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Review

Capillary electrophoresis coupled to biosensor detection

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Abstract

The present review highlights some modern aspects of biosensor revelation, a detection method which has already found a large number of applications in healthcare, food industry and environmental analysis. First, the concept of bio-recognition, which is at the heart of biosensor technology, is discussed, with emphasis on host–guest-like recognition mechanisms. This detection device has been successfully coupled, in its first applications, to chromatographic columns, which allow a high resolution of complex mixtures of analytes prior to interaction with the biosensing unit. The properties of the transducing elements, which should generate a signal (e.g., electrochemical, thermal, acoustic, optical) of proper intensity and of relative fast rise, are additionally evaluated and discussed. The review then focuses on potential applications of biosensing units in capillary electrophoresis (CE) devices. CE appears to be an excellent separation methodology to be coupled to biosensor detection, since it is based on miniaturized electrophoretