminescence induction and measurements as applied to immunoassays, in the development of sensors, and, even more important, in the fast developing area of micromachining (sub-droplet-sized capillary electrophoresis) are discussed.

Introduction

Luminescence phenomena have been known for a long time in nature, being reflected since Antiquity in the early scientific literature. In fact, all of these first observations were related mainly to living organisms that emit light such as the fireflies, luminous bacteria, fungi, fishes, or insects [1]. Although the phenomena of chemiluminescence being the light emission produced in a synthetic way was observed for the first time by Radziszewski [2] in 1877, more specifically the green light emitted from lophine when reacting with oxygen**,** the term "chemiluminescence" was not introduced until 1888. Wiedemann defined the term "luminescence" (Greek: Lucifer, light bearer) in order to distinguish between light emission from thermally excited substances and light emission from molecules excited by other mechanisms without increasing their average kinetic energy [3]. He classified luminescence phenomena into six different kinds, according to the way of excitation: *photoluminescence* caused by the absorption of light, *electroluminescence* produced in gases by an electric discharge, *thermoluminescence* produced by slight heating, *triboluminescence* as a result of friction, *cristalloluminescence* as a result of crystallization, and *chemiluminescence* caused by a chemical reaction.

In this sense, chemiluminescence (CL) is defined as the production of electromagnetic radiation (ultraviolet, visible or infrared) observed when a chemical reaction yields an electronically excited intermediate or product, which either luminesces or donates its energy to another molecule, which then luminesces. If radiation is emitted by energy-transfer, the process is normally called *chemi-excitation*; likewise when the chemiluminogenic reaction is enzymatic and/or occurs within a organism living, the phenomenon is named bioluminescence (BL).

Exploration of CL for analytical applications has just been ongoing since the 1970s for gas-phase and from the 1980s for liquid-phase reactions. In this decade, general chapters and reviews have been published, related to the characteristics of CL as analytical technique [4-6], mainly in liquid phase [7-10], and its use as detection mode in flowing streams [11-13]. Extensive reviews have reported the specific application of CL reactions according to the nature of the analyte (inorganic species, enzymes and nucleotides, acids and amines, carbohydrates, steroids, polycyclic aromatic compounds and drugs) and covering the literature from 1983 to 1991 [14] and from 1991 to mid-1995 [15]. The number of reactions producing CL cited in literature is increasing in different fields of interest. Some recent reviews have reported the analytical applications in several disciplines such as biomedical [16-18], food [19,20], environmental and toxicological [21] analysis.

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General principles

In general, a CL reaction can be generated by two basic mechanisms. In a *direct* reaction, two reagents, usually a substrate and an oxidant in presence of some co-factor, react to form a product, then some fraction of the product will be formed in an electronically excited state which can subsequently relax to the ground state with emission of a photon. On the contrary, *indirect or sensitised* CL is based on a process of transference of energy of the excited species to a fluorophore. This process makes it possible for those molecules that are unable to be directly involved in CL reactions to transfer their excess of energy to a fluorophore that in turn is excited, releasing to its ground state with photon emission. For a chemical reaction to produce light, it should meet some essential requirements:

1) The reaction must be exothermic to produce sufficient energy to form the electronically excited state. In this sense, for CL to occurs the reaction must be sufficiently exothermic such that:

$$-\Delta G \geq \frac{hc}{\lambda_{ex}} = \frac{2{,}86 \times 10^4}{\lambda_{ex}}.$$

Since the creation of the electronically excited state and the generation of CL in the visible region require around 40-70 Kcal.mol^{-1}, the occurrence of CL is tied with exothermic processes, being limited to the reactions that meet this requirement, such as redox reactions using oxygen and hydrogen peroxide or similar potential oxidants.

2) The reaction pathway must be favourable to channel the energy for formation of an electronically excited state.

3) Photon emission must be a favourable deactivation process of the excited product in relation to other competitive non-radiative processes that can appear in low proportion. In the case of sensitised CL, both the efficiency of energy transfer from the excited species to the fluorophore and the fluorescence efficiency must be good.

In all luminescent processes, the intensity of the emission depends on the efficiency in the generation of molecules in excited state, which is represented by the quantum efficiency (or quantum yield) and the rate of the reaction. In the case of CL reactions, the intensity can be expressed as:

$$I_{CL} = \phi_{CL} \frac{-\mathrm{d}A}{\mathrm{d}t}$$

being I_{CL} the CL emission intensity (photons/seconds), ϕ_{CL}, the CL quantum yield and $-\mathrm{d}A/\mathrm{d}t$, the rate at which the CL precursor A is consumed. CL quantum yield is expressed as a product of two efficiencies:

$$\phi_{CL} = \phi_{ex}\, \phi_L$$

where ϕ_{ex} is the efficiency of production of the excited species (the fraction of the CL precursor A that produces an excited molecule) and ϕ_L is the luminescence efficiency of the luminescent species. For sensitised CL, ϕ_{ex} also includes the efficiency of the energy transfer process. Higher values of quantum yield are usually associated with BL reactions whereas in most of the CL reactions used for analytical purposes, ϕ_{CL} is ranging from 0.001-0.1.

CL measurements are strongly modified by experimental factors including temperature, pH, ionic strength, solvent and solution composition, and, because of the dependence of the reaction rate on the concentration, CL techniques may be satisfactorily used for quantitative analysis. Due to the emission intensity being linearly proportional to the concentration of any of the reagent, the technique is versatile for the determination of a wide variety of species that can participate in the CL process, such as: CL substrates or CL precursors responsible for the excited state; the required reagent for the CL reaction (usually an oxidant); some species that affect the rate or efficiency of CL reaction: activators such as a catalysts (enzymes or metal ions) or inhibitors such as reductants that inhibit the CL emission; fluorophores, in the case or sensitised CL; some species that are not directly involved in the CL reaction but that can react with other reagents in coupled reactions to generate a product which is a reactant in the CL reaction; species that can be derivatized with some CL precursors or fluorophores, being determined by direct or sensitised CL.

As advantages inherent to CL techniques, one can indicate the basic instrumentation required and the simplification of the optical system because no external light source is needed. CL is often described as a dark-field technique: the absence of strong background light level, such as found in absorptiometric techniques, reduces the background signal and leads to improved detection limits. Instrumentation for the CL measurements ranges from simple to more complex, and it is worth mentioning that simple fluorometers can be used by turning off the excitation source, or more sophisticated systems, mainly using photomultiplier tubes (PMT) or photon counting systems [22], photodiodes, scintillation counters or films. The technical evolution in the field of microelectronics and opto-electronics has allowed the use of charged coupled devices (CCD) detectors, fiber optics, assembly techniques and robotics, which resulted in the introduction of a new generation instruments, commercially available, with increased performance, speed and ease of handling [23].

However, some limitations have to be considered in CL analysis, such as the above-mentioned dependence of the CL emission on several environmental factors that hence must be controlled, the lack of selectivity because a CL reagent is not limited to just one unique analyte, and finally, like other mass flow detection approaches, since CL emission is not constant but varies with time (light flash composed of a signal increase after reagent mixing, passing through a maximum, then declining to the baseline), and this emission *versus* time profile can widely vary in different CL systems, care must be taken so as to detect the signal in the flowing stream at strictly defined periods.

Main chemiluminescence systems for analytical purposes

Gas-phase CL reactions

The development of CL methods for determining components of a gas largely originated from the need to determine atmospheric pollutants, allowing the development of instruments for pollutant monitoring with sensitivities ranging at the ppb-level. The production of CL emission in the UV-visible spectral region requires highly exothermic reactions such as atomic or radical recombinations, or reaction of reduced species such as hydrogen atoms, olefins, and several sulphur and phosphorous compounds with strong oxidants such as ozone, fluorine and chlorine dioxide. Several commercial CL analysers have been developed for the determination of sulphur, nitrogen or phosphorous compounds. In the case of sulphur dioxide and other sulphur compounds the emission is due to the electronically excited sulphur produced [24,25]:

$$SO_2 + 2\ H_2 \rightarrow S + 2\ H_2O$$

$$S + S \rightarrow S_2^*$$

$$S_2^* \rightarrow S_2 + h\nu\ (300\text{-}425\ nm).$$

The most widely used gas-phase CL reagent is ozone [26]. Some analytical methods have been proposed for the determination of NO, sulphur monoxide and unsaturated hydrocarbons, such as alkenes, alkynes, aromatics and alkanes at high temperatures, based on the CL emission produced in their reaction with ozone. Several of these are as follows:

$$NO + O_3 \rightarrow NO_2^* + O_2 \rightarrow NO_2 + h\nu\ (600\text{-}2800\ nm)$$

$$C_2H_4 + 2O_3 \rightarrow HCHO^* + O_2 \rightarrow h\nu\ (350\text{-}520\ nm)$$

$$SO + O_3 \rightarrow O_2 + SO_2^* \rightarrow SO_2 + h\nu\ (260\text{-}480\ nm).$$

In atmospheric research, the most useful detectors are applied in the detection of oxides of nitrogen using the above-proposed reaction. The detector, named "NO_x box" measures both NO and the sum of NO and NO_2 (*i.e.*, NO_x), as well as NO_y (*i.e.* total reactive oxides of nitrogen) [27]. These reactions with NO and ethylene are the basis of the Fast Ozone Detector, used to measure ozone in the atmosphere. In heterogeneous gas-solid phase, a fast-response detector allows a very sensitive and selective determination based on the CL induced when rhodamine B, adsorbed on silica gel, is exposed to ozone [28,29]. This procedure has been used for the study of vertical distribution of ozone in the atmosphere using miniaturised sondes [30]. The former reaction constitutes the basis of the named "pyroluminescence method", in which organic and inorganic nitrogen (including nitrate) are oxidised in air at 1000 °C to form nitric oxide, which makes possible to determine harmful nitrogen as a criterion of beet quality [31]. Sulphur-selective CL detection can also be achieved by converting sulphur-containing compounds to sulphur monoxide which allows the analysis of sulphur-containing pesticides [21].

Oxygen atoms have also been applied as strong oxidants when reacted with a wide range of analytes, although not so frequently used in analytical applications. The reaction with nitric oxide produces a yellow-green CL emission that has been used for measuring concentration of O atoms in kinetics experiments:

$$NO + O\cdot \rightarrow NO_2^* \rightarrow NO_2 + h\nu\ (450\text{-}1800\ nm)$$

$$NO_2 + O\cdot \rightarrow NO + O_2$$

Also, F is used in CL as the stronger oxidant to produce CL emission in gas-phase when it reacts as F atoms or F_2. F atoms react with hydrocarbons and F_2 reacts with certain reduced sulphur compounds such as thiols, sulphides, disulphides and trisulphides, phosphines, alkyl phosphine and monophosphinate esters to produce intense CL emission [32,33].

Some CL reactions are based on the light emission produced in a flame, rather than the "cold" CL emission abovementioned [34]. In this case, the high temperatures of the flame promote chemical reactions that form key reaction intermediates and provide additional thermal excitation of the emitting species. This methodology has been used for selective detection of compounds containing sulphur, nitrogen, phosphorous, boron, antimony, arsenic and halogens. As example, the mechanism for the detection of organophosphorous compounds can be cited involving the formation of PO, which subsequently reacts with H atoms in a fuel-rich flame to produce the excited species [35]:

$$H\cdot + PO \rightarrow HPO^* \rightarrow HPO + h\nu\ (\approx 526\ nm).$$

Liquid-phase CL reactions

Acyl hydrazides

The best known example in direct reactions is the oxidation of luminol (5-aminophthalylhydrazide) in alkaline medium, to produce excited 3-aminophtalate ion (Fig. 1). Oxidants such as permanganate, hypochlorite, or iodine can be used but the most useful is hydrogen peroxide. The reaction is catalysed by metal ions (Fe(II), Cu(II), Co(II) amongst others), ferricyanide or some metallocomplexes (hemin, haemoglobin and peroxidases). In this sense, this reaction has been applied for the determination of catalysts such as metal ions or enzymes (peroxidases, hematic compounds, etc.), certain

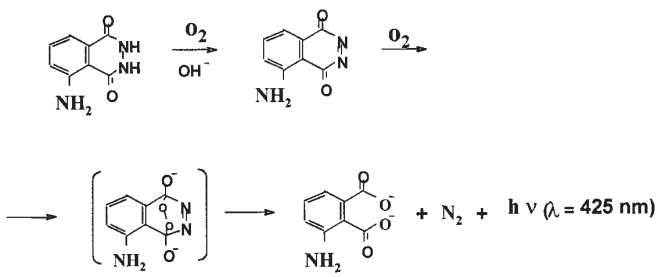

Figure 1. Proposed mechanism for the luminol CL reaction.

