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Review

Derivatization of biomolecules for chemiluminescent detection in capillary electrophoresis

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Abstract

An overview is presented on the power and drawbacks of the relatively unfamiliar chemiluminescence-based detection technique applied in analysis by capillary electrophoresis, for determining chemically derivatized biomolecules. Examples of the most common systems are given for many series of biologically active compounds as well as for some pharmaceuticals. The most common chemiluminescent systems include the application of peroxyoxalate ester chemiluminescence, acridinium esters, luminol and derivatives, detection based on the tris(2,2′-bipyridine)ruthenium(III) system, the huge potentials offered by direct oxidations—though often with still unelucidated reaction mechanisms—and the powerful area of bioluminescence techniques, revealing as well the fast developing area of microchip-based analysis employing this specific luminescence principle.

Keywords: Reviews; Derivatization; Biomolecules

Contents

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1. Introduction

Since the early 1980s, capillary electrophoresis (CE) has become one of the most powerful, growing and conceptually simple separation techniques for the analysis of complex mixtures. Its high resolution, relatively short analysis times and low operational cost when compared to high-performance liquid chromatography (HPLC) have been the main reasons for this development. The ability to analyze ultra-small volume samples in the picoliter to nanoliter ranges makes it an ideal analytical method for extremely volume-limited biological microenvironments.

The separation of biomolecules in CE, mainly proteins and polypeptides, can be carried out using different modes of operation [1–4], being capillary zone electrophoresis (CZE) the most commonly used technique due to the simplicity of operation and versatility. Successfully validated CE methods are nowadays routinely applied in clinical diagnosis comprising biological sample preparations prior to CE analysis [5–10], as well as in pharmaceutical quality control laboratories where applications include purity testing, quantitative assays, separation of enantiomers, determination of drug stoichiometry and DNA identification [11,12].

Due to the ultra-small sample volumes introduced in the system and because of the small internal diameter requirements in CE, together with the fact that in general the analytes of interest are present in low concentrations, poor detection limits are encountered, limiting the usefulness of the technique. For this reason, relatively concentrated analytical solutions, on-line preconcentration methods and a variety of sample injection techniques for the preconcentration of analytes have been developed so as to improve sensitivity [13,14]. Also, specific and non-specific bioaffinity and molecular recognition CE methods for preconcentrating and characterizing analytes present in a wide concentration range in diluted liquids, fluids or complex matrices have been developed. The methods combine the low as well as the high binding selectivity of the sorbing molecules with the efficient resolution abilities of CE. Detection limits of parts-per-trillion or -quadrillion can be reached, which allow a significant impact in biomolecular analysis [15–17].

The selection of detection techniques capable of providing detection improvements in CE has been a principal issue of research, being the main performance criteria for the adequate choice of selectivity and sensitivity, followed by the linearity of signal response, linear detection range and reproducibility. These performance criteria are related to the independence of the detector response of buffer composition and of the physical devices (cells, joints, fittings, connectors, etc.), which must not contribute to extra-column zone broadening, being compatible with the separation conditions.

According to the detection cell architecture, two different detection modes can be considered. On-column detection is most commonly used as in this case the detection cell makes part of the electrophoretic capillary, thus eliminating the broadening effect and producing high separation efficiencies. When using off-column detectors, the band broadening is generally increased.

Based on the specificity of the detection principle, the detectors are divided into two categories, i.e., ‘‘bulk-property’’ and ‘‘specific-property’’ series [18]. The former evaluates differences between a physical property of the solute relative to the buffer alone, such as the refractive index, conductivity and the application of indirect methods. In spite of their universal character, lower sensitivities and dynamic ranges are obtained. Specific-property detectors measure inherent physico-chemical properties of species, for example ultraviolet (UV) absorption, fluorescence emission, and mass spectral behavior. Hence

only the analytes possessing these properties are detected, minimizing background signals and increasing sensitivity and width of linear ranges for these determinations. Detection may occur in the migration process (UV, fluorescence, conductivity and refractive index) or as the components elute from the capillary (post-column derivatization before detection, and electrochemical and mass spectrometric methods).

UV (and much less frequent, visible) absorbance detection is the most widely used detection technique for CE due to its ability to detect nearly all species without derivatization and to the easy adaptability of UV detectors originally designed for HPLC work; several commercially available CE equipments offer some basic types of absorbance detectors. The main limitations of UV detection are its relatively low sensitivity and dynamic ranges. The concentration limits of detection (between 1 and 10 μM) are usually limited by the short pathlength of on-column detection systems (inner diameter of 25 μm or greater) and the limited time available to observe the sample as it passes the detector. Various methods have been employed to increase the pathlength for optical detection in small capillaries, including the use of axial illumination, Z-shaped flow-cells, multi-reflection cells and the use of tubing with non-circular cross-section. Diode-array detectors provide additional spectral information from the separated analytes that can be used to assist in peak purity assessment, analyte identification and pre-run screening to determine the wavelength setting for optimum detection sensitivity.

Fluorescence detection is the second mode quite frequently applied in CE analysis because of the low detection limits, not strictly pathlength depending, and the easy adaptability of fluorescence detectors to the smaller diameter capillary. The high sensitivity obtained is due to the fluorescence emission inherently at higher wavelengths than the excitation wavelength, yielding low background signals. Moreover, a single fluorescent analyte may emit multiple photons. As with UV-detection, depending upon the needs of each specific application, direct and indirect (the latter being much less common) detection methods can be considered. Also, a wide variety of reagents exist for pre-, post- and on-column derivatization in CE in order to convert analytes into products with more favorable detection characteristics. Some reviews related to derivatization in CE have been published [19,20]. Laser-induced fluorescence (LIF) has also been utilized as a highly sensitive detection principle for CE [21–24]. However, while the LIF detector is nowadays able to achieve zeptomole (10^{-21}) detection limits, conventional derivatization techniques are inefficient at these exceptional levels [25].

Also, CE has successfully been coupled with mass spectrometry (MS) [26], nuclear magnetic resonance (NMR) [27,28], near infrared fluorescence (NIRF) [29,30], radiometric [31], flame photometric [32], absorption imaging [33] and electrochemical (conductivity, amperometric and potentiometry) [34–36] detectors.

Chemiluminescence (CL) constitutes a powerful detection mode under actual investigation in CE. This technique is based on the production of electromagnetic radiation (ultraviolet, visible or infrared) observed when a chemical reaction yields an electronically excited intermediate or product, which either luminesces (direct CL) or donates its energy to another molecule which then luminesces (indirect or sensitized CL). 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 living organisms, the phenomenon is named bioluminescence (BL). Minimal instrumentation is required and because no external light source is needed, the optical system is quite simple. Hence strong background light levels are excluded, as occurring in absorption spectroscopy, reducing the background signal, the effects of stray light and the instability of the light source, leading to improved detection limits. For this reason, CL has been defined as a ''dark-field technique'' as this technique produces a signal against a dark background, making it easier to detect and thus to acquire the CL signal by a photomultiplier tube (PMT) which must obviously be sufficiently sensitive in the spectral region of interest.

From the past decade, several books, chapters and reviews have been published related to the characteristics of CL and BL [37–41], mainly in the liquid phase [42–47], and the use of CL as detection mode in flow injection analysis (FIA) [48,49], in liquid chromatography (LC) [50,51], in gas-chromatog-

raphy (GC) [52] and in immunoassays [53,54]. Extensive reviews reported on the specific application of CL reactions in different disciplines such as medical and biochemical [55–59], food [60,61], environmental and toxicological [62] analysis. An extensive monograph on CL has recently been published by our groups including historical evolution, principles, reactions, instrumentation, and combinations as detection technique with FIA, HPLC, GC, CE, immunoassay and specific applications of CL in Analytical Chemistry [63].

Due to the advantages of CL detection in terms of low detection limits, wide dynamic ranges and high sensitivity and its potential when combined with the high separation ability offered by CE, research in this area has significantly increased from the 1990s, allowing the resolution and quantification of various analytes in complex mixtures. One of the main advantages for this coupling is the fast kinetic nature of CL reactions which allows a short detection time using relatively large volume flow-cells avoiding the expected band broadening with these kinds of volumes and also, due to the light emission ceasing before the analyte diffusing significantly into the surrounding reagents in the bulk volume of the interface, relatively narrow peaks can be obtained from CE separation using CL as detection technique, which is known as the chemical band narrowing effect. In recent years, some review articles and book chapters have appeared, including the principles, instrumental designs and applications of CL as a detection system in CE [19,64–71].

Practical considerations can be taken into account for the cited coupling, such as the dependence of the CL emission on several environmental factors, which should be dealt with during the CE separation procedure. In this sense, a compromise between the required and optimized separation and detection conditions should be worked out for each developed procedure. Also, 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 widely varying in different CL systems, care must be taken so as to detect the signal in the flowing stream at strictly defined periods. From 1990 onwards, different instrumental developments for CL detection in CE were developed, which involve the design of CE-CL interfaces for the introduction of the CL reagents into the system with the purpose of initiating the CL emission.

Huang and Fang have reviewed the different designs and modes of operation for CL detection in CE [68]. Considering the fact that most published papers include the reagent to be added to the CE system in a postcolumn mode so as to ensure optimum CE separation conditions up to the detection zone, a classification of the interface design is established based on the mode of postcolumn flow mixing, including merging flow, coaxial flow and reservoir mixing. Also, based on the site of detection and whether the detection is isolated from CE high voltage supply, the interface designs are classified into ‘‘off-’’, ‘‘on-’’ and ‘‘end-’’column.

The present review provides an up-to-date overview on chemiluminescent derivatization reactions for the detection of biomolecules separated by CE, including the progressive development of instrumental setups for coupling CL detection with CE separation.

