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Journal of Pharmaceutical and Biomedical Analysis
17 (1998) 941–953

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Chemiluminescence-based detection: principles and analytical applications in flowing streams and in immunoassays

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Received 25 July 1997; accepted 17 November 1997

Abstract

The present paper provides the principles of chemiluminescence (CL) and its powerful applications in analytical chemistry, mainly in the area of flow injection analysis, column liquid chromatographic and capillary electrophoretic separating systems, and its potential in immunoassays. CL is light produced by a chemical reaction. The most common advantages of chemiluminescent reactions are the relatively simple instrumentation required, the very low detection limits and wide dynamic ranges, which have contributed to the interest of CL detection in flow injection analysis, high performance liquid chromatography, including miniaturized systems, and, most recently, the exploding area of capillary electrophoresis. The latter powerful microanalytical separation technique offers high numbers of theoretical plates and relatively short analysis times requiring only small sample volumes, the migrating system comprising aqueous buffer solutions. In non-isotopic immunoassays, covering a great variety of applications in human and veterinary medicine, forensic medicine, agriculture and food industry, the radioisotope is replaced by a fluorescence or chemiluminescent label. The use of CL as a detection principle permits quantitative determination of various compounds at low concentrations. Disadvantages of the CL-based technique may include lack of sufficient selectivity and sensitivity to various physicochemical factors. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Chemiluminescence; Liquid chromatography; Flow injection analysis; Capillary electrophoresis

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PII S0731-7085(98)00062-4

1. Introduction

In slightly over three decades, luminescence spectrometry has transcended its origins as a curiosity in the physical laboratory to become a firmly established and widely employed branch of analytical chemistry. Owing to elegant new instrumentation and especially to new techniques, some of which entirely new and some borrowed from other disciplines, fluorescence, phosphorescence, CL and bioluminescence spectrometries can be routinely applied to qualitative and quantitative analytical problems.

Molecular luminescence techniques have several characteristics that make them useful for many kinds of analyses. Their advantages include low limits of detection (for highly luminescent molecules), amenability to remote detection (by means of a laser or fiber optic probes), applicability to complex samples, and the generation of several types of information—excitation and emission spectra, decay times, polarization data—useful for molecular identification. In liquid chromatography (LC) luminescence detection represents an interface between the selectivity of an elegant separation method and an ultrasensitive detection method.

Due to the general trend in analytical sciences to study smaller samples at increasingly lower concentrations, the need for improved detection technology increases. Moreover, the problem of waste disposal is gradually forcing analytical separating systems into miniaturization, e.g. narrow-bore and capillary liquid chromatography versus conventional HPLC, miniaturized high performance thin layer chromatography (HPTLC), and, during recent years, capillary electrophoresis (CE). As CL (and fluorescence) may help solve the problem of detection limits in column liquid and in capillary electrophoretic miniaturized set-ups, not using hazardous labels, the technique is bound to be thoroughly explored for the coming decade.

The principles of CL and some of its powerful applications in the wide area of analytical chemistry, often biomedical and environmental analysis, including direct methods and detection of CL emission in flow injection, chromatographic and CE separating systems as well as in immunoassays will be treated in the present paper.

2. Chemiluminescene: Principles and considerations in separation systems [9,31,36,37,47]

CL is light produced by a chemical reaction; the energy levels are identical with those involved in fluorescence phenomena, the only difference being the mode of excitation. The distinction between CL and bioluminescence lies in the origin of the reactions. Reactions that are biological in origin (e.g. the firefly luciferin–luciferase–ATP reaction) are called bioluminescence, the remainder, CL.

CL reactions generally yield a product in an electronically excited state producing visible light. As a general rule, only quite exothermic reactions can generate the required energies. Therefore, most CL reactions use oxygen, hydrogen peroxide, or similar potential oxidants. As a principle in bioluminescence and CL reactions, at least two reagents, A and B react to form a product C, some fraction of which is present in an electronically excited state, C*, which may subsequently relax to the ground state emitting a photon:

$$A + B \rightarrow C^* \rightarrow C + h\nu$$

Hence, the luminescence process takes place at the rate of a chemical reaction and the factors that affect emission intensity are in fact a combination of chemical reaction rate and luminescence considerations. Most common cited advantages of CL reactions are the relatively simple instrumentation required, the low detection limits and wide dynamic ranges, having contributed to the interest in CL detection in HPLC and in flow injection analysis (FIA).

CL is often described as a dark-field technique: the absence of a strong background light level, as in absorption spectroscopy, reduces the background signal, improving detection limits. Small changes in a small signal are more easily observed than small changes in the presence of a large signal. As a principle, it is possible to measure CL in a fluorometer by turning off the excitation source. What is actually needed, is a photomultiplier tube, sufficiently sensitive in the spectral region of interest.

However, some disadvantages are to be considered as well. A CL reagent may yield significant

emission not just for one unique analyte, i.e. a lack of selectivity may occur. Moreover, CL emission intensities are sensitive to a variety of environmental factors such as temperature, solvent, ionic strength, pH, and other species present in the system. As a result, separation conditions (HPLC, CE,...) may not always match the optimum CL emission conditions. Third, as the emission intensity from a CL reaction varies with time (light flash composed of a signal increase after reagent mixing, passing through a maximum, then decreasing back to the base-line), the CL emission versus time profile differs widely from one compound to another and care has to be taken to detect the signal in the flowing stream during strictly defined periods. For many CL systems, there is a low background level of emission in the absence of analyte. Hence, CL signals in flow systems, increasing proportionally to the analyte concentration, appear as sharp peaks superimposed on a low constant blank signal, measured as viewed by the time window when the mixture of analyte and reagent(s) pass through the detector cell. Due to the small portion of CL emission that is only measured from this time profile, reactions with complex kinetics can give nonlinear plots of response versus analyte concentration [3].

There exists a wide variety of typical CL labels and reactions that have been used for analytical purposes, amongst which chemical, biochemical, biomedical, pharmaceutical, food, environmental and toxicological applications, for which the reader is referred to the specific literature [3,6,15,26,37,38,45,46,48–52]. In HPLC systems, several chemiluminescent reactions have been utilized, including peroxyoxalate (oxalate ester + hydrogen peroxide), firefly luciferase, lucigenin and luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) reactions. The classical peroxyoxalate reaction (Fig. 1), most commonly used for post-column detection in conventional and microcolumn HPLC, however, requires the use of organic solvents, due to the limited aqueous solubility of most oxalate esters, which may cause precipitation problems when dealing with hydrophilic reversed-phase eluents. On the other hand, in the presence of a suitable fluorophore, high quantum efficiencies may be reached, and, importantly, a wide range of 'excitable' fluorophores can be analyzed when compared to other CL systems. TDPO (bis[4-nitro-2-(3,6,9-trioxadecyloxycarbonyl) phenyl] oxalate), for example, has been used to sensitively determine fluorescent compounds separated with an acidic mobile phase [39]; femtomole concentrations of catecholamines were determined with this reagent after on-column fluorogenic derivatization with ethylene diamine and subsequent post-column CL reaction detection [22]. In CE separations applying peroxyoxalate CL detection, extra problems occur due to, on one hand, the influence of organic solvents on the migration behaviour of the analytes in the electrophoretic buffer, and, on the other hand, to the possible effects of high electric field strengths on the stability of the oxalate reagent mixture. Luminol, in the presence of a catalyst, reacts with hydrogen peroxide in alkaline solution to emit light of about 425–435 nm; when adding the catalyst and hydrogen peroxide in a post-column mode, CL can be measured in an HPLC system. Luminol derivatives such as isoluminol and *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI), may also be applied to label carboxylic acids and amines (e.g. amino acids); after separation and post-column reagent addition, the labeled analytes may be detected via their CL emission. Continuous flow CL-based detection of various analytes, including many drugs, has been extensively studied for the determination of tetra-

Ar–O–C(=O)–C(=O)–O–Ar + H_2O_2 ⟶ [1,2-dioxetane-3,4-dione] + 2ArOH

aryl oxalate

1,2-dioxetane-3,4-dione

+ Fluorophore ⟶ [Charge-transfer complex] ⟶ Fluorophore* + $2CO_2$

Fluorophore + light ⟵ Fluorophore* + $2CO_2$

Fig. 1. Principle of aryl oxalate–peroxide reaction generating CL emission from fluorophores.

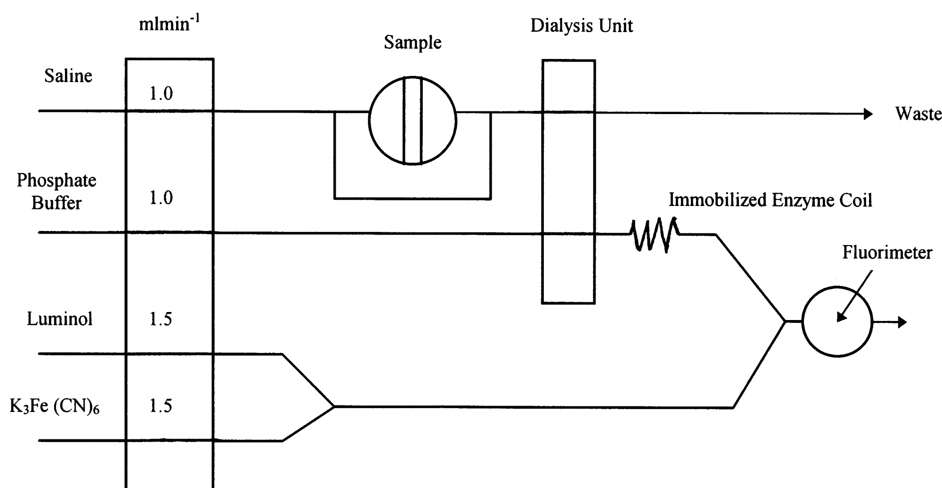

Fig. 2. FIA manifold for the determination of glucose in plasma.

cyclines [19,40], cyclamate [34], and catecholamines [15].