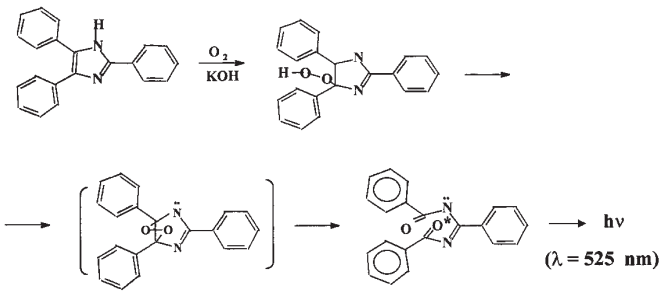

Figure 2. Proposed mechanism for the lophine CL emission.

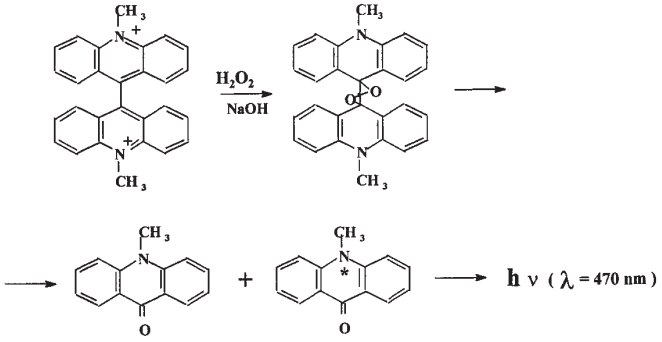

Figure 3. Proposed mechanism for the lucigenin CL reaction.

oxidants, inhibitors or substances that are easily oxidised and are determined indirectly by measuring the decreased CL emission. Also, luminol has been extensively used in medico-legal investigations in presumptive tests to detect trace quantities of blood, which are not visible to the naked eye, *e.g.*, areas intentionally wiped clean of blood, washed clothes, dark surfaces, etc. [36]. In fact, the application of fresh luminol by spraying – after allowing the previous applications to dry – can reactivate the luminescence, and hematin can be detected in a dilution of $1{:}10^8$. Recently, some developments of luminol-based CL in relation to the use of reductants, or its application to time-resolved or solid surface analysis have been summarised [37].

Imidazoles

Lophine (2,4,5-triphenylimidazole) is the most representative of the imidazole CL precursors. A yellow light is produced by oxidation of lophine with peroxide-hypochlorite or peroxide-hexacyanoferrate(III) and O_2, in aqueous alkaline medium, to the hydroperoxide, from which excited state diaroylarylamidines are formed *via* a dioxetane structure that then emits light (Fig. 2) [38]. Determination of Co(II), Cr(III), Cu(II), $Fe(CN)_6^{3-}$, MnO_4^-, ClO^-, amongst others have been carried out using this system. Recently, hydroxylammonium chloride was found to enhance the CL emission of the lophine-Co(II)-H_2O_2 system, allowing a very sensitive determination of Co(II) [39].

Acridinium esters

Lucigenin (10,10'-dimethyl-9,9'-biascridinium nitrate) is one of the more efficient CL substances which emits an intense green emission when oxidised in an alkaline medium [40]. Other acridinium derivatives have been shown to produce CL emission upon hydrogen peroxide oxidation of aqueous alkaline solutions. The main reaction product is N-methylacridone, acting as an active intermediate in the mechanism proposed by Rauhut *et al.* [41,42] (Fig. 3). Due to the reaction product being water-insoluble, the addition of a small amount of a surfactant, such as sodium dodecyl sulphate, prevents precipitation [43]. The application of this reaction has permitted the determination of several ions, oxidants or reductors, such as ascorbic acid [44].

Tris (2,2'-bipyridine) ruthenium (II)

Another CL system frequently applied involves the use of $Ru(bpy)_3^{2+}$ which produces an orange emission at 610 nm from excited state $[Ru(bpy)_3^{2+}]^*$ that can be obtained by different reactions which imply electron transfer and regeneration of the $Ru(bpy)_3^{2+}$ species:

* By reaction of $Ru(bpy)_3^{3+}$ and $Ru(bpy)_3^{+}$

$$Ru(bpy)_3^{3+} + Ru(bpy)_3^{+} \rightarrow [Ru(bpy)_3^{2+}]^*$$

* By reaction of $Ru(bpy)_3^{+}$ with certain oxydants

$$Ru(bpy)_3^{+} + ox \rightarrow [Ru(bpy)_3^{2+}]^* + ox^-$$

* By reaction of $Ru(bpy)_3^{3+}$ with certain reductants

$$Ru(bpy)_3^{3+} + red \rightarrow [Ru(bpy)_3^{2+}]^* + red^+$$

For these reactions the CL intensity is linearly proportional to the concentration of any of the reagents, allowing their determination by suitable adjustment of the remaining reagent concentrations. The solvent usually applied in CL determinations based on these reactions include acetonitrile-water, methanol-water and acetone-water [45]. Recently, the higher CL emission generated by a similar complex, $Ru(phen)_3^{2+}$ (phen = 1,10-phenanthroline) during oxidation of $Ru(bpy)_3^{2+}$ by Ce(IV) in sulphuric acid medium was investigated, allowing the establishment of a CL method for the analysis of nucleic acids, which enhance the CL emission [46]. $Ru(bpy)_3^{2+}$ is the most studied and exploited inorganic compound used in electrogenerated chemiluminescence (ECL) and its analytical usefulness as an ECL label has been summarised in two extensive reviews [47,48]. This alternative is based on the production of CL emission directly or indirectly as a result of electrochemical reactions: reactive species formed electrochemically, diffuse from the electrode and react, either with each other or with chemicals to produce light from a CL reaction in the vicinity of the electrode. $Ru(bpy)_3^{2+}$ can undergo ECL in aqueous buffered solutions, in the presence of dissolved oxygen and other impurities, at room temperatures, at easily attainable potentials with very high efficiency. Analytical applications of ECL have only been reported since the last few years [49-51].