2. Chemiluminescent systems for detection in capillary electrophoresis

2.1. Chemiluminescent detection based on peroxyoxalate CL system

The peroxyoxalate chemiluminescent (PO-CL) reaction is an indirect CL system based on the hydrogen peroxide oxidation of an aryl oxalate ester in the presence of a fluorophore [72]. 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 transfer complex with the fluorophore, donating one electron to the intermediate [73]. This electron is transferred back to the fluorophore raising it to an excited state and liberating light characteristics typical for the fluorophore nature, as shown in Fig. 1. 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 aryl oxalates in water (though newer reagents overcome

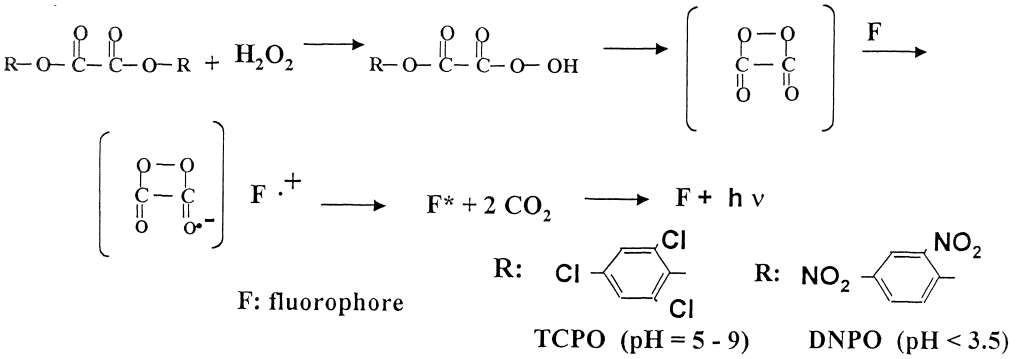

Fig. 1. Possible reaction pathway for the PO-CL system.

this limitation) 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 and analytes converted to hydrogen peroxide via either an enzymatic or a photochemical post-column reaction, for compounds that are highly fluorescent (e.g., polycyclic aromatic hydrocarbons) or compounds that do not exhibit native fluorescence but can be derivatized chemically using fluorophoric labels [74] such as 5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl chloride) (amino acids, steroids, aliphatic amines, carboxylic acids, etc.) or fluorescamine (amines) and analytes that efficiently quench or enhance the CL reaction. The high sensitivity (detection limits in the low femtomoles and improvements of 10–100 times compared to conventional fluorescence), high versatility of the CL reaction and the relative absence of interferences (more selective than fluorescence because of the inability to excite all fluorescent compounds) make this system useful for a wide range of analytical applications [75].

2.1.1. Reactions for labeled amino acids, peptides and proteins

Zhu and Zhao reported in 1990 the first combination of CL detection in CE, using this high efficient PO-CL system [76]. They detected a protein, bovine serum albumin, labeled with dansyl chloride, using as CL reagent bis-pentachlorophenyl oxalate ester (PCPO), showing a detection limit of 10 fmol. An off-column merging flow interface was employed, using a 70-cm×0.65-mm I.D., 1.0-mm O.D. glass capillary coiled as flow cell for detection of the CL reaction emission with a photon counter. CL reagent and CE effluent were mixed by simply merging the flows at the end of the separation capillary through the arms of a four-way connector and directed to the reaction capillary though the third arm, as can be seen in Fig. 2. Good sensitivity was obtained for the large size of the non-conventional separation capillary employed (400 μm I.D. teflon tubing), as well as by the high capacity of the flow-cell.

Hara's group reported the combination of CZE

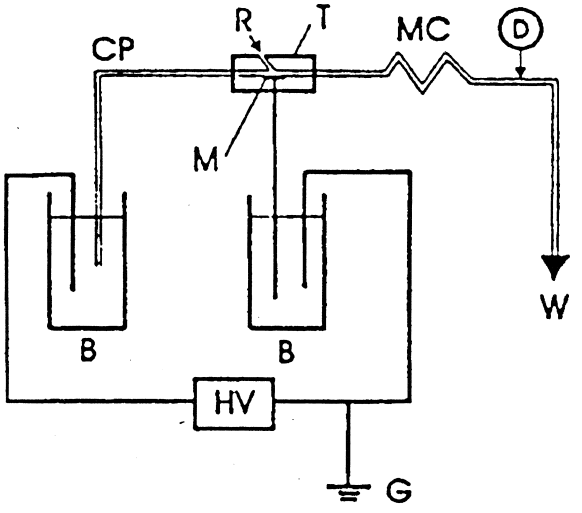

Fig. 2. Schematic diagram of off-column merging flow interface firstly used in CE-CL. CP, separation capillary; HV, high voltage power supply; G, grounding; M, semipermeable membrane; D, PMT detector; R, CL reagent, B, buffer reservoir; T, four-way connector; MC, mixing coil; W, waste (adapted from Ref. [68]).

and CL detection using an on-column coaxial flow interface for the detection of proteins using the PO-CL reaction [77]. The CL detector was coupled to the capillary by burning off the polymer coating (Fig. 3). In this application the dyestuff Eosine Y (EY) comigrates with the proteins as a supramolecular binary or ternary complex in the presence of molybdate, tungstate, silver (I) or mercury (II). Similar results were obtained using Bromopyrogallol Red. The use of EY provided the possibility to overcome several problems encountered in the separation of biopolymers owing to the appearance of plural peaks in the fluorescence spectra of the protein labeled with a fluorophore and adsorption of the protein onto the inner wall of the capillary tube. Quite an improved determination in terms of sensitivity was obtained by measuring EY in the supramolecular compound mentioned above. The EY complex with the protein was determined by measuring the CL intensity of the TCPO-H_2O_2-EY system using an interface between CZE and CL detector. The CL-reagent obtained by mixing 1 m*M* TCPO and 75 m*M* hydrogen peroxide in acetonitrile, and the migrating buffer solution were fed into a four-way joint by the use of two pumps. The protein-EY complex passing through the capillary tube from the upper to the lower parts was mixed with the feeding solution.

As the use of EY did not achieve the adequate sensitivity for protein detection, other fluorescent xanthene dyestuffs including Rose Bengal, Erythrosine B, Phloxin B, etc., were studied in later work [78] using the same experimental device. The addition of a fluorinated cationic surfactant (FC-135) to the migration buffer improved the CE resolution by forming a bilayer via a hydrophobic interaction between the apolar chains, resulting in a reversal of the EOF and avoiding, in this way, the above mentioned inconvenience encountered in the separation of proteins and other biological compounds. When labeling the proteins with the dyestuff by mixing both species, Rose Bengal was found most sensitive and gave the highest CL intensity. Using this dye, 5×10^{-7}–10^{-4} *M* of bovine serum albumin (BSA) was determined in about 20 min, showing a detection limit of 4 fmol.

However, this proposed apparatus, in which two streams comprising a buffer solution and a CL reagent solution are applied, presented some disadvantages such as a lowering of CL intensity due to the buffer solution and the difficulty of maintaining steady-state mixing. To overcome these problems, this research group developed a new on-line CL detector [79]. The stream of solution buffer is eliminated as a definitive migration current and a good resolved electropherogram for the separation of proteins is obtained using a stationary flow of fluorosurfactant. The complicated mechanical operation for preparing the cited four-way joint is avoided, using a three-way joint, commercially available, held

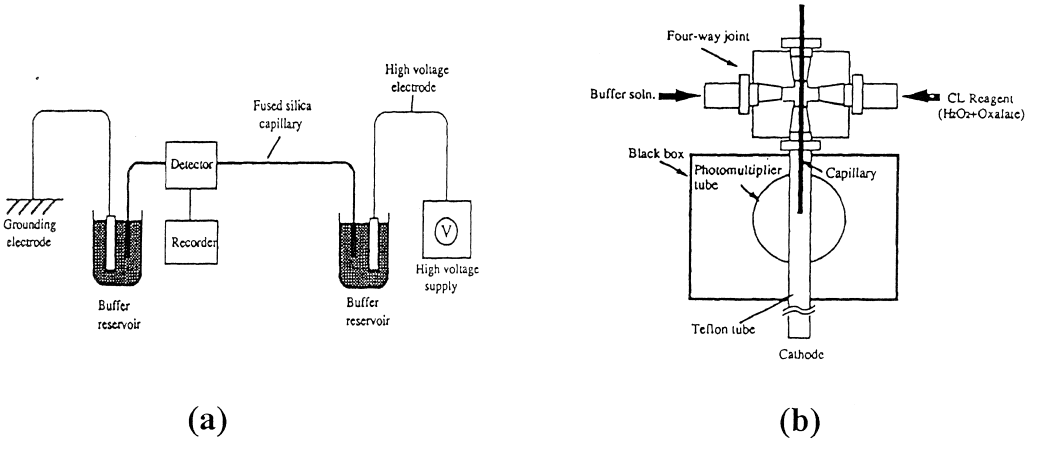

Fig. 3. Schematic diagram of (a) main part of the CE-CL device; (b) on-column coaxial flow interface for CL detection (from Ref. [77], with permission).

at 10 cm below the horizontal surface of a reservoir solution in order to prevent any back-flow of the CL reagent into the capillary tube. Using this on-line CL detector, an improved detection limit of 1.7 fmol for BSA was obtained by measuring the CL intensity of the complex with EY.

In this system, the sensitivity was increased by replacing EY by rhodamine B isothiocyanate (RITC) dye in the binary complexes formed with the proteins BSA or human serum albumin (HSA) and using a different imidazole buffer solution of pH 6 [80]. However, best detection limits for these determinations were found employing the tetramethylrhodamine isothiocianate isomer (TRITC) dye, left for 4 h with a standard solution of BSA in acetonitrile followed by introduction into the capillary. For BSA, a detection limit of 6 n*M* was reached [81].

The introduction of the application of the fluorogenic reagent fluorescamine (FR) to the system TCPO-H_2O_2, for the analysis of proteins achieved high sensitivity and very short labeling times, as well as an undisturbed electropherogram in which a possible peak due to excess of labeling reagent did not appear as the excess of fluorescamine is quickly hydrolyzed in aqueous solution [82]. The reagent reacts readily and rapidly with primary amines under alkaline conditions to form fluorescent substances, providing the basis for a rapid assay of amino acids, peptides, proteins and other primary amines (Fig. 4). In this case, the former apparatus was improved with respect to the attachment of the grounding electrode, which was directly immersed in the solution of the buffer reservoir, avoiding the formation of bubbles that could interfere with the CL measurements.

The need for using organic solvents in the PO-CL system due to the low solubility and the instability of oxalate derivatives in aqueous solution presents several problems when applying this reaction in CE. The organic solvents may influence the migration behavior of the analytes and their mobility in the aqueous electrophoretic buffer, and the stability of the PO-CL reagents may be affected by the high electric field strength inherent to CE systems. In this sense, the previously described CE-CL system using untreated fused-silica capillaries was recently improved by the introduction of a polyacrylamide-coated capillary and the attachment of a flow line of an aqueous buffer solution and a mixing filter in order to provide the conductivity required for CE [83]. The stable and constant electric current in the system allowed the separation and detection of a mixture of the proteins lysozyme, cytocrome C and ribonuclease A labeled with FR. The CL detection was about 3- and 30 times as sensitive as fluorescence and UV detection, respectively.