In principle, it should be feasible to chemically excite various fluorescent species, native fluorescers as such, or fluorescent derivatives generated after suitable labeling reactions, as described for various liquid chromatographic [4,5,7,8,16,18,23,24,53,54] and CE [12,55] separations.

3. Flow injection analysis and liquid chromatography

As mentioned above, the major attractions of CL reactions for analytical applications are the excellent detection limits and the wide dynamic ranges that can be achieved with relatively simple instrumentation. CL reactions are being more widely exploited in analytical chemistry as better sample handling and on-line separation techniques become available, in the first place FIA and HPLC. Both are unsegmented, liquid phase, continuous flow techniques that are used in conjunction with flow-though detectors, compatible with CL reactions [3,25,26].

The use of CL detection in a flowing stream requires a method to deliver and mix the CL reagents with the analyte stream or column effluent and a suitable flow-cell that allows detection of the CL emission at an appropriate time period after the initiation of the reaction.

FIA is a simple and elegant analytical technique in which a discrete liquid sample is injected into a liquid carrier stream which transports it to a flow-through detector. During transport, the sample can undergo on-line physical or chemical treatment. In its simplest form, FIA is used to solely transport a sample to a detector. The first reaction that was adapted to a FIA procedure was the copper(II)-catalyzed oxidation of luminol for the determination of hydrogen peroxide; later on, other metal ions and catalysts, for example cobalt, were determined. Signal-to-noise ratios need to be maximized by simplex optimization of some key variables, such as reagent concentrations, flow rates, pH, sample volume and length of mixing coils. Fig. 2 shows a more complex chemical reaction and sample matrix via a four-line manifold incorporating an on-line dialyser and immobilized enzyme reactor for the determination of glucose in blood plasma [42].

As a given CL reagent may yield significant emission not for just one unique analyte, but for a variety of compounds, lack of selectivity may occur, an obvious disadvantage for FIA applications. Selective reactors, such as enzyme reactors, positioned before the CL reaction may overcome

this problem. For HPLC applications, on the other hand, lack of detector selectivity is not a real issue: detector response to one single substance might possibly be considered as a restriction as well. In a flow system, the entire emission versus time profile is not constant but varies with time. The CL intensity detected in a flowing stream application would be the integrated portion of the emission profile that occurs during the time interval intersected by the observation cell. To maximize detector sensitivity, adjustment of solution flow rate (and/or detector cell volume) is required so that the observation time window is near the peak in the CL emission versus time profile [42].

FIA is most useful for optimizing the reaction conditions for CL emission, and, as such, is an important tool for the quantitative analysis of real samples. Some compounds may be directly determined by simple oxidation reactions. Metal ions, catalysts, ligands, oxidants and reductants, hydrogen peroxide, inorganic chlorine compounds can be determined by this approach. For complex samples, however, it is obvious that selectivity may remain a problem and, therefore, optimized FIA procedures should be applied in post-column derivatization reactions established for HPLC procedures.

In the HPLC area, probably the most widely used analytical technique nowadays for the separation and analysis of mixtures, the need for a highly sensitive and simple separation technique has been particularly obvious in biochemical and biomedical research, where progress has often been hampered by time-consuming, tedious or inadequately sensitive or specific methodology. The explosive growth and great popularity of HPLC was catalysed by advances in column (silica) technology and instrumentation. The reversed-phase mode of HPLC (RPLC), using a non-polar stationary phase and a polar mobile phase, has emerged as the most widely applied HPLC technique. It is estimated that $>80\%$ of the current HPLC separations are performed using this technique. Operational simplicity, high efficiency, column stability, and the ability to simultaneously analyse a broad spectrum of both closely related and widely different compounds have made this technique the most universal mode of HPLC. Progress is still continuing, particularly in the area of micro-HPLC.