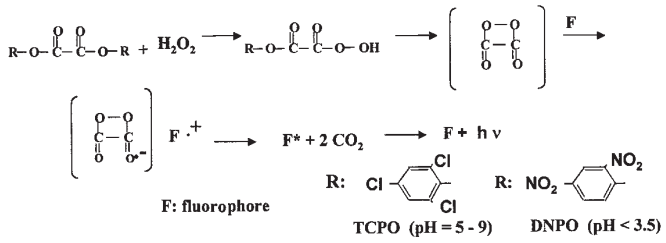

Figure 4. Possible reaction pathway for the PO-CL system.

Peroxyoxalates

In relation to indirect CL, one of the more efficient non-biological system that are frequently used is based on the so-called peroxyoxalate CL reaction (PO-CL), which involves the hydrogen peroxide oxidation of an aryl oxalate ester in the presence of a fluorophore. The reaction is suggested to follow a CIEEL (chemically initiated electron exchange luminescence mechanism) *via* a high-energy intermediate, 1,2-dioxetanedione, which forms a charge complex with the fluorophore, donating one electron to the intermediate [52]. This electron is transferred back to the fluorophore raising it to an excited state and liberating light characteristics typical for the fluorophore nature (Fig. 4). Bis-(2,4,6,-trichlorophenyl)oxalate (TCPO) and bis-(2,4-dinitrophenyl)oxalate (DNPO) are commonly used oxalates. The main disadvantage of this system resides in the insolubility of the above-mentioned compounds in water and their instability towards hydrolysis, which requires the use of organic solvents such as acetonitrile, dioxane, tert-butanol and ethyl acetate. This reaction can be used to determine a great number of species such as hydrogen peroxide, compounds that are highly fluorescent (*e.g.* polycyclic aromatic hydrocarbons) or compounds that do not exhibit native fluorescence but can be derivatized chemically using dansyl chloride (amino acids, steroids, aliphatic amines, carboxylic acids, etc.) or fluorescamine (catecholamines) [53]. Recently, Irgum's group has studied the use of 1,1'-oxalyldiimidazole (ODI) to determine hydrogen peroxide, and the results show that ODI is about 10 times more sensitive than TCPO [54,55].

Bioluminescent reactions

The analytically most widely applied BL systems are based on the firefly luciferin-luciferase reaction and the systems derived from the firefly *Photinus pyralis* and certain marine bacteria *(Vibrio harvey* and *Photobacterium fischeri*). Luciferases are stabilized by protein such as albumin. In the former system, the hydrophobic enzyme luciferase catalyses the air oxidation of luciferin in the presence of ATP, which is consumed as a substrate to yield light emission at 562 nm [56]. The presence of Mg(II) is necessary for the luciferase activity to be triggered. A detailed scheme is reported in figure 5. Considering the stoichiometry of the reaction, for one ATP molecule consumed, approximately one photon is emitted. This property, together with the high nucleoside specificity of the enzyme, makes this reaction an ideal analytical system for assaying ATP presence, ATP production or consumption in dependence of enzymatic activity, and for quantification of substrates linked to the ATP metabolism. ATP detection is an alternative to detect contamination from micro-organisms, food residues or human contact on surfaces. The advantage of ATP measurements in the evaluation of cleanliness lies in the ability to detect this product's residues and organic debris in addition to living micro-organisms. This system is being widely applied in the Quality Management and Assurance field to increase safety

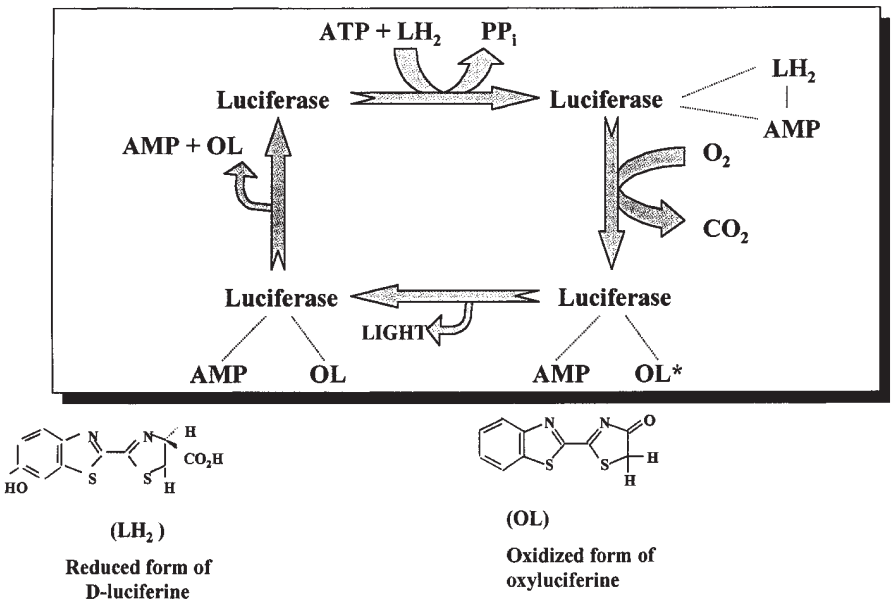

Figure 5. Schematic representation of the firefly luciferase reaction. ATP, adenosine triphosphate; PP_i, pyrophosphate; AMP, adenosine monophosphate.

of foods [57] and in a wide range of fields such as environmental quality control [58], monitoring systems at sludge and sewage water plants [59], pharmaceutical and cosmetics industries [60,61], vitality and quality of biomasses involved in fermentation processes [62], bacterial contamination in sterile areas (surgical rooms) or micro-organisms contribution to alterations affecting artistic works [63]. Several commercial kits are available and also directly usable on the field with portable instruments [64].