Wu and Huie [84] avoided the disadvantages related to the incompatibilities between mixed aque-

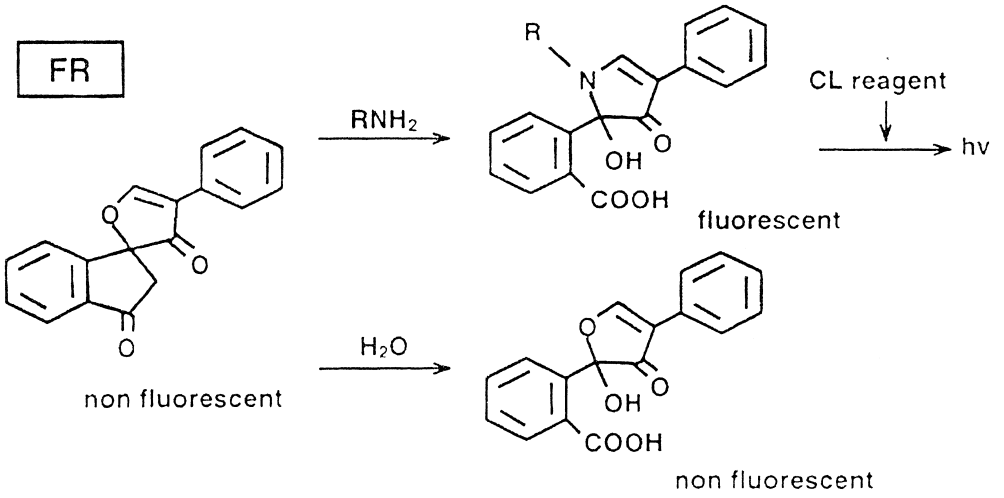

Fig. 4. Structure of fluorescamine and its fluorigenic reaction with primary amines.

ous–organic solvents and electrically driven systems by switching off the CE power supply at an appropriate time and connecting the CE capillary to a syringe pump to carry out dynamic elution. They developed a post-column detector equipped with various fused-silica capillaries held within a stainless-steel tee and a detection cell (Fig. 5). By burning off 2 mm length of the polyimide coating, a detection window was created to detect CL emission generated within the post-column mixing region. The light was collected via one end of an optical fiber bundle situated directly above the detection window and the other end interfacing to the detection system using a PMT. The proposed system was applied to the determination of three dansylated amino acids, showing a significant improvement in detection limits when compared to UV absorption detection.

2.1.2. Reactions for compounds labeled with dyestuff-containing liposomes

The liposome structure, a synthetic lipid bilayer vesicle, has been used for model biomembranes, as drug carrier, and for other purposes such as immunoassays, in which dyestuff-containing liposomes play an important role as a probe and are used for characterizing liposomes in terms of homogeneity, capture volume, stability, permeability, etc. However, the methods for measuring the probe require tedious treatments and, in general, provide only indirect information. In this sense, Tsukagoshi et al. examined for the first time the electrophoretic behavior in a capillary [85,86]: when a solution of Eosin Y-containing liposome was subjected to the CZE-CL method above described for proteins, two peaks, being Eosin Y entrapped in liposome and free Eosin Y in the bulk solution were successfully observed in the electropherogram, from which various types of information about the liposomes can be provided rapidly, CL detection—only—being effective due its high sensitivity. In these studies, the Eosin Y-containing liposome was migrated to the cathode by EOF in the capillary and was mixed with the CL reagent (TCPO+H_2O_2) at the tip of capillary, where the liposome structure was broken by an

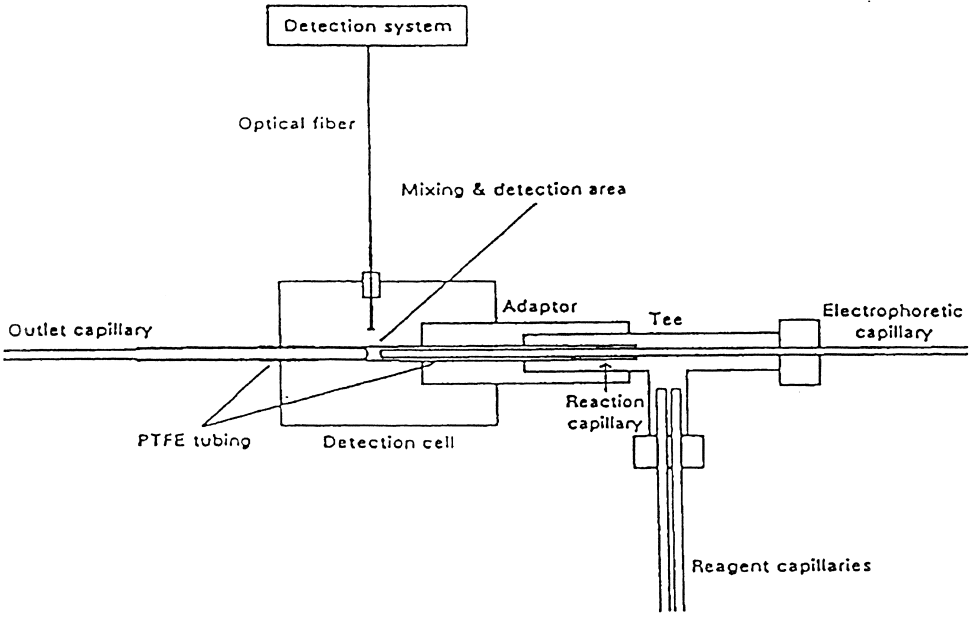

Fig. 5. Schematic diagram of a post-column reactor. One arm of the tee contains the electrophoretic capillary which is inserted in the reaction capillary situated at the opposite arm of the tee. The tee is connected to the detection cell via an adaptator and both the electrophoretic and reaction capillaries are inserted into the detection cell through the inner core of a PTFE tubing. Two reagent capillaries inserted into the central arm of the tee are used to deliver the TCPO and H_2O_2 reagents into the mixing area through the small gaps that exist between the outer surface of the electrophoretic capillary and the inner surface of the reaction capillary (from Ref. [84], with permission).

organic solvent (acetonitrile) containing the reagent to induce a stable and reproducible CL emission intensity over all liposome concentrations. Under an alkaline condition the free Eosin Y in the bulk solution migrated slower than the liposome due to the negative charge. The changes of retention times and ratios of both peaks offered useful information as to the permeability and surface charge of the liposome membranes. Other dyestuffs such as rhodamine B have been efficiently used [87]. This system is the basis of a new immunoassay CL detection of dyestuff-containing liposomes as a labeling reagent for the determination of human serum albumin in serum after separation by CE over the range of 1×10^{-6}–5×10^{-4} *M* [88], as is shown in Fig. 6.

2.2. Chemiluminescent detection based on acridinium ester CL reaction

The acridinium CL reaction is based on the oxidation of an acridinium ester by hydrogen peroxide in alkaline medium to produce *N*-methylacridone (10-methyl-acridan-9-one) in the excited state, which, upon relaxation, emits a photon, making it suitable as a derivatizing agent for amino acids, peptides and proteins in CE analysis (Fig. 7). This reaction has a high efficiency, yielding improved detectability and presents several advantages for its application as detection system in CE, such as, e.g., the possibility to adjust its rate for measurements in flowing systems that require complexation reactions in a few seconds so as to minimize overlapping bands, the easy modification of the acridinium esters to include functional groups suitable for the derivatization of biomolecules and the positive charge of the quaternary nitrogen atom in the ring structure, which provides greater mobility in the applied electric fields.

2.2.1. Reaction for labeled amino acids, peptides and proteins

Ruberto and Grayeski developed in 1992 a new detection interface for the addition of post-column reagents to evaluate the efficacy of this CL system as a detection method for CE [89]. The experimental configuration is shown in Fig. 8(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 Fig. 8(b). The CL reagents

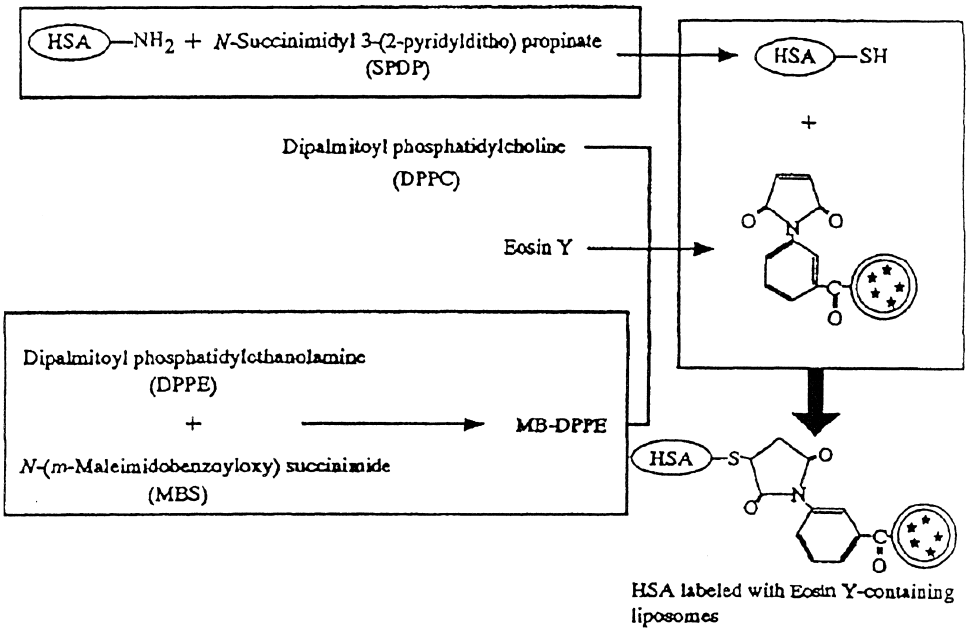

Fig. 6. Preparation of HAS labeled with EY-containing liposomes (from Ref. [88]).

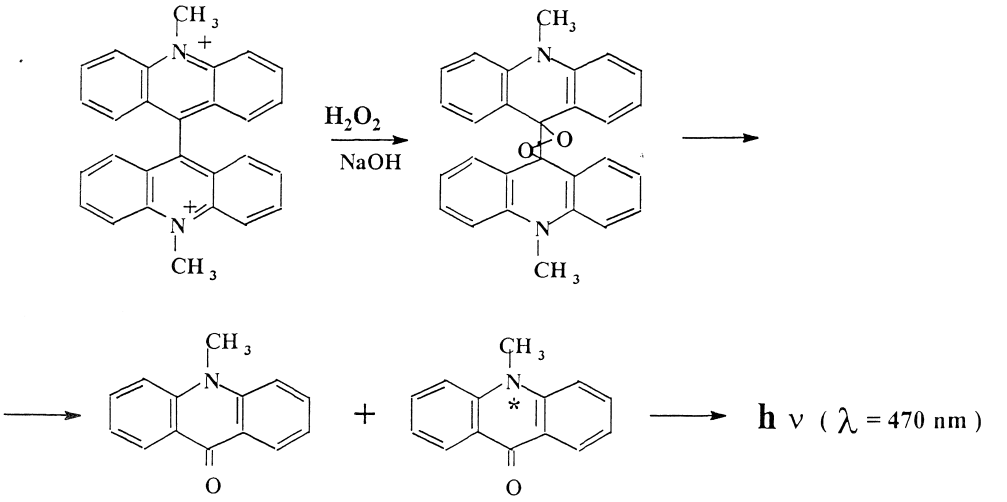

Fig. 7. Proposed mechanism for the CL reaction of a representative acridinium ester (lucigenin, 10,10′-dimethyl-9,9′-bisacridinium dinitrate).