An ideal detector should have good sensitivity to all eluting components. It should be reasonably linear so that it can be used in quantitative analysis. It should not significantly degrade the separation obtained in the column and should be reliable and easy to operate. As unfortunately, a practical universal detector for HPLC has not yet been developed, a device must be chosen providing adequate sensitivity for each particular problem. For the liquid chromatographer interested in trace analysis, there are few detectors capable of measuring picogram or femtogram quantities of material in the column effluent. Conventional absorption detectors can measure chromophores in the nanogram range, but these measurements are frequently confounded by matrix interferences. Among the optical methods of detection, fluorescence represents a means of increasing both the selectivity and the sensitivity of analysis. Selectivity is enhanced, since not all compounds that absorb radiation will emit.

When eliminating the light source from the fluorimeter and employing chemical excitation, the factors that limit the sensitivity of classical fluorescence measurements, including straylight, background emission, light source instability, are reduced, resulting in extraordinary limits of detection, at least for several fluorophores. The beneficial effects of substituting chemical excitation for photoexcitation, thus achieving CL, can be used for the excitation, thus, the determination of many classes of fluorophores, and also for the detection of small amounts of hydrogen peroxide produced by photochemical or enzymatic reactions.

Commonly used reagents for post-column CL reaction in HPLC include luminol, whose reaction has been used in conjunction with low capacity cation-exchange resins for the determination of transition metal ions catalyzing the CL reaction [6,42]. The peroxyoxalate reaction, using bis-(2,4,6-trichlorophenyl)oxalate (TCPO) and, to a lesser extent, bis-(2,4-dinitrophenyl)oxalate (DNPO), still comprises a useful, versatile and efficient CL system for the detection of LC elu-

ates. In general, fluorophores having low oxidation potentials are most efficiently excited in the peroxyoxalate reaction. The amino-substituted polycyclic aromatic hydrocarbons, for example, apparently as a result of their low oxidation potentials and ability to form charge transfer complexes, are among the most efficient fluorophores to be chemically excited.

Under optimal conditions and for efficient fluorophores, limits of detection can be improved by more than two orders of magnitude by substituting chemical excitation for light excitation. The major disadvantage for post-column CL detection in column effluents is the requirement to add the oxalate ester and the hydrogen peroxide reagents separately in order to excite the eluting fluorophores. Apart from mixing problems, the fact that the additional pumps must be as pulse-free as possible and that the mixing tees should be designed so as to contribute as little as possible to band broadening, it should be kept in mind that CL reactions display a luminescence growth curve followed by a decay of the signal intensity that is caused by the exhaustion of the light-generating agent(s), as described earlier. In a flow system, the half-life of the CL signal is a very important parameter. For given values of the various flow rates (that obviously should be extremely constant), the dead volume between the mixing tee and the flow-cell, and the volume of the flow-cell itself, the CL half-life determines the percentage of the emitted light that will be measured. The relative short CL half-life of the DNPO as compared with the TCPO system should be taken into account when constructing a detection set-up.

With respect to the proper solvent selection in peroxyoxalate CL systems, TCPO being the most commonly used aryl oxalate, it should be mentioned that esters and ethers are the best solvents for this reagent. However, because ethers react with oxygen to form peroxides, TCPO decomposes rather rapidly if dissolved in an ether. Most commonly used esters are not readily miscible with water. In other words, CL monitoring of partly aqueous solutions—most HPLC eluents—requires the presence of a third solvent to create a homogeneous system. In practice, ethyl acetate is often selected as solvent for TCPO, and hydrogen peroxide is dissolved in tetrahydrofuran or acetone. Conventionally, the two reagent solutions are mixed on-line, and the mixture is then added to the HPLC effluent [27] (Fig. 3). Occasionally, TCPO and hydrogen peroxide are premixed; however, the sensitivity of such a system does not remain constant for over 1 day.

Application of microcolumn LC in conjunction with peroxyoxalate CL detection of fluorophores in effluents was suggested, Fig. 4 showing a schematic representation of a measuring device [2].

In principle, the conversion of a FIA/CL set-up to an HPLC/CL set-up only requires the inclusion of an HPLC column between the injector and the CL reactor, taking into account the practical necessities of switching tubing, connectors, pumps and the higher pressures applied in the latter systems.

4. Capillary electrophoresis

CE is a most powerful separation tool in analytical sciences because of the high number of theoretical plates and the relative short analysis times offered. It is a fundamental microanalytical technique (extremely small sample requirement) that is more than complementary to conventional chromatographic techniques, and well suited for biomedical, biochemical, pharmaceutical, food, environmental, toxicological research, protein analysis, DNA sequencing, chiral analysis, purity testing, etc.

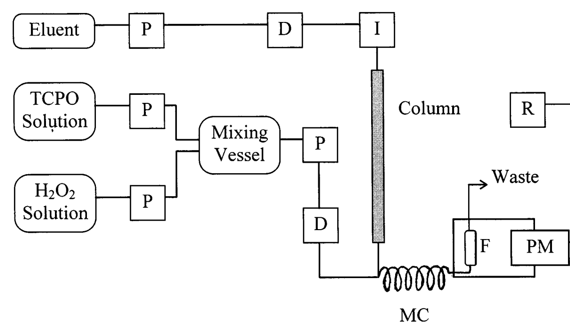

Fig. 3. Schematic diagram for a post-column CL-based (TCPO) detection system in HPLC (D, damper; F, flow-cell; I, injector; MC, mixing coil; P, pump; PM, photomultiplier; R, recorder).

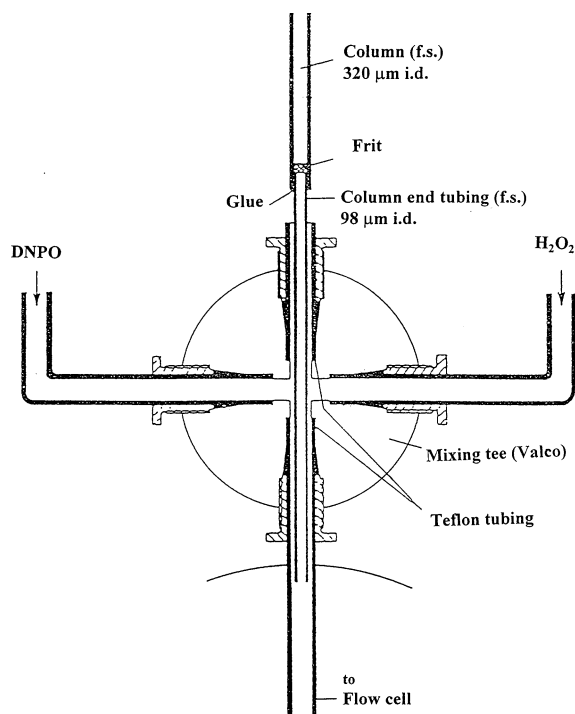

Fig. 4. Scheme for the mixing of column eluate with reagents required for CL generation. A capillary of about 100 μm i.d. is inserted against the end-frit of the column and brought through a Valco mixing cross by which the TCPO and hydrogen peroxide solutions are added. The glass 74-μl flow cell is also fixed to the mixing cross, the column end tubing ends into the flow-cell and the reagents and the LC eluate are premixed in the first part of the flow-cell.

Validated CE methods are applied routinely in many pharmaceutical analytical laboratories, where applications include purity testing, quantitative determinations of formulation content, and chiral analysis [1]. As in liquid chromatography, pre-electrophoretic labeling may be performed so as to have access to a different detection technique, e.g. fluorescence [28–30]. Post-separation treatments or labeling procedures are possible as well [28], though instrumentally complicated due to the high voltage bridge installed over the separating capillary. Post-capillary fluorescence detection devices have been described for capillary zone electrophoresis systems, comprising a capillary reactor to mix the tagging reagent with the migrating zones, avoiding zone broadening. A reactor in which the reagent buffer is pumped into the capillary by differential electroosmotic flow has been mentioned in literature, as well as a system applying sample injection by a rotary injector, three syringe pumps and two mixing parts where the fluorescent reagent is mixed with the buffer in a three-way connector [28].

Thanks to the many modes of CE, the most diverse classes of molecules—not restricted to charged species—can nowadays be investigated, the lack of sensitive detection originally having been a serious limitation. As a result of the small capillary and cell dimensions, and due to the nanoliter sample volumes, relatively concentrated analytical solutions ($> \mu g\ ml^{-1}$) or pre-concentration methods are to be used, absorptiometric detection (UV) being most common. The potentials of CL detection are currently being considered for CE purposes, apart from other (often home-made) detecting principles [56]. In principle, any fluorescing substance or appropriately labeled molecule can be measured in CE experiments after suitable chemical excitation, implying the detecting device to add the chosen 'chemical system', solubility problems and decay curves of the light flash to be taken into account, but removing, on the other hand, the light source for excitation which influences detection limits and often causes base-line problems. As discussed earlier, it is not possible for any given fluorescing species to be chemically excited by any of the existing chemiluminescent systems. As in biomedical liquid chromatography and flow injection analysis, highly sensitive detection ($< 10^{-7}$ M injected solutions) is envisaged considering the use of peroxyoxalate, firefly luciferase, luminol, acridinium ester or various oxidative reactions. Reactor design for post-column derivatization in CE is clearly under development which will be of value for the development of CL detectors. Many CL-based reactors are still in the optimization phase due to reproducibility problems when measuring the light-flash, not to speak of the band-broadening problem. Moreover, the importance of micromachining in CE instrumentation—the technology used to produce integrated circuits—is being cited in recent literature.

As in HPLC work, the needs and priorities of analysis will determine the derivatization mode:

application of existing separating systems for the intact species, stability of the analyte before or after labeling, availability of reacting devices, sensitivity and so on. As detectors for CE should be selective and sensitive, the response signals reproducibly related with the solute concentration over a wide linear range, independent of the buffer, the 'cell' not contributing to extra-column broadening, the system being reliable and easy in use, it is clear that some of these performance criteria are to be considered when choosing a detector for a particular application. Selectivity will be most important (unless a 'universal' detecting device is envisaged), followed by sensitivity, linearity range and noise.

As described above, in CL detection the analytes (native fluorescers, labeled compounds, key species in a more or less complex CL reaction) are introduced into a system that chemically brings along the required excitation energy so as to emit light, without the need for any excitation source (lamp, laser) as in (photo) fluorescence-based set-ups. Although CL reactions are, in general, rather inefficient in the production of light, the lumigenic reaction can be monitored over its entire course, and the resulting light output can be integrated. Consequently, chemiluminescent determinations can be very sensitive.

Hara et al. [20,21], applied high performance CE of proteins in a pH 3.5 phosphate buffer using 50 m i.d. fused silica capillary tubes, and found Eosine Y to migrate together with protein as a supramolecular complex in the presence or absence of molybdate, silver(I) and mercury(II). This finding provided the authors not only the possibility to overcome the problems encountered in protein estimation, such as the appearance of multiple peaks in the fluorescence detection mode after protein labeling with a fluorophore, low sensitive detection and adsorption of the protein onto the inner wall of the capillary tube, but also to measure Eosine Y instead of protein much more sensitively. Their experiments were carried out starting from a standard HPCE set-up with on-column detection (by burning off the polymer coating) via UV-visible or fluorescence emission measurements, the latter by using a specially designed detection holder, and by peroxyoxalate-induced CL detection (TCPO–H_2O_2). Although the system showed some promise, the sensitivity obtained was not sufficient, prompting the authors to further improve sensitivity, which allowed them to reach, for example, a bovine serum albumin detection limit of 4 fmol (applied) using Rose Bengal as a dye.

Dadoo et al. [13] described highly sensitive CL detection in a CE set-up based on the luminol reaction. The authors were stimulated by CL detection results obtained in HPLC work and applied the luminol CL reaction in a CE set-up, so as to provide to CE analysis the essential sensitivity, several orders of magnitude greater than that available from UV absorption measurements. A photon counting system was used for detection in order to measure the low light levels generated in the capillary column. Detection limits of 400 amol and lower were obtained for some compounds.

Ruberto and Grayeski [35] presented a detection interface designed for the addition of post-column reagents to evaluate CL as a detection method for CE. Their interface utilizes a reactor that introduces the reagents into the migrating system in a sheathing flow profile. They studied reaction conditions including pH, concentration and flow rates of the reagents for acridinium CL to evaluate the effect on the detector response. They estimated detection limits for the interface to be in the low fmole to upper amole range for acridiniums. The interface was evaluated as a potential source of zone dispersion by investigating its effects on bandwidth. 'Chemical band narrowing' due to the fast kinetics of the applied CL reaction was observed. The CL-producing oxidation reaction of the acridinium ester by hydrogen peroxide in the presence of a base was considered suitable as a derivatizing tag for amino acids, peptides and proteins in CE analysis. It is clear that the positive charge will provide greater mobility in the applied electric fields. It is worth mentioning that the acridinium reaction has a high CL efficiency yielding improved detectability; its rate can be adjusted for measurements in flowing systems, which require reaction completion in a few seconds to minimize overlapping bands. Moreover, acridiniums have been modified to in-

clude functional groups suitable for the derivatization of biomolecules. The authors conclude that increased sensitivity can be achieved due to the larger detection volumes provided by the interface without the band broadening expected from these volumes. If the kinetics of the CL reaction are fast, light production ceases before any significant diffusion occurs; low detection limits can be obtained with acridinium CL due to the reduced background present from chemical excitation.

Wu and Huie [43] employed peroxyoxalate CL detection in a home-made CE apparatus using a two-step approach for the CE separation and dynamic elution (elution under pressure) of the analytes. In this way, the authors avoided the problems associated with incompatibilities between mixed aqueous-organic solvents and electrically-driven separations by switching off the CE power supply at an appropriate time and connecting the CE capillary to a syringe pump to effect dynamic elution. They separated three dansylated amino acids and examined the effects of dynamic flow-rate and reagent concentration on the CL signal intensity employing a post-column CL detection reactor which consisted of various fused-silica capillaries held within a stainless-steel tee and a detection cell. Dynamic elution of the electrophoretic buffer and transport of the CL-reagents under pressure were achieved using two syringe pumps. The CL emission generated within the post-column mixing region was detected in a window by burning off 2 mm length of the reaction capillary polyimide coating, the light being collected via one end of an optical fiber bundle, and detected using a photomultiplier tube. Average detection limits for dansylated amino acids of about 1.2 fmol were reported.