BL reaction from luminous bacteria can likewise be applied for analytical purpose. The BL enzyme system from marine bacteria consists of a NAD(P)H:FMN oxidoreductase and a luciferase (E.C. 1.14.14.3) which emits light at 490 nm in the presence of FMN, NAD(P)H, a long chain aliphatic aldehyde and molecular oxygen [65,66]. Marine bacteria also contains a NAD(P)H:FMN oxidoreductase cofactor which supplies the reduced flavin mononucleotide ($FMNH_2$), according to the general mechanism presented hereafter:

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

$$FMNH_2 + RCHO + O_2 \xrightarrow{\text{Luciferase}} FMN + RCOOH + H_2O + h\nu$$

Bacterial luciferase is highly specific for $FMNH_2$, but the enzyme also shows weak activity towards other flavins. Only aliphatic aldehydes with a chain length of eight or more carbon atoms are effective in the luminescent reaction. The total light production is proportional to the amount of each of the substrates (O_2, $FMNH_2$, RCHO) when they are present in limiting quantities. Various substances of biological interest and enzyme activities can be analysed by coupling the luciferase and the oxidoreductase to a third reaction, which produces or consumes NADH or NADPH. Using this reaction, an automated bacterial luminescent test for biomonitoring of environmental contaminants was established [67]. Applications of these bioluminescent systems to biochemical analysis have been reviewed [68,69].

Direct oxidations

In the last years, there has been a development of new CL reactions by testing the analyte with a wide range of strong oxidants, such as MnO_4^- (in acidic and alkaline medium), ClO^-, Ce(IV), H_2O_2, IO_4^-, Br_2, N-bromosuccinimide, and reductants, under different ranges of chemical conditions [70]. Usually, if oxidation of the molecule is known to give a fluorescent product, or if the analyte itself has a typical structure that might be fluorescent, there is a possibility that oxidation of the analyte will produce CL emission. Some of the first applications were the CL determination of morphine [71], buprenorphine hydrochloride [72] and the benzodiazepine loprazolam [73], using a flow injection analysis (FIA) assembly. In these cases, the sample containing the drug was injected into a phosphoric acid stream that was subsequently merged with the oxidant stream, offering an extremely simple, economic and effective experimental procedure. Since then, a wide range of such reactions have been reported mainly in the analysis of drugs [17]. Sensitizers, micellar media or catalysts can be added to these systems in order to enhanced the CL emission. As example, penicillamine [74], tiopronin [75] and cefadroxil [76] have been determined in presence of quinine sulphate; folic acid [77], captopril [78], hydrochlorothiazide [79], phenothiazines [80] or furosemide [81] using rhodamines 6G or B and hydrazine in presence of dichlorofluoresceine [82]. Quinine sulphate and Rhodamine 6G were simultaneously used for the determination of tiopronin [83]. Also, in the presence of the cationic surfactant cetyltrimethyl ammonium bromide (CTAB), tetracycline was determined with high sensitivity [84]. These reactions have been mainly applied to developed CL-based flow injection methods.

CL as detection technique

Gas-chromatography

Applying the known CL reactions in the gas-phase, CL has been coupled in gas-chromatography as detection technique and several commercial CL detectors have been developed [85]. The Flame Photometric Detector (FPD) for sulphur and other compounds, the Sulphur Chemiluminescence Detector (SCD), which uses the above-mentioned reaction with ozone, the Thermal Energy Analysis (TEA) Detector, which applies the NO + O_3 reaction for the analysis of N-nitrosamines, recently in cosmetic products [86], the Redox Chemiluminescence Detector (RCD), which selectively detects compounds containing oxygen, nitrogen, sulphur, etc., capable of reducing NO_2 to NO that is detected by the reaction with ozone, or the Fluorine-Induced Chemiluminescence Detector (FCLD) for S, Se, Te and P compounds, are the most widely applied [87]. Figure 6 shows a typical device for CL detection in gas chromatography.

Flow injection analysis

Flowing stream methods involve delivering and mixing the CL reagent with the analyte stream or column effluent and the use of a flow-cell for the detection of the CL emission at a fixed time after mixing [88]. FIA includes a simple methodology offering robustness, feasibility, excellent precision and rapid response making it suitable to monitoring liquid phase CL reactions. This alternative allows the sample to undergo in-line chemical and physical treatment to obtain species suitable for CL detection. The sample is injected into a flowing stream and mixed with the reagent(s) in close proximity to the detector. Emission occurs in a cell placed in front of the optical window of the detector. The sensitivity of this detection can be optimised by controlling some experimental variables such as dimension of the mixing and detector coils, flow rates, temperature, pH and reagent concentrations. Using this approach and adapting different reactions above-mentioned, a wide range of compounds has been determined and the reproducible peaks

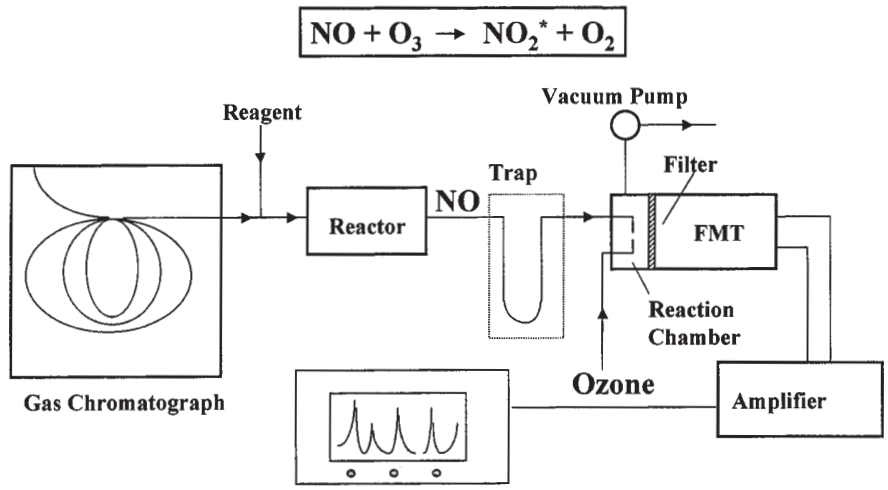

Figure 6. Schematic diagram for CL detection in gas chromatography.

obtained assure an adequate relation between the CL intensity recorded and the concentration of the analyte. Important applications have been carried out using flow injection [14,15], mainly in quality control in pharmaceutical analysis [17,18]. More recently, the development of the immobilisation techniques has provided the introduction of enzyme reactors, which can be positioned before the CL reaction takes place thus avoiding the lack of selectivity that may occur when a given CL reagent yields emission for a variety of compounds. In this alternative, the analyte is the substrate of the enzymatic reaction and one of the products will sensitively participate in the CL reaction. Substrates detected in this way include glucose, cholesterol, choline, uric acid, amino acids, aldehydes and lactate which generate H_2O_2 when flowing through a selective column reactor with immobilized oxidase enzymes in the presence of the necessary oxidant, usually O_2, present in the samples. Luminol in the presence of a peroxidase catalyst is the best system so far for this post-column H_2O_2 determination. Figure 7 shows a typical manifold for glucose determination based on the CL emission produced when hydrogen peroxide formed by immobilized glucose oxidase reacts with luminol in the presence of potasium hexacyanoferrate (III) [17]. Using the luminol reaction, glucose has recently been determined sensitively by immobilization of pyranose oxidase [89] and hydrogen peroxide [90] or sulphite [91] by immobilisation of luminol and Co(II).