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. By diffusion and radial migration, the reagents are mixed 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 at a distance of 1 cm and the photons emitted from the CL reaction are detected by the later PMT. The end portion of the reaction capillary exits the detector and enters a buffer reservoir to complete the circuit. Several factors influencing the detector response have been taken into account, such as the flow rate of the post-column reagents, which requires an exhaustive control to procure an adequate mixing of the reagents and a completed reaction in the time interval of the analyte being present in the proximity of the detector. A good separation of different acridinium esters at the optimum experimental conditions could be reached. The possible

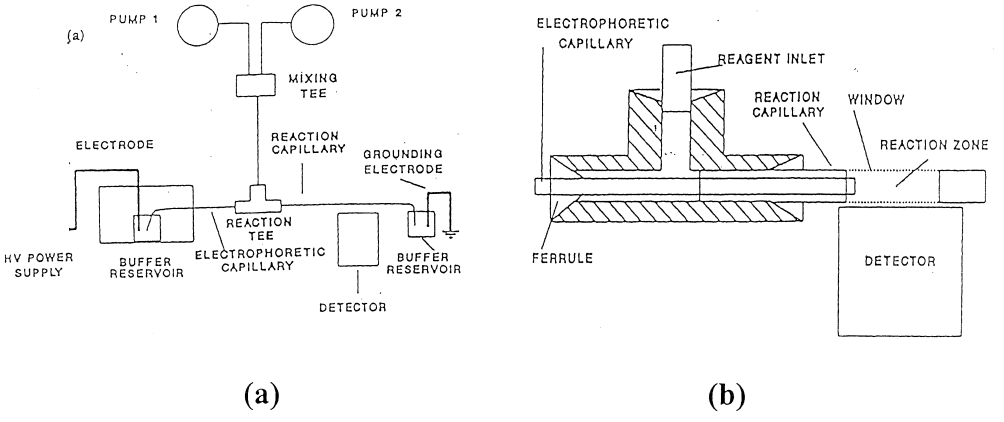

Fig. 8. Post-column CE-CL system. (a) Experimental configuration. The hydrogen peroxide is introduced by pump 1 and pump 2 provides base; (b) cross sectional scheme of the coaxial CL detection interface (from Ref. [89], with permission).

hydrolysis presented by the acridinium esters above pH 3 limits the working pH range, because this reaction is one of the competing processes to the photon-generating mechanism, decreasing the CL signal by more than 99% if the pH is increased up to 4. Nevertheless, biological species such as amino acids and proteins can be separated under these conditions.

A synthetic acridinium ester, 4-(2-succinimidyloxycarbonylethyl)phenyl - 10 - methylacridinium-9-carboxylate fluorosulfonate (acridinium NHS) can be used to label unhindered primary amine functionalities (Fig. 9), and using this interface for CL detection, it was later satisfactorily applied for performing trace peptide CE separation with CL detection [90].

2.3. Chemiluminescent detection based on luminol CL reaction

Oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) in alkaline medium is the most representative example for direct CL reactions, producing the excited 3-aminophthalate ion (Fig. 10(a)), avoiding the inconvenience typical for PO-CL reactions which require the use of organic solvents. 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, hemoglobin and peroxidases). This reaction has been applied for the determination of catalysts such as metal ions or enzymes (peroxidases, hematic compounds, etc.), certain oxidants, inhibitors or substances that are easily oxidized and are indirectly determined by the decreasing CL emission, as well as species labeled with the catalyst, peroxide or species that may be converted into peroxide, luminol, or species labeled with luminol. One drawback of the luminol based CL systems is that the reaction is only efficient over a narrow pH range (approximately 10–11), and this can limit the pH at which CE separation can be carried out.

The unsubstituted phthalic acid hydrazide and several non-aromatic cyclic hydrazides such as maleic acid hydrazide or succinic acid hydrazide are either non-chemiluminescent or show extremely weak CL. However, the 6-amino isomer of luminol (isoluminol) is chemiluminescent to the same extent

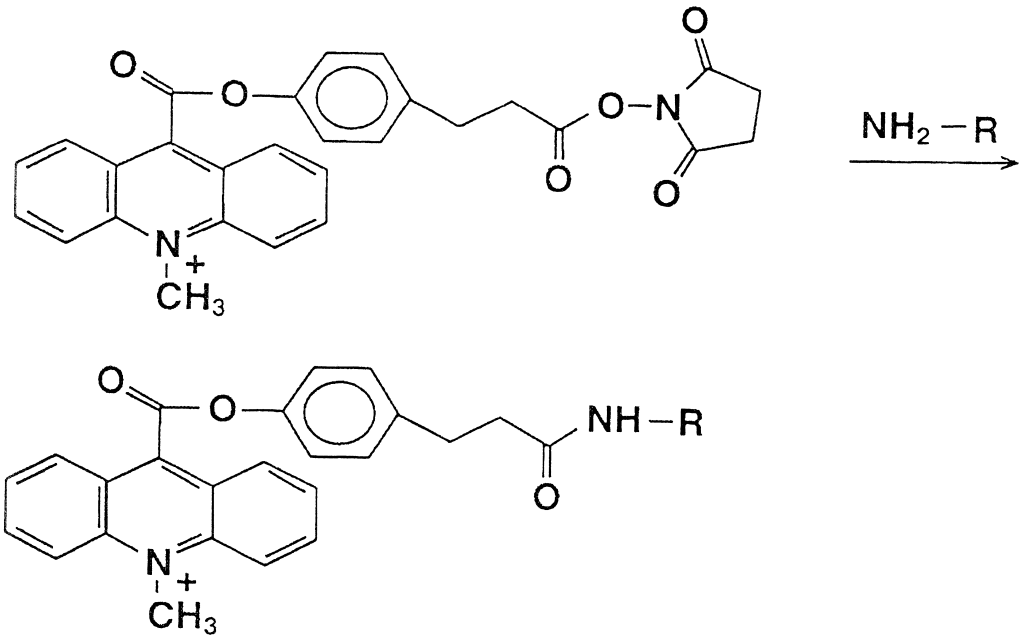

Fig. 9. Acridinium tagging reaction employing acridinium NHS.

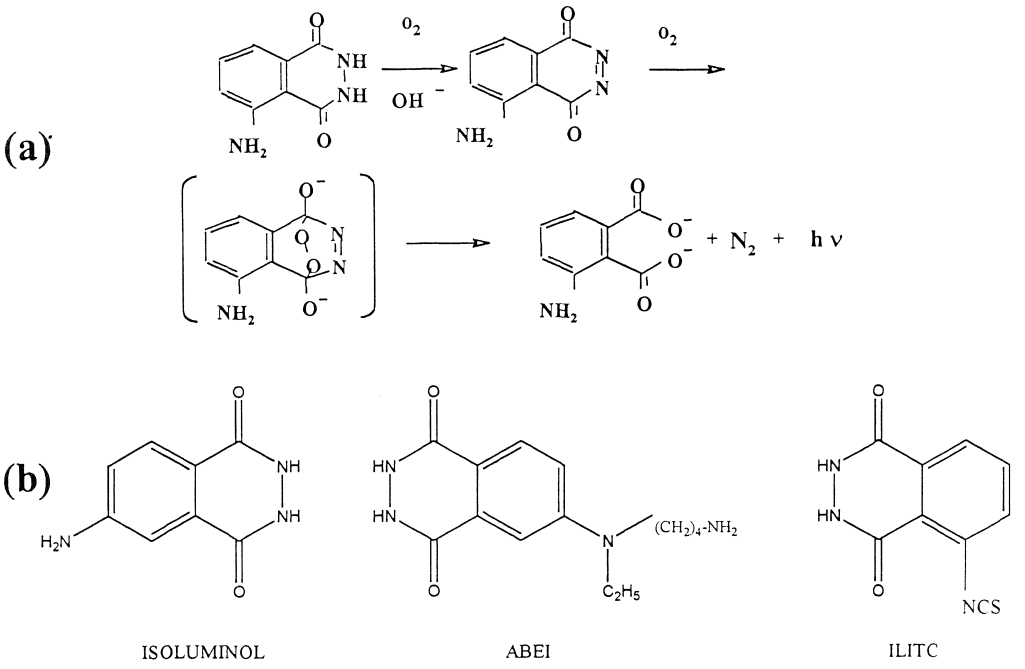

Fig. 10. (a) Proposed mechanism for the luminol CL reaction; (b) luminol derivatives.

as luminol. Isoluminol has been widely used in CL reactions, and because of the amino group it is less sterically hindered than luminol; it has been derivatized for chemiluminescent labeling far more often than luminol (Fig. 10(b)). Luminol-type CL derivatization reagents used mainly in liquid chromatography and CE have been recently reviewed [91].

2.3.1. Reactions for luminol derivatives and labeled compounds

Dadoo et al. reported in 1992 a successful study involving luminol CL detection by using an on-column coaxial flow interface [92] similar to that proposed by Hara et al. in 1991. This preliminary work demonstrated well the use of CL as a highly sensitive and selective detection method in CE by its application to the separation of luminol and *N*-(4-aminobutyl)-*N*-ethylisoluminol (ABEI). Detection limits of 100 and 400 amol were obtained for the compounds mentioned, respectively, achieving an improvement in sensitivity of 2–3 orders of magnitude with respect to the ones obtained using UV-absorption for detection.

Zhao et al. [93] designed a post-column reactor for CL detection in the capillary electrophoretic separation of isoluminol thiocarbamyl derivatives of amino acids, because, like other isothiocyanates, isoluminol isothiocyanate (4-isothiocyanatophthalhydrazide, ILITC) has potential applications in the protein sequencing area. The determination was performed using a post-column sheath flow cuvette as the mixing chamber and a sheath stream from a syringe pump to carry the analytes away from the detection zone once emerging from the column (Fig. 11). The mixing of the labeled analytes with hydrogen peroxide and microperoxidase as a catalyst is required because if these three components are introduced as separated streams, the peroxide will be destroyed by the catalyst before ever reacting with the analyte hence no CL emission would be observed. In this paper, microperoxidase is added directly to the separation buffer, producing an intimate contact between the catalyst and analyte when mixed with peroxide, avoiding the problem of bubble formation in the separation capillary, which could perturb the separations. The separation of labeled amino acids was improved by the addition of a low concentration of the anionic surfactant sodium

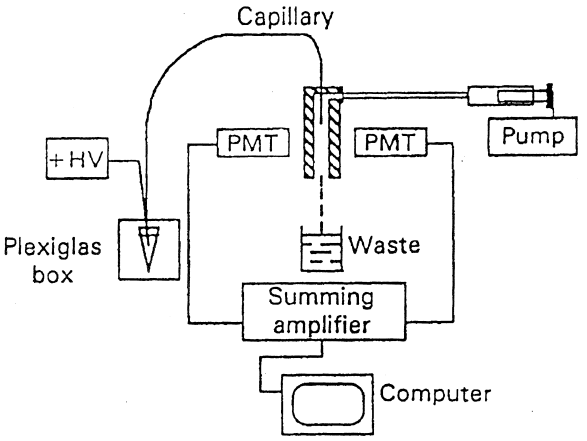

Fig. 11. CE-CL device using a post-column sheath flow cell. A low pressure syringe pump introduces H_2O_2 solution into the flow cuvette. Analyte mixes with the oxidant in the cuvette producing the CL emission (adapted from Ref. [93]).

dodecyl sulfate (SDS) to the running buffer, taking into account that an increase in the concentration of the micellar medium may denature the microperoxidase, destroying the catalyst and inhibiting the reaction. The elimination of turbulent mixing in the flow chamber and the short residence time of the reaction mixture in the detection chamber provided a high separation efficiency of 100 000 theoretical plates for labeled amino acids, yielding good resolution in comparison with previous CL detectors.