Zhao et al. [44] designed a post-column reactor for CL detection in the CE separation of isoluminol thiocarbamyl derivatives of amino acids. A detection limit of 500 attomoles for derivatized valine was reported.

It is worth mentioning that most CL detectors only offer poor separation efficiencies which actually limit full exploitation of this detection technique in CE. Increased band broadening caused by turbulences at the column end often are at the basis of these drawbacks. Zhao et al. [44] were capable of producing a high separation efficiency; therefore it needs to be mentioned that—at least taking into account the few relevant CE-CL publications available so far—only carefully designed and optimized CL detectors may contribute to acceptable plate numbers making the technique more attractive to the analytical scientist.

Dadoo et al. [14] designed a CL detector for CE in which the signal is generated at the column outlet. The analytes emerging from the column react with the reagents to produce visible light that is carried by a fiber optic to a photomultiplier tube. They adapted the luminol and firefly luciferase reactions for use in their detection scheme, yielding detection limits of 2×10^{-8} M for luminol and 5×10^{-9} M for ATP, approximately 3 orders of magnitude lower (better) than those obtained with absorbance. The authors state that concentration detection limits in the nanomolar range should be routinely possible using a proposed end-column detector.

5. Immunoassays [11,26,37,46]

Immunoassay methods cover quite an important field of analytical chemistry. Their power to quantitate rather specifically an almost limitless number of analytes has proved their value in the area of biomedical analysis. Nanomolar and picomolar amounts of large biopolymers present in biological matrices, unassayable by other techniques, have provided biochemists with much essential information. Diagnostic methodologies have evolved, with immunoassays becoming centrally important in the analysis of drugs, pesticides, hormones and proteins.

Immunoanalytical methods are based upon the competitive binding hat occurs between a labeled and an unlabeled ligand for highly specific receptor sites on antibodies. Analysis is effected by measuring some physical or chemical property associated with the label, thereby allowing the construction of a standard curve that represents a measured physical signal as a function of the concentration of the unlabelled ligand. Unknown ligand (analyte) concentrations are extracted from this calibration curve.

Distinction of the signal corresponding to either the bound or free labeled analyte from that of the total labeled analyte population can be accomplished in two ways. The first involves physical separation of the bound fraction from the free fraction of labeled analyte. This can be accomplished by chemical precipitation, using a salt such as ammonium sulfate or a polymer such as poly(ethylene glycol), followed by centrifugation. Alternatively, one might employ 'solid-phase' chemisorption, where the analyte or antibody is attached to a solid surface (beads, tube wall, dip sticks, etc.) and distribution of reactants between the liquid phase and the solid phase is followed by their physical separation. These techniques, known as heterogeneous immunoassays, are required in radioimmunoassay, where the radiactivities of bound and free labeled analytes cannot be distinguished from one another. Also, the majority of immunoassay techniques in which the label is an enzyme, such as ELISA (enzyme-linked immunosorbent assay), are heterogeneous methods. Homogeneous immunoassays make up the second category, where physical separation of bound from free labeled ligand is not required. A signal that is related to bound and free labeled distribution is obtained from the solution that contains all of the participating analytical species. The signal-producing species may be derived enzymatically when the label is an enzyme whose substrate turnover rate is reduced upon ligand–antibody association, or the label can be a fluorophore (fluoroimmunoassay) or a chemiluminogenic-active entity (CL immunoassay). Separation-free, homogeneous immunoassay protocols offer several advantages over heterogeneous methods. Since no separation is involved, the number of procedural steps is reduced, decreasing the time required per assay. Additionally, since the physical transfer step is circumvented, potential sample loss related to this step is eliminated.

CL immunoassay has proved to be a good alternative to radioimmunoassay, especially in the field of clinical chemistry, and a large number of specific applications have been cited in the literature, including veterinary and food analysis [26].

About five components are required for CL reactions: the CL substrate which reacts to form the light emitting species, oxidants, cofactor(s), inorganic ions, and a catalyst. As a principle, any of these components can be coupled to an antibody or antigen. The labeled reagent can be used in a competitive or non-competitive binding assay, CL being initiated by the addition of the remaining components of the reaction. For use as a CL label, a compound must fulfil four requirements: it must be capable of participation in a CL reaction; it must be attachable it 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.