Liquid chromatography

Promising perspectives introduced the first applications of CL reactions as detection mode in liquid chromatography (LC), combining the high efficiency in separation and the low detection limits inherent to several CL systems. Although fluorescence is the most useful detection technique for trace analysis, the elimination of the excitation source in CL mode can reduce straylight, background emission or light source instability, improving the sensitivity for some fluorophores to a 10-100-fold over fluorescence detection. In LC, luminol and isoluminol have been used for post-column CL detection in the analysis of different transition metal ions which are separated using cation-exchange resins and act as catalyst in the CL reaction, for the determination of ligands (amines, amino acids and proteins) which complex the metal catalysts and for the analysis of several species that are pre-column derivatized with luminol or some derivatives [14]. Using reverse phase and ion-exchange columns, $Ru(bpy)_3^{2+}$ system has been used for the post-column detection of oxalates, amines amino acids and some antibiotics [92]. The most commonly used system involves peroxyoxalate CL as post-column reaction using mainly

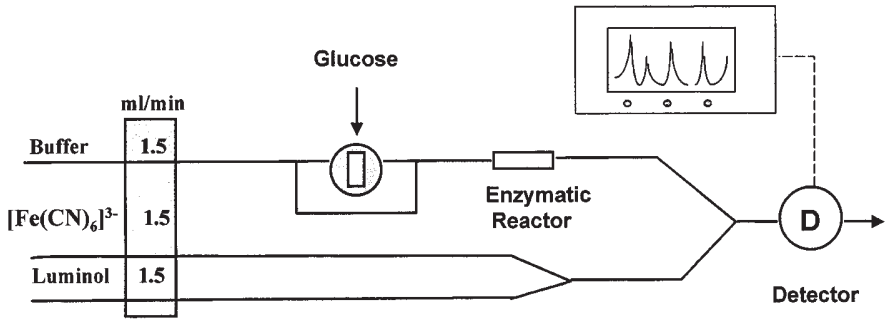

Figure 7. Schematic diagram for a FIA manifold for the analysis of glucose incorporating an enzymatic reactor with detection based on the luminol reaction.

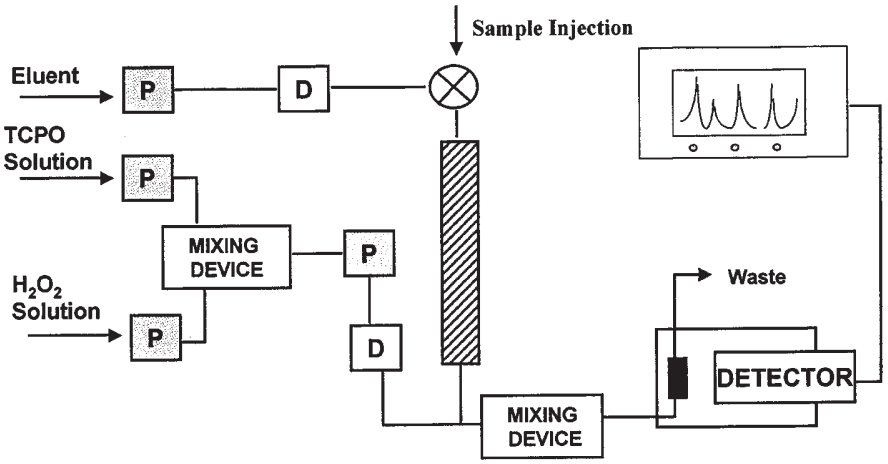

Figure 8. Schematic diagram for a post-column CL-based detection system used in HPLC (D, damper; F, flow-cell; I, injector; MC, mixing coil; P, pump; PM, photomultiplier tube; R, recorder).

TCPO and DNPO for determination of fluorophores. Applications of this system where extensively reviewed [93] and a model mechanism as applied detection in LC was proposed [94]. An HPLC-CL detection system must be constructed considering the conditions for HPLC separation, efficiency of CL reaction and the stability of the reagents. Figure 8 shows a typical manifold for CL-detection using TCPO in HPLC. The aryl oxalate and H_2O_2 solutions are premixed on-line and added post-column to the chromatographic effluent to start the CL reaction. This system has been applied in the sensitive analysis of fluorescent analytes such as polycyclic aromatic hydrocarbons and polycyclic aromatic amines and fluorescent derivatives using dansyl chloride (for amino acids, amines, catecholamines, steroides and amphetamines); o-phthalaldehyde and naphthalene dialdehyde (for amines); fluorescamine (for catecholamines) or N-[4-(6-dimethyl-amino-2-benzofuranyl)phenyl]maleimide (for thiols) [95,53]. Analytical use of TCPO in LC has been reported [96]. Recent use of HPLC-coupled CL-flow injection analysis has been applied to the analysis of thiolic drugs based on CL produced by a Ce(IV) oxidation system sensitised by quinine and rhodamine B, such as hydrochlorothiazide and captopril [97,98] and the determination of tiopronin and its metabolite 2-mercaptopropionic acid in human urine [99].