An end-column interface was proposed in 1994, in which the signal was generated at the column outlet [94]. Based on the same CE system previously used [92] and removing a 1–3-mm section of the polyimide at the outlet end of the capillary by burning, the modified CL apparatus was coupled to the CE set-up as can be seen in Fig. 12. The outlet end of the separation capillary is immersed in the reservoir containing the electrolyte and the reagent for the CL reaction. When the analytes emerge from the column they react with the CL-reagents in a reservoir producing visible light that is transported by a fiber optic to a PMT tube. Immersing a platinum wire for the grounding electrode in the reservoir completes the CE electrical circuit. The device was applied to the separation and detection of luminol and ABEI and also for the separation of arginine and glycine derivatized with ABEI. In this separation of luminol and ABEI, the efficiency obtained was between 10 000 and 20 000 theoretical plates, indicating an improvement with respect to the one obtained for the previous design but with a detection limit for luminol (500 amol) lower than the one previously reported. The factors responsible for the decrease in sensitivity include the inefficient light collection as obtained

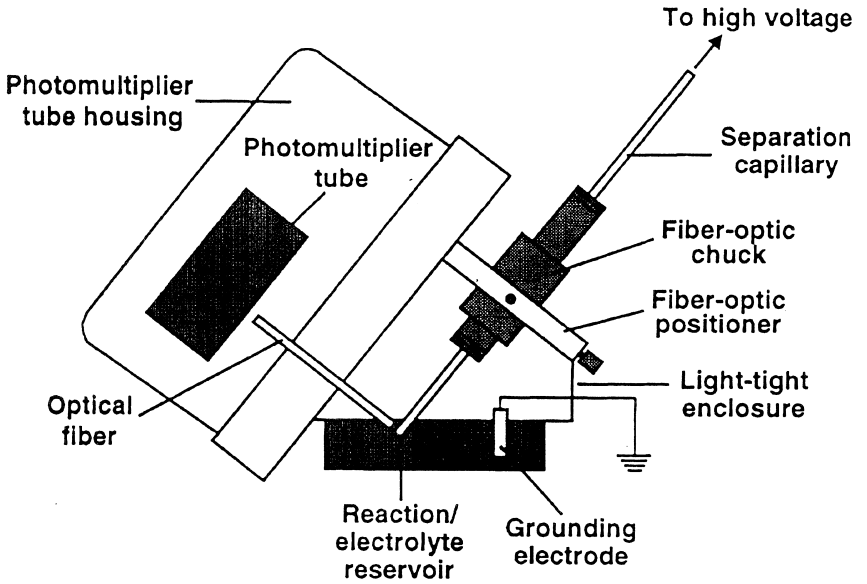

Fig. 12. End-column CL detector for CL (adapted from Ref. [94]).

using the fiber optic in comparison with the application of the parabolic reflector and, also, the absence of cooling of the PMT. Although this end-column detector produces band broadening as a result of the mixing at the column outlet, and in spite of the slow CL reaction kinetics and a large detection zone which provides relatively low numbers of theoretical plates, the simplicity and the capability to obtain detection limits in the nanomolar range make the set-up suitable for routine work. Another drawback of this device could be the possibility of contamination via the outlet buffer reservoir, although this contamination would be very little, considering the volumes implied in the CE separation. Replacing the buffer reservoir frequently could solve this problem.

Based on the reaction of luminol and hydrogen peroxide, the detection by electrogenerated CL (ECL) was also applied in CE [95]. In this detection technique the production of light is followed by an oxidation or reduction reaction at an electrode which serves the purpose of the catalyst used in luminol-based CL detection, offering the advantage to generate luminescence in a defined position of the electrode surface. This means of photon detection emitted from ECL provides an extreme sensitivity, which implies a close control of the placement of the electrode due to the confinement of generation of CL to a specific area as defined by the position of the electrode. Once luminol is separated using a conventional apparatus, it electrophoretically migrates into the detection cell containing hydrogen peroxide. A microelectrode is positioned immediately outside the bore of a fused-silica capillary and when a potential is applied, the light produced upon electrochemical oxidation of luminol and hydrogen peroxide is generated at the microelectrode. This small size of electrode employed allows an easy alignment with the separation capillary and an optimum isolation of ECL generated in a reduced area. Subsequently, two optical fibers positioned 180 degrees apart collect the generated light, which is detected at the PMT. In this way, the postcolumn device for addition of a reactant in the CL reaction, as is the case with the CL detectors previously proposed, can be suppressed. The efficacy of this methodology was proved in the analysis of amines derivatized with ABEI coupled to *N*,*N*-disuccinimidylcarbonate (DSC), used as condensing reagent. Labeling of amines is carried out in two steps: the ABEI-DSC intermediate is firstly prepared and then, amines react with ABEI-DSC using triethylamine as catalyst (Fig. 13). Detection limits of 2.0 and 0.96 fmol for *n*-octylamine and *n*-propylamine were provided, respectively. Also, ABEI-DSC was used successfully to label the tripeptide Val–Tyr–Val and, applying micellar electrokinetic capillary chromatography (MEKC) with ECL detection, the separation of labeled amines was achieved. The main advantages of this ECL detection are the highly enhanced sensitivity by reducing interferences from solution impurities due to replacement of the catalyst, added in the luminol reaction, by the electrode and the elimination of complicated post-column reactors needed when CL detection is combined with CE.

Most of the CE-CL detectors reported have involved variations of a postcapillary reactor to mix the reagents. The reactor requires the insertion of the separation capillary into the reaction/detection capillary. These procedures are manually intensive and it is difficult to reproducibly control reagent concentrations at the reactor. In order to overcome these problems, a novel compact CL detection cell, made of PTFE was recently designed for CE [96]. This detection cell is equipped with an optical fiber, a fused-silica capillary and a grounding electrode and it could be easily combined with CE equipment without any complex construction. The CL light generated at the capillary outlet was transported by the optical fiber to a PMT. The luminol CL system was adapted for the use of this cell, carrying out detailed optimization of the concentration of hydrogen peroxide and catalyst, providing a detection limit for luminol of 5×10^{-10} *M*, that is the most sensitive result reported up to date. Also a mixture of glycine, glycylglycine and glycylglycylglycine, which was labeled with ILITC, was sensitively detected and baseline-separated.

The same group has developed a simpler and more convenient batch-type cell without an optical fiber for CE, to which only a fused-silica capillary and a grounding electrode were inserted [97–99]. The cell also works as an outlet reservoir including the migration buffer and it is placed just in front of a photosensor module that captures directly the light generated at the capillary (Fig. 14). Luminol, ILITC-labeled compounds, H_2O_2 and the catalyst are

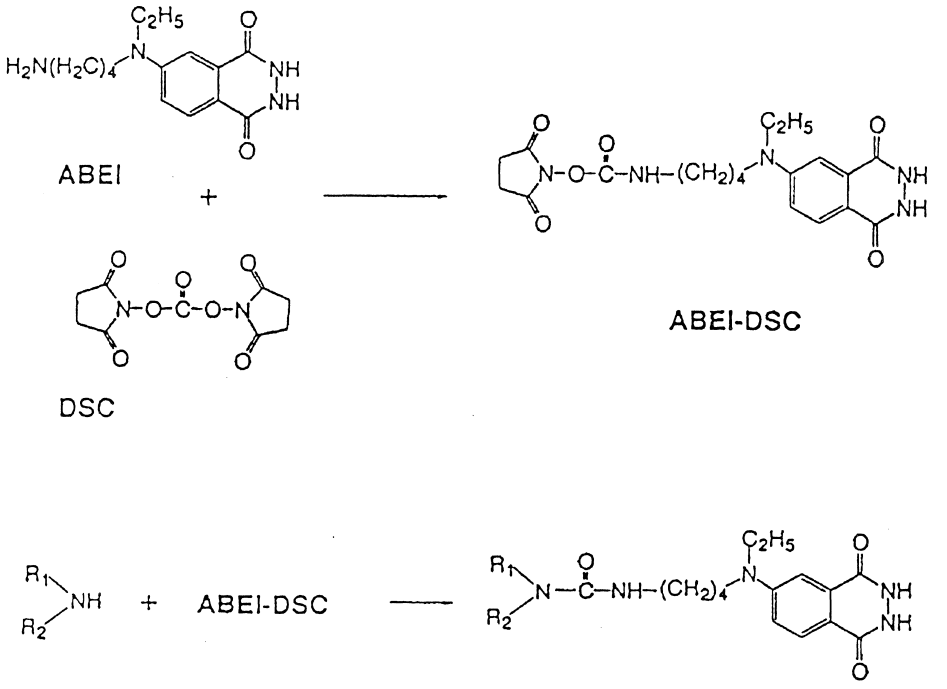

Fig. 13. Labeling reaction of primary and secondary amines with ABEI.

dissolved in a 10-m*M* phosphate buffer that is used as electrophoretic buffer. The catalyst is added to the inlet reservoir and H_2O_2 to the outlet reservoir (detection cell). The sample migrates in the migration solution toward the CL detection cell and is mixed with reagents generating the CL emission which is captured by the detector. Recently, peak shape and apparent theoretical plate numbers have been examined as a function of sample injection times by using this batch-type detection cell, showing that the injection time considerably influences the peak shape as well as the sensitivity in the CL detection cell for CE [100].