The following groups of compounds, amongst many others, have been investigated extensively to meet the cited requirements: synthetic organic compounds (e.g. phthalazine diones, acridinium esters); cofactors in bioluminescent reactions (NAD and ATP); enzymes (peroxidase, oxidases, kinases, luciferases). Covalent linking to either the antigen or antibody is carried out by chemical modification of the label (e.g. diazotisation, isothiocyanate, *N*-hydroxysuccinimide, hemisuccinate, imidoesters), by chemical modification of antigen or antibody (e.g. hemisuccinate, glutaraldehyde), or by conjugation using bifunctional reagents (e.g. mixed anhydride, carbodiimide, bis (*N*-hydroxy succinimides), azido-succinimides).

The growing success of CL in immunoassays is based on the fact that CL labels (mostly aryl hydrazides or acridinium derivatives) can replace radioactive labels in almost all cases without less performance, offering low detection limits and good precision.

The instrumentation for CL light detection is rapidly developing, as was demonstrated at the 1993 Banff (Canada) bioluminescence and chemiluminescence meeting [41] (including the earlier event [42]) and the Ghent (Belgium) biomedical quantitative luminescence spectrometry [32] meetings. Recent advances of biological imaging techniques for example may provide important aspects of signal transduction pathways within living systems. Receptor–ligand and antigen–an-

tibody coupling may serve as striking examples of these reactions. Although classical radioligand-binding studies are still successful, nonisotopic strategies are of increasing impact because of several advantages. Besides the problems of radiolabeled ligand handing, waste disposal and limited shelf-life, some non-isotopic labels e.g. chemiluminogenic, may provide dynamic signals that are more jointly related to the target biological attribute. For example, light-emitting sources that decompose as conjugates of target molecules signal the place and rate of degradation of the substance being measured. Special enzyme-ligand conjugates (e.g. alkaline phosphatases or horseradish peroxidase) are frequently used to induce an enzymatic decomposition of a luminogenic substrate (e.g. 1,2-dioxetanes or luminol). If the amount of available substrate does not limit these reactions, the light emission intensity becomes a direct measure of enzyme conjugates.

Imaging cameras for CL ELISA and for monitoring bacterial bioluminescence, fast and non-polluting CL imaging and quantify systems as an alternative to X-ray film approaches, quantitative photon imaging techniques for CL measurements of single cells and for simultaneously measuring multiple samples in arrays (e.g. immunoassays and screening of drugs and toxic agents), the use of calibration standards for microtiter plate and tube chemiluminometers employed in both research and clinical laboratories have been described extensively in the literature. The number of chemiluminescent assays with applications in human disease (e.g. tumor diagnostics, protein and hormone measurements), DNA sequencing, genetic and environmental research is rapidly growing [11,26]. Moreover, taking into account the increasing importance of CL in drug assay procedures as surveyed in recent years [10,33], many more applications are to be expected in the coming years.

6. Conclusion

From the various detection techniques nowadays available for detection in flowing streams and in immunoassays, CL measuring systems and devices are of growing importance with respect to the low detection limits that may be reached using selected techniques.

Reactor design for post-column derivatization as taken from liquid chromatography and FIA is clearly under development which certainly will be of value for the development of CL detectors. Many devices are still in the optimization phase due to reproducibility problems when measuring the CL light flash. Moreover, CL detectors often produce poor separation efficiencies which limit their usefulness in CE. It should be mentioned that CL enhancement techniques, from the analytical electrophoretic point of view, will likewise be focused on in further research. Detection sensitivity obviously may also be improved by techniques of sample pre-concentration during injection, apart from the development of suitable derivatizing agents.

The importance of micromachining in CE instrumentation—the technology used to produce integrated circuits—is to be cited as originally mentioned by Dovichi at the Orlando HPCE '93 meeting [17] and ever since further developed, as reported most recently by Ewing [57]. Not only can separation channels be etched into a glass substrate, but mixing and reaction chambers can also be incorporated. Moreover, micromachining allows mass production, and glass substrates with a complex manifold of channels with cross-sectional dimensions of 10×30 mm have been used.

CL-based immunoassay methods are in full development mainly due to the various restrictions encountered when using radioisotopes.

Finally, it should be mentioned that the rapid evolution of immobilization techniques has improved the application of CL-based measuring systems, especially in FIA and liquid chromatographic set-ups. Immobilization of enzymes can be of use in CL detecting systems. For example, immobilization of luciferase in a flow-cell in front of the photomultiplier tube, so that light is emitted as the substrates flow over the enzyme, allows very selective and sensitive measurements. Immobilization of the enzyme may occur within the flowing stream in order to produce a product which participates in a CL reaction, e.g. the action of enzymes on various analytes to produce

hydrogen peroxide which is determined by the luminol reaction. Also, all applications of immobilized reagents in CL can easily be adapted to biosensing systems, an area which has tremendously increased after the introduction of fiber optics in analytical research. Immobilization of enzymes on the fiber is focussed on since immobilization of chemiluminogenic reagent(s) (e.g. luminol) would cause problems due to fast reagent consumption.

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