CL immunoassay

CL immunoassay constitutes a powerful alternative to radioisotope immunoassay in the analysis of very low levels of biological substances, especially in the field of clinical chemistry and food analysis [12,100]. Assays using CL compounds as labels are increasingly used because of the their high sensitivity and wide dynamic range [101]. As a principle, any of the species that participate in a CL reaction can be coupled to an antibody or antigen. The labelled reagent can be used in a competitive or non-competitive binding assay, CL being initiated by the addition of the remaining component(s) of the reaction. To be used as a CL label, a compound must fulfil some requirements: it must be capable of participating in a CL reaction, it must be attachable to the antigen or antibody to form a stable reagent until the reaction is triggered, the label should retain a high quantum yield and the necessary reaction kinetics after coupling; it should not significantly alter the physico-chemical properties of the molecule to which it is attached, in particular its immunological activity. Compounds usually employed in CL immunoassay include synthetic organic compounds (phthalylhydrazides and acridinium esters), cofactors in bioluminescent reactions (NAD and ATP) and enzymes (peroxidases, oxidases, kinases, luciferases). The PO-CL detection system was also applied in immunoassay to measure the CL from fluorescent labels, but solvent restrictions significantly affected the precision. A recent review reported CL investigations of some acridinium esters and derivatives of luminol and isoluminol as powerful markers in CL immunoassay due to their high quantum yield [102]. Covalent linking to either the antigen or the antibody is carried out by chemical modification of the label (*e.g.* diazotisation, isothiocyanate, N-hydrosuccinimide, hemisuccinate, imidoesters), by chemical modification of antigen or antibody (*e.g.* hemisuccinate, glutaraldehyde), or by conjugation using bifuntional reagents (*e.g.* mixed anhydride, carbodiimide, bis (N-hydroxysuccinimides), azido-succinimides). The use of enzymes as label in immunoassays potentially affords much more sensitive tracer detection in comparison with radiolabels because of the great catalytic power resulting in the generation of many product molecules from one enzyme molecule through turnover. The most widely used enzyme for CL immunoassay is horseradish peroxidase (HRP). In the last years, a serendipitous discovery was observed when firefly luciferase was accidentally added to a mixture of HRP, luminol and hydrogen peroxide. The light yield of the HRP-catalysed peroxidation of luminol was substantially enhanced. This fact marked the initiation of a most successful analytical tool for immunoassay and all kinds of blotting applications (protein and nucleic acids), named enhanced CL [103]. Recently, certain aromatic acridan esters were used as part of a signal

reagent for the very sensitive detection of HRP, which constitutes a major improvement over the commercial luminol-based enhanced CL reagent [104-106]. The usefulness of these compounds has been recently probed in the CL immunoassay detection of atrazine and clenbuterol offering potential advantages in sensitivity and on-site screening applications [107].

CL sensors in liquid phase

A CL sensor is a device incorporating an active material with a transducer, with the purpose of detecting in a continuous, selective and reversible way, the concentration of chemicals in diverse kinds of sample, employing CL emission. There are several ways of classifying CL sensors in relation to the manner of bringing the sample in contact with the sensing surface: batch and flow CL sensing systems. In the former, the sensing surface is immersed in the analyte solution and an optical fiber is sometimes used as light transducer. This type of CL sensor is also called a CL optrode. Flow CL sensors are more popular because a flow system allows the management of additional reagent solutions, and improves the analytical speed and repeatability, thus easing automation. Two alternatives have been developed in relation to the immobilisation of reagents involved in enzymatic or non-enzymatic systems using chemiluminescence detection, which have been reported in a recent review [108]. The first enzyme-based CL sensor was based on the immobilisation of HRP at the end of an optical fiber with a detection limit of 2×10^{-6} mol/L for H_2O_2 [109]. A recent modification implied the use of silicate-glasses obtained by the sol-gel method [110], which represents an alternative for immobilisation of biological entities due to its low-temperature preparation, providing an adequate supporting matrix in which HPR is immobilised by microencapsulation. This sol-gel biosensor permits the determination of hydrogen peroxide in the range of 0.1-3.0 mM by measuring CL in a cuvette and through an optical fiber modified at its end with immobilised HPR gel. Bioenzyme sensors for glucose have been produced using this HRP-catalysed H_2O_2/luminol reaction and co-immobilised glucose oxidase. High sensitivity has been obtained in the analysis of glucose in plasma using the luminol reaction with H_2O_2 produced by immobilized pyranose oxidase within a flow-through cell containing immobilised peroxidase [111], because this enzyme oxidises α- and β-anomers of D-glucose to the same extent stability and with sensitivity about twice those for the methods with immobilised glucose oxidase. Other enzymes have been co-immobilised with peroxidase allowing the analysis of other compounds such as aminoacids, choline and acetylcholine, cholesterol, lactate and xanthine, phosphate, oxalate, ethanol, etc. The immobilisation of non-enzyme reagents in the performance of CL-sensors has been extensively studied in the last years. As example, Zhang's group [112] presented recently a novel CL sensor combined with FIA for ammonium ion determination. It is based on reaction between luminol, immobilised electrostatically on an anion-exchange column, and chlorine electrochemically generated on-line *via* a Pt-electrode from hydrochloric acid in a coulometric cell. Ammonium ion reacts with chorine and decreases the produced CL intensity. The system responds linearly to ammonium ion concentration in a range between 1.0-100 μM, with a detection limit of 0.4 μM. A complete analysis can be performed in 1 min, being satisfactorily applied to the analysis of rainwater. Organic and inorganic analytes have been determined using sensors prepared electrostatically by the same group [113], by immobilizing luminol and some metal ions such as Co(II), Cu(II), $Fe(CN)_6^{3-}$, etc., on anion/cation exchange columns. The analytes (H_2O_2, ClO^-, CN^-, Co^{2+}, ascorbic acid, etc.) are sensed by CL reaction of luminol and metal ions bleeding from the ion exchange columns by hydrolysis. Some important contributions in relation to the simplification of detection devices, reduction of the consumption of reagents, cyclic use and immobilization, regeneration and miniaturisation of CL sensors for special applications are expected as further developments.