2.3.2. Reactions for amino acids, neurotransmitters, heme proteins and metal-porphyrins

Another detection mode, commonly used in LC and in FIA, and recently adapted to CE separations is the indirect detection, based on the detection of a non-chemiluminescently-active analyte that produces interference or suppression of a given CL reaction, the analyte being detected indirectly as an inverted peak, where the CL intensity decreases from a normally high background level. Liao et al. [101,102] demonstrated for the first time the feasibility of this indirect CL detection technique in CE. Their contribution shows the utility of the Cu(II)-catalysed luminol CL system for the determination of five amino acids without previous pre- or post-column derivatization, allowing the detection of a wide range of biomolecules able to strongly and rapidly complex with Cu(II), as amines, catechol, catecholamines and proteins. To carry out the separation and detection, the interface used is slightly modified with respect to the one proposed by Dadoo et al. [92]. Cu(II) catalyses the luminol CL reaction, the CL emission intensity being proportional to the concentration of free Cu(II). In the presence of amino acids, the catalytic activity of free Cu(II) is decreased due to the post-capillary formation of Cu(II)-amino acid complexes, and the CL intensity is considerably reduced. This new detection system in CE is simpler than direct CL detection and reaches higher sensitivities, allowing detection limits for five amino acids, arginine, leucine, serine, cysteine and

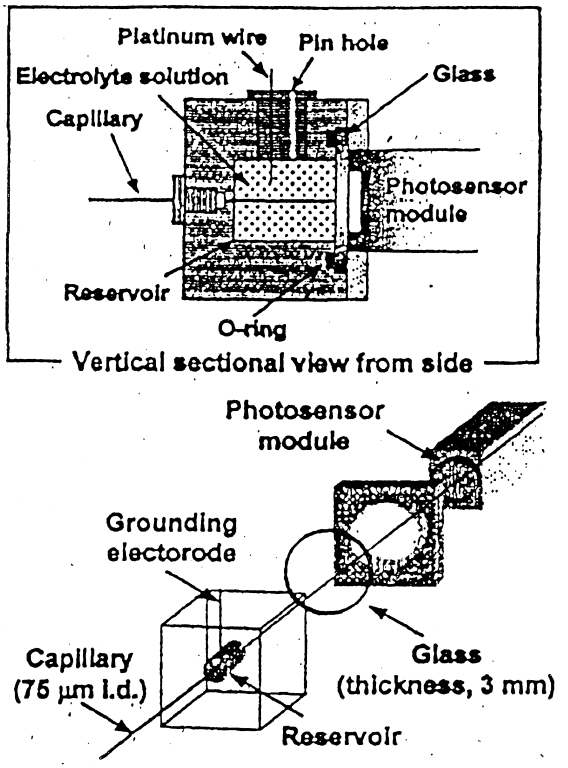

Fig. 14. Schematic diagram of the CL detection cell (from Ref. [97], with permission).

aspartic acid, in the range between 100 and 400 fmol.

Zhang et al. have firstly used on-line indirect detection for the analysis of catecholamines (CAS) and catechol (CAT) [103]. In this case, the high and constant CL background is obtained by the CL reaction of luminol enhanced by Co(II). The analytes complex with Co (II) and reduce the free Co(II) concentration, and thus the CL intensity decreases. The authors propose a new mixing mode of the analytes with the CL reagent in which luminol is used as a component of the electrophoretic carrier; H_2O_2 and Co(II) are introduced post-capillary. In this way, luminol, H_2O_2 and Co(II) meet just at the detection window simultaneously. Against the mixing mode previously reported by Liao et al. [101], who used luminol and H_2O_2 as electrophoretic carrier and catalyst solution of a carbonate buffer containing copper sulfate, the formation of bubbles produced by H_2O_2 in the presence of base is impeded, which prevent the electrophoretic current and make CL background unsteady, as well as causing an increase in the noise and a decrease in the separation efficiency. Sodium dodecyl sulfate was used in the separation of CAT, epinephrine, norepinephrine and dopamine, providing detection limits of 87, 51, 22 and 38 fmol, respectively. Six amino acids such as Arg, Hyp, Lys, His, Glu and Asp were detected, yielding better limits of detection than the ones reported by Liao et al. in their first study.

Tsukagoshi's group developed a new CE apparatus with on-line CL detection using the luminol–H_2O_2 system for analyzing heme proteins [104]. It was found that iron (III), sulfate, hematin as an iron (III) porphyrin complex, and various heme proteins migrated and could be detected. They used the same three-way joint for mixing a CL reagent solution with an eluate from the capillary and the cell structure for detecting a low CL signal as previously described [82]. However, treatment of the CL solution (luminol+H_2O_2) was considered due to the drastic change of the CL intensity of the solution upon standing. In this case, the CL intensity quickly decreased and became about one-tenth of that of the initial solution within 1 h; an almost constant CL intensity was observed after about 12 h. For this reason, a CL reagent solution after being left for more than one night was used in this study because a fresh CL reagent solution provided high baseline noise levels and hence no reproducible results.

Though CE has shown excellent performances so far for the separation of many compounds having various molecular masses, it is not always satisfactory for the separation of biopolymers, such as proteins, glycoproteins and lipoproteins due to the adsorption of proteins onto the inner wall of a capillary tube (and the low sensitivity in the detection of protein), the absorption phenomenon being promoted due to the high concentration of the protein sample. However, Tsukagoshi's group obtained good resolved peaks for a mixture of proteins, in spite of turbulent mixing of the analyte with a CL solution at the end of the separation capillary. These good results could be explained as follows: (i) all protein samples were migrated at pH 10, which is higher

than or equal to the isoelectric point values of the protein, reducing in this way the interaction between protein surfaces and the negative charges of silanol groups on the inner wall of the capillary; and (ii) the concentration of protein samples was considerably reduced. This CE-CL method was about 10^4-times as sensitive as the conventional CE-absorption detection system for the detection of hemoglobin.

The measurement of metal-porphyrins by on-line CL detection has been successfully implemented using an improved apparatus developed by Cheng's group [105]. Some synthesized metal-porphyrins complexes catalyse the reaction between luminol and hydrogen peroxide and their low relative molecular mass facilitates their coupling to proteins and nucleic acids. They could be potentially implemented as labeling reagent in CL immunoassay with CE, being an inexpensive, sensitive and reproducible alternative to the use of natural substances as catalyst in CL immunoassay using luminol. In the proposed CL device, the grounding electrode of the power has been inserted in the four-way joint, not in the eluate reservoir and the reaction capillary with greater inner diameter has been set at a slope down, as can be seen in Fig. 15. This configuration in which the reaction capillary is not a part of the electrophoretic circuit permits that both the bubbles and convergence of CL reagents with the electrophoresis electrolyte in the reaction capillary could not influence the current stability, allowing adjustment of the conditions for the CL reaction without affecting the CE process, yielding much sharper and more symmetric peaks with higher detection sensitivity.

2.4. Chemiluminescence detection based on tris(2,2′-bipyridine)ruthenium(II) ($Ru(bpy)_3^{2+}$)

$Ru(bpy)_3^{2+}$ (bpy=bipyridine) 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, such as:

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

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

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

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. Solvents usually applied in CL determinations based on these reactions include acetonitrile–water, methanol–water and acetone–water [106]. $Ru(bpy)_3^{2+}$ is the most studied and exploited inorganic compound used in ECL and its analytical usefulness as an ECL label has been summarized in extensive reviews [107,108]. $Ru(bpy)_3^{2+}$ is the stable species in the solution and the reactive species, $Ru(bpy)_3^{3+}$, can be

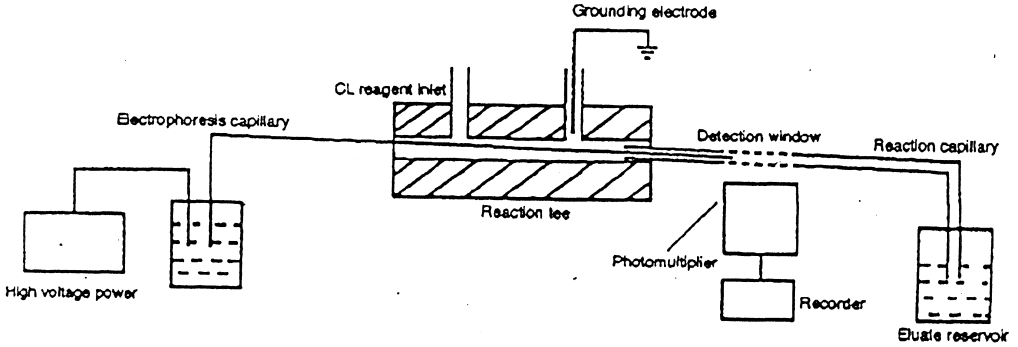

Fig. 15. Schematic diagram of an improved post-column CL detector for CE (from Ref. [105], with permission).

generated from $Ru(bpy)_3^{2+}$ on the electrode surface by oxidation at about +1.3 V.

Adding $Ru(bpy)_3^{2+}$ to the electrolyte and using and end-column electrode to convert the $Ru(bpy)_3^{2+}$ into the active $Ru(bpy)_3^{3+}$ form allow a simple and sensitive ECL detection mode. The reaction lends itself to electrochemical control due to the electrochemically-induced interconversion of the key oxidation states:

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

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

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

In this CL system, the reagent is regenerated and it can be recycled and derivatization is not required for many classes of compounds. The oxidant, $Ru(bpy)_3^{3+}$, generally reacts best with tertiary, next secondary and primary alkyl amines, allowing the determination of amino acids, proteins, antibiotics having a tertiary amine, such as erythromycin and clindamycin, NADH, amongst others that can participate in this reaction, which is compatible with FIA and HPLC solvent systems. ECL employing $Ru(bpy)_3^{2+}$ offers an attractive detection scheme for CE because of the solubility and stability of the reagents in aqueous media [109] and the high efficiency over a wide pH range, making it compatible with most buffer systems commonly used in CE.

2.4.1. Reaction for β-blockers

In 1997, Nieman's group used for the first time this system in CE introducing inactive $Ru(bpy)_3^{2+}$ into the electrophoretic buffer and generating electrochemically, on-line, the active $Ru(bpy)_3^{3+}$ species just inside the outlet of the capillary [110]. This was done by applying +1.25 V vs. Ag/AgCl to a Pt wire inserted 3 mm into the outlet of the capillary. In this way, analyte bands exiting the separation capillary react with the $Ru(bpy)_3^{3+}$ and produce light. The outlet is placed within a parabolic mirror that directs the emitted photons to a photon counting PMT. Concentration of $Ru(bpy)_3^{2+}$ in the electrolyte is an important parameter to be optimized because of its large impact on the background CL signal and dynamic range. The performance of the system was demonstrated using a series of β-blockers, a class of amine compounds that block the effect of norepinephrine on the adrenergic receptors. Detection limit for oxprenolol was 0.6 μg ml^{-1} with a separation efficiency of 15 000 plates.

2.4.2. Reactions for non-labeled amino acids, peptides and proteins

Another simple apparatus for post-column ECL detection [111] consists of a working electrode (either a fine platinum wire or carbon fiber), which is very close to the end of the CE capillary. The end of the capillary and working electrode are placed within a reservoir holding a few millimeters of buffer, forming the ECL cell, which is housed within a light-tight enclosure. Other conventional reference and counterelectrodes are also placed within the reservoir. A conductive joint is employed to isolate the high voltage used to carry out the CE separation from the potential need to drive the ECL due to the electric currents generated in capillaries that greatly affect the faradaic currents at microelectrodes. This is accomplished by constructing a porous joint in the separation capillary and encapsulating it in a buffer-filled reservoir that is connected to ground. The high voltage is then between the sample injection end of the capillary and this ground buffer reservoir. Bulk flow of the liquid then carries the separated analytes onto the ECL cell, which houses the capillary, ECL reagent, Pt working, Pt auxiliary and Ag/AgCl reference electrodes, fabricated from a Nalgene bottle cap and filled with 1 n*M* $Ru(bpy)_3^{2+}$ and Na_2HPO_4 (pH 9). The ECL signal is detected by a PMT positioned directly above the working electrode. The device is shown in Fig. 16.

In this case, post-column and pre-column reagent addition were compared, showing that although the addition of the CL reagent to the running buffer and on-column detection ideally would lead to higher efficiency separations than the post-column arrangement, differences in migration between analytes and $Ru(bpy)_3^{2+}$ lead to zone broadening. Moreover the absorption of $Ru(bpy)_3^{2+}$ onto the silica walls of the separation capillary with an equilibration time of several hours and the perturbation in equilibrium produced when the capillary is flushed with dilute NaOH, water and finally buffer, hinder the use of on-column detection, suggesting the post-column

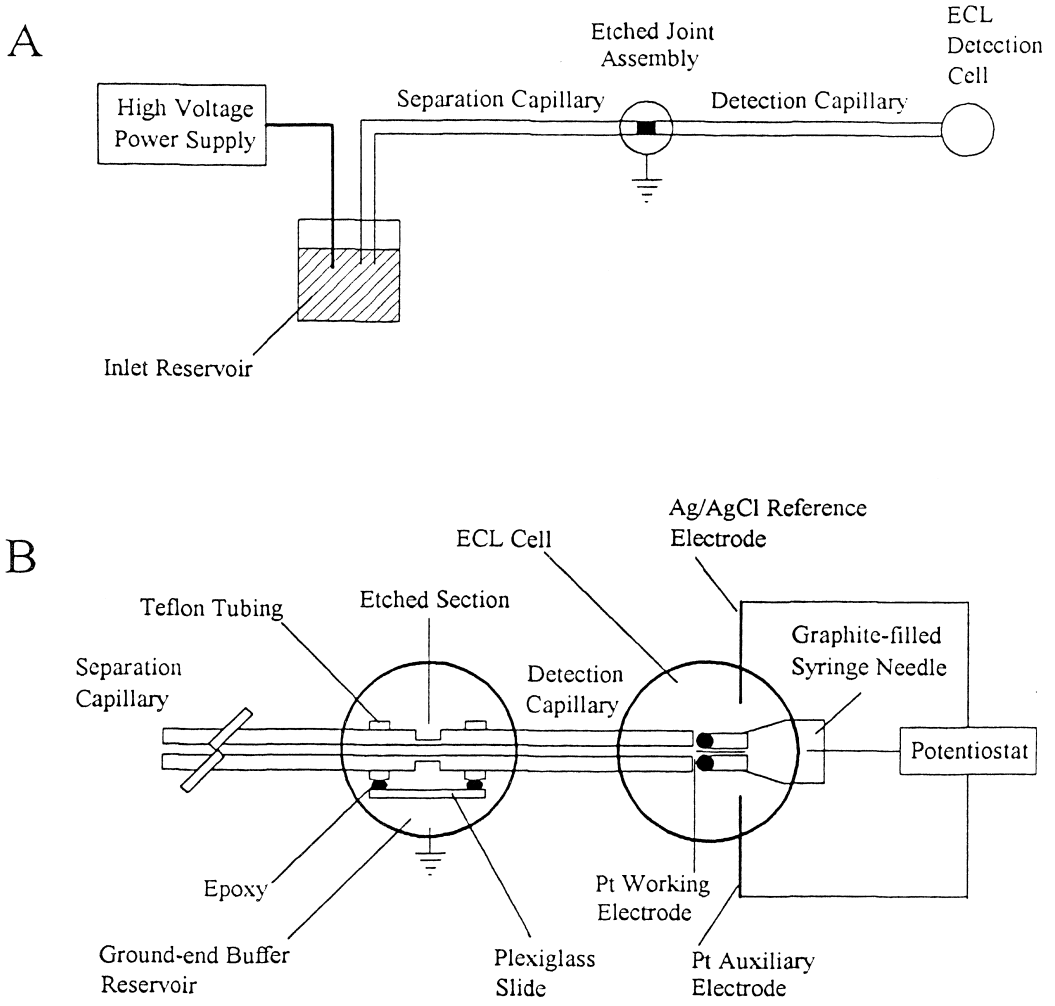

Fig. 16. Schematic diagram of post-capillary ECL detection in CE. (A) overview of the apparatus; (B) detailed view of the etched joint and ECL detection cell. The PMT is positioned directly over the working electrode and the entire apparatus placed inside a light-tight housing (from Ref. [112], with permission).

addition of CL reagent to overcome these problems. In this sense, a post-capillary reservoir of $Ru(bpy)_3^{2+}$ has been used for in situ generation of $Ru(bpy)_3^{3+}$ [112]. $Ru(bpy)_3^{2+}$ is added post-capillary as a small reservoir ($\approx$100 μl) at the interface of the separation capillary and the detection electrochemical cell and is then converted to $Ru(bpy)_3^{3+}$ at a carbon microfiber for reaction with eluting amines or amino acids. This detection approach has been found to provide a reproducible electrophoretic separation compatible with the nanoliter detection volumes required to maintain CE separation efficiencies, being the major advantage that the electrophoresis will not be inhibited by the presence of $Ru(bpy)_3^{2+}$ in the running buffer. This system was applied to the separation and CL detection of the amino acids triethylamine (TEA), proline, valine and serine at pH 9.5. Detection limits range from approximately 100 n*M* for TEA and proline, the most efficient luminescent species, to approximately 100 μ*M* for serine.

In order to eliminate the problems associated with the reservoir-based design, a new in situ generated $Ru(bpy)_3^{3+}$ CL cell was proposed [113]. In this design, $Ru(bpy)_3^{2+}$ is continuously delivered to the

cell and $Ru(bpy)_3^{3+}$ is then generated at the interface of the separation capillary and the working electrode. Electrochemical control of the production of $Ru(bpy)_3^{3+}$ at the distal end of the separation capillary without interference from the CE current is provided and finally the ECL process is coupled to an optical system for monitoring light emission. The authors used a CE homemade apparatus to perform electrophoretic separation and electrokinetic injections. A 2–3-mm detection window at the end of the separation capillary was formed by thermally removing the polyimide coating and this separation capillary was inserted into the reaction tube. $Ru(bpy)_3^{3+}$ reagent was delivered by a syringe pump to the reaction cell at a flow rate of 10 μl/min. For precise electrochemical control of the conversion of $Ru(bpy)_3^{2+}$ to $Ru(bpy)_3^{3+}$, the CE current is isolated from the detection capillary using an on-column fracture. The efficacy of this approach was shown in the detection of a mixture of the amino acids TEA, proline, lysine, methionine, phenylalanine and valine, showing limits of detection of 4, 10 and of 9 femtomoles for Pro, Val, and Phe, respectively. Also the system has been evaluated as a separation and detection methodology for the compositional analysis of peptides and proteins, avoiding the complexity of derivatization procedures. These must be previously hydrolyzed using a commercially available instrument and then the resulting amino acids are identified based on their CE migration time and their relative luminescence, which depend on the electron-withdrawing character of the *R*-group attached to the α-carbon. Two tripeptides were separated and detected, showing detection limits of 2.5 pmol for the peptide Gly–Phe–Ala and 80 fmol for Val–Pro–Leu, with an analysis time of less than 25 min [114]. This method, although not fully optimized, is less complex that other methods which require derivatization, and shows as advantages short analysis times and high sensitivity.

2.5. Chemiluminescence detection based on direct oxidations

Strong oxidants, such as MnO_4^- (in acidic or alkaline medium), ClO^-, Ce(IV), H_2O_2, IO_4^-, Br_2, *N*-bromosuccinimide, and reductants have been tested under different chemical conditions in order to produce a CL emission from different analytes. 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 exhibit CL. Since then, a wide range of such reactions has been reported mainly in the field of drug analysis by FIA [56,115].

2.5.1. Reaction for neurotransmitters

Oxidation of catecholamines by potassium permanganate in an acidic medium is known to produce CL, developing for the first time an off-column CL detection device in CE for this application in CE [116]. A porous polymer joint is easily constructed by fracturing the capillary followed by covering the fracture with a thin layer of cellulose acetate membrane. This porous joint, rather than the end of the capillary, was submerged in a buffer reservoir along the cathode (Fig. 17). The applied voltage was dropped across the capillary prior to the porous joint and the resulting EOF acted as a pump to push the analytes through the short section of capillary after the joint. The analytes were mixed with permanganate and emitted CL in a field-free region at the column outlet. The feasibility of this off-column CL detection mode was demonstrated in the CE of serotonin, catecholamines and catechol. This detection mode is useful in case where the CL reagent or catalyst added at the column end must not stream back into the separation capillary and degradation and decomposition of analytes may occur inside the capillary before they reach the column end. However the only limitation is that at least some EOF is needed to push the analytes past the grounded joint.

2.6. Chemiluminescence detection based on bioluminescent reactions

The most widely applied bioluminescence (BL) analytical 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*).

Two steps can be considered in the highly efficient firefly luciferin–luciferase reaction:

$$\text{Luciferin} + \text{ATP} \rightarrow \text{Adenyl-luciferin} + PP_i$$

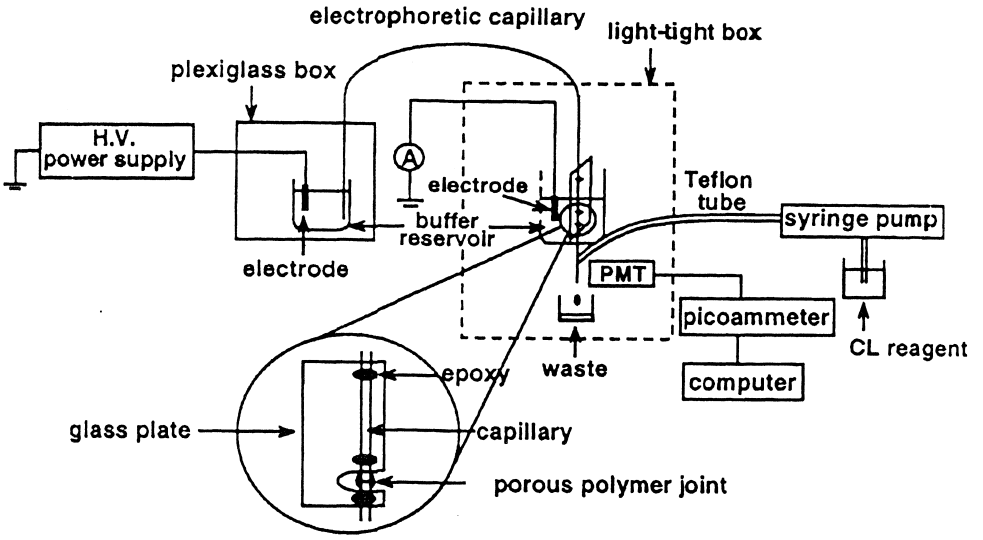

Fig. 17. Schematic diagram of the CE with off-column CL detection system (from Ref. [116], with permission).

$$\text{Adenyl-luciferin} + O_2 \rightarrow \text{Oxyluciferin} + \text{AMP} + CO_2 + \text{light}$$

The hydrophobic enzyme luciferase catalyses the air oxidation of luciferin in the presence of adenosine triphosphate (ATP), which is consumed as a substrate to yield light emission at 562 nm [117]. The presence of Mg(II) is necessary for the luciferase activity to be triggered. 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 with diverse surfaces.