Capillary electrophoresis

In last decade, the combination of CL as a detection method with CE as prior separation methodology started to be explored. Due to the advantages of CL detection in relation to the high sensitivity and its potential when combined with the high separation ability offered by CE, and considering the limitation of the latter technique with respect to, amongst others, the low volume of sample to be injected, research in this area is bound to offer a powerful analytical tool to the resolution and quantification of biomedical analytes in complex matrices. CL-reactions involving peroxyoxalates, firefly luciferase, luminol, acridinium esters, acidic potassium permanganate and $Ru(bpy)_3^{2+}$ are being used for post-capillary detection of different species using home-made manifolds for the coupling technique [114-116]. In 1991, Hara *et al.* [117] reported for the first time the CL detection in the analysis of proteins using peroxyoxalate reaction in a pH 3.5 phosphate buffer due to the co-migration of the dyestuff Eosine Y (EY) with a protein as a supramolecular complex in presence of molybdate, tungstate, silver (I) or mercury(II). A standard CE apparatus was used, providing on-column detection by burning off the polymer coating, and the EY complex with the protein was determined by measuring the CL intensity of the (TCPO)-H_2O_2-EY. Improvements in these studies have been achieved using different dyestuffs such as Rhodamine B, isothiocyanate and tetramethylrhodamine isothiocyanate isomer R. More recently a CE apparatus with an on-line CL detection *via* the luminol-H_2O_2 system was developed for analysing heme proteins [118], being 10^4 times as sensitive as the conventional CE-absorption detection method for the detection of hemoglobin. Indirect CL detection has been adapted to CE to carry out the analysis of amino acids without derivatization using the luminol system with Cu(II) as catalyst. In presence of amino acids, the catalytic activity of Cu(II) is decreased because of the post-capillary formation of Cu(II)-amino acid complexes, hence the CL emission is reduced and the analyte is detected indirectly as an inverted peak [119]. Acridinium esters have also been used as CL detection system in CE [120]. Their

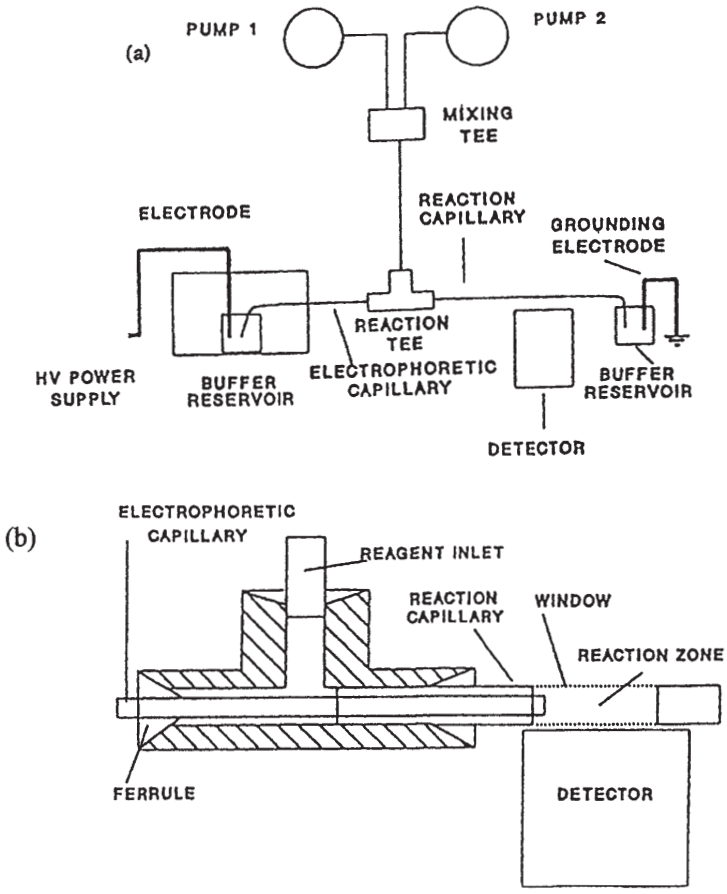

Figure 9. Example of a CE-CL system. (a) Experimental configuration. The hydrogen peroxide is introduced by pump 1 and pump 2 provides base. (b) Cross sectional scheme of the CL detection interface (with permission from réf. [120]).

oxidation by hydrogen peroxide makes them suitable as derivatizing agent for amino acids, peptides and proteins in CE analysis, being possible to detect attomole quantities. The experimental configuration used is shown in figure 9(a). The detection interface uses a coaxial reactor consisting of two concentric fused silica capillaries in which the smaller diameter 50-cm electrophoretic capillary is inserted into the larger diameter 35-cm reaction capillary as illustrated in figure 9(b). The CL reagents enter the reaction tee and flow as a sheath around the electrophoretic capillary and its effluents. The hydrogen peroxide delivered by pump 1 is combined with the base from pump 2 by the mixing tee, the outlet of which leading to the reaction tee of the detection interface. Diffusion and radial migration mix the reagents with the acridinium ester in a specific section of the reaction capillary called reaction zone. This zone is placed in front of the detector PMT. The end portion of the reaction capillary exits the detector and enters a buffer reservoir to complete the circuit. Recently CL detection based on the HRP-catalysed reaction of luminol with peroxide has been integrated as a post-separation detection for microchip-based CE [121], illustrating the suitability of on-chip CL detection of an immunological reaction or HRP-labelled proteins.

Conclusion

Recent advances have been achieved, more specifically in this last decade, with respect to the use of CL reagents as labels to derivatize and sensitively determine analytes containing amine, carboxyl, hydroxy, thiol and other functional groups, including their application in HPLC and CE [53,95], the synthesis and application of new acridinium esters [102,122], the development of enhanced CL detection of HRP labels [123], the use of immobilisation techniques for developing CL-based sensors [69,124,125], or the analytical application of ECL [126], amongst others.

It is clear from the actual literature [127], and as stressed on various occasions, that CL measuring systems applied to detection in flowing streams and immunoassays are of growing importance, based on the advantages amongst which low detection limits and the relatively simple instrumentation. Also, CL-based detection appears to be of utmost importance in the area of CE-micromachining in which microchannel-based mixing of reagents and the creation of reaction chambers open new analytical perspectives. However, one should not forget that this very "exotic" measuring technique also suffers from some drawbacks, which sometimes include lack of selectivity – not a real problem when applied to separational set-ups, insufficient sensitivities (mostly when fluorophores cannot be excited in a physico-chemically efficient way), to name a few. It should be emphasised as well that the fast evolution of immobilisation techniques (*e.g.*, employing beds of CL-reagents or enzymes) improves the application of CL detection, amongst others, in separational streams and in the biosensing area.

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