Using the proposed device, Dadoo et al. [94] adapted this BL reaction to determine ATP. A selective and sensitive determination is achieved because the use of CE as a separation technique minimizes the effect of several interfering substances such as some anions (e.g., SCN^-, I^-), which inhibit the reaction decreasing the luminescence emission, and even some nucleotides generating light in this reaction but with lower intensity. A detection limit of 5 n*M*, approximately 3 orders of magnitude lower than using UV-detection, was obtained.

3. Miniaturized systems for CL detection in CE

Recent trends are focused on the use of micromachining techniques (photolithography and chemical etching) in the fabrication of a complex manifold of flow channels on a microchip, capable of sample injection, pretreatment and separation [118,119]. These super-miniaturized systems are being considered to overcome the problem of unsatisfactory detection limits characteristic for standard CE setups. The nature of electrokinetically driven systems in CE makes it suitable for integration on a planar device, giving highly efficient separations in short capillaries together with a considerable reduction of analysis times and not requiring high-pressure pumps or gas supply. As electroosmotic flow velocity and electrophoretic migration only depend on the strength of the applied field, the separation efficiency is exclusively related to the voltage installed across the separation capillary and not on its length. Several materials as planar glass, fused-silica wafers and quartz have been used to construct devices with different geometry and sizes. Since the first contribution by Manz's group in 1991 [120], introducing the

concept of CE-μTAS system (Capillary Electrophoresis micro-Total Analysis System) further advances have been achieved, reducing considerably the microchip size and extending the field of applications [121–123]. LIF has been the most common detection mode used in miniaturized CE system, however, due to the characteristics of CL, it could be one of the most promising detection methods in CE-μTAS because it does not require any light source for excitation. Some applications have recently been developed in this field [124–126].

CL detection based on the horseradish peroxidase (HRP)-catalysed reaction of luminol with peroxide was the first contribution of a post-separation CL detection scheme for microchip-based CE [127]. In this contribution, an integrated injector, separator and post-separation reactor was fabricated on a planar glass wafer. Post-column derivatization manifold was used as a tool for mixing CL reagent and the studied analytes. In this case, the fluorescein conjugate of HPR (HPR-Fl) was used as a sample for the optimization of the CL detector response. The detection limit obtained, 7–35 n*M* for HRP-Fl for on-chip CL are about a 50–100-fold lower than could be achieved by absorbance detection [128]. Using this microchip, separation and CL detection of the products of an immunological reaction of a fragment of the HRP conjugate of goat anti-mouse immunoglobulin G (IgG) with mouse IgG was performed.

Dansyl amino acids (lysine and glycine) have been detected using a proposed on-line quartz microchip CE-CL device by means of the PO-CL reaction [129]. In this case, bis [(2-(3,6,9-trioxadecanyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) was used as CL reagent with H_2O_2 in the microchip, which consists of a bottom and cover plate, two main channels, four reservoirs, a cross-shaped injector and the absence of the mixing junction for CL reaction. After the channels were filled with migration elec-

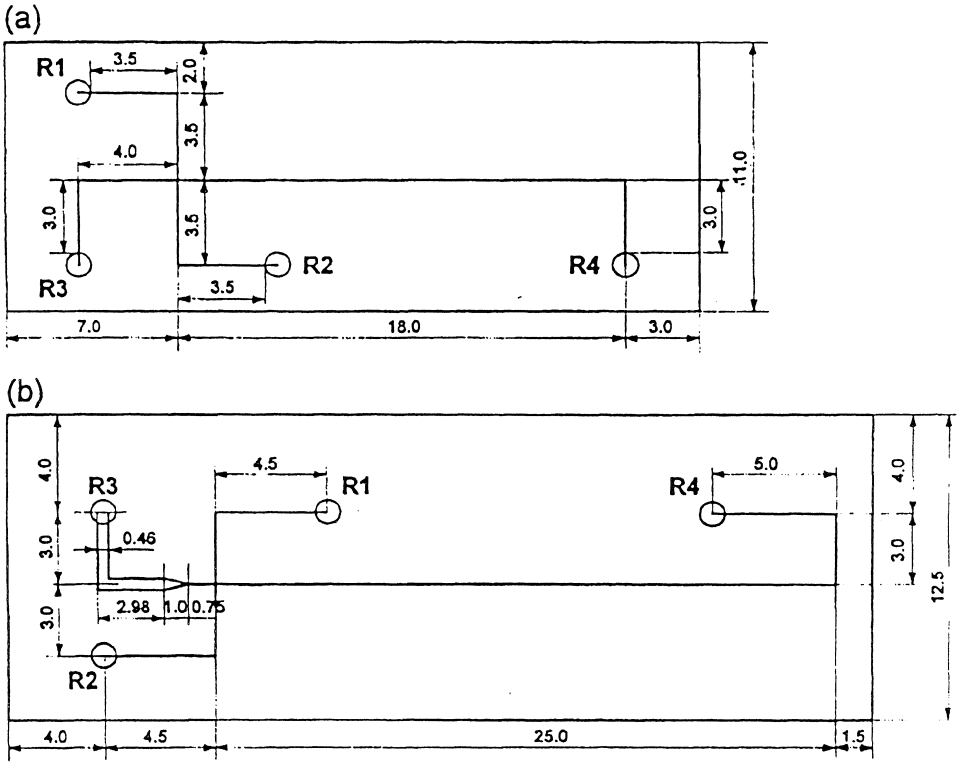

Fig. 18. Schematic layout and dimensions (in millimeters) of microchips (a) and (b). R1–R4 indicate reservoirs. Solutions distribution to the respective reservoirs: R1, sample; R2, buffer; R3, buffer; R4, CL reagent (from Ref. [129], with permission).

trolyte using a disposable syringe, the sample was placed in R1, buffer in R2 and R3, and CL reagent in R4, respectively. Platinum wires as electrodes were inserted into these reservoirs (see Fig. 18).

A program was used to control the power supplies and relay device used with the aim to control the sample load and separation, as shown schematically in Fig. 19. The sample (Dns–Lys and Dns–Gly), which was electrokinetically delivered and migrated toward R4, came into contact with the CL reagent to produce visible emission at the interface between the separation channel and CL reagent-containing reservoir, which was collected by a photosensor module located under R4 and connected to an integrator to produce electropherograms. The present system has advantages as concerns rapid separation times, the small and accurate sample injection method, the simplification and miniaturization. However, more studies are needed with the aim to improve the concentration sensitivity by some useful techniques, such as on-line sample stacking.

Recently, miniaturization of batch- and flow-type CL detector for CE have been proposed using glass and PTFE tubes as detection cells, respectively, and light-tight boxes which includes these cells together with a photosensor module [130]. In both cases, aminoacids labeled with dansyl chloride were separated and detected with good repeatability, showing detection limits of 0.43 and 1.3 fmol for Dansyl–Trp.

4. Final remarks

The advantages offered by the CL as sensitive detection system offer interesting perspectives with coupling with CE in the analysis of biomolecules, increasing the advantages presented by both methodologies. Nevertheless, the coupling CE-CL is still in development, being the improvement and development of post-capillary reactors and CL detectors that can be coupled to the capillary of separation, the main aims, since commercial devices are not yet available. The main drawback of the CE-CL cou-

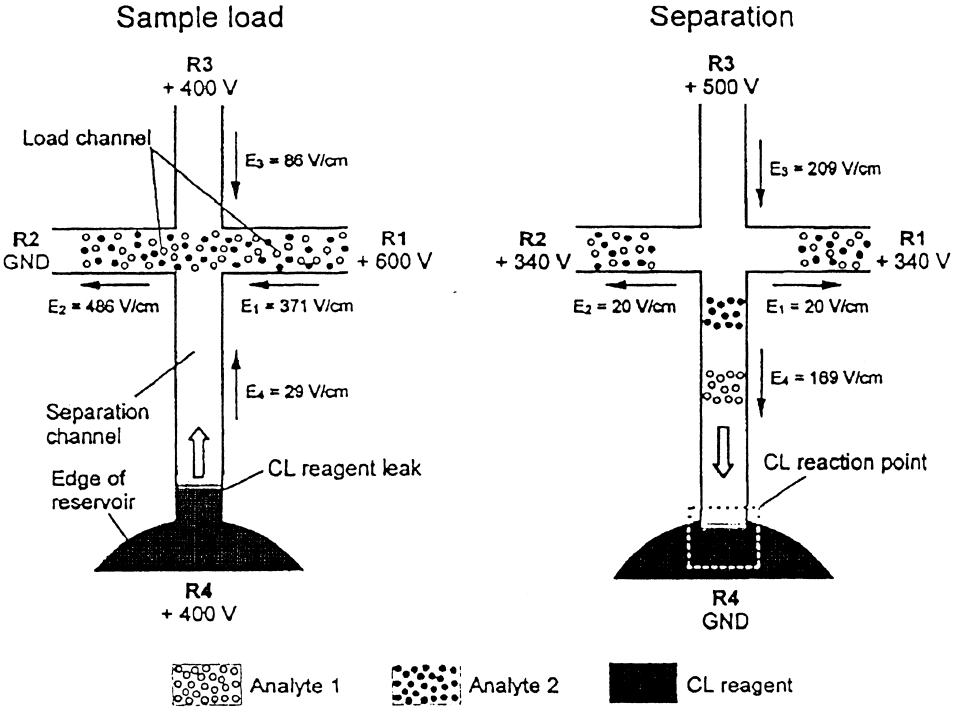

Fig. 19. Schematic layout of CE procedure on the microchip for CL detection, with the potentials and electric fields. Arrows depict direction of flow (from Ref. [129], with permission).

pling is the fact that, in contrast with the chromatographic systems where the detector is placed in the exit of the column, here it is necessary to incorporate the detector in the capillary of separation itself, together with the reactors where the CL reaction takes place. Further research is needed in this sense.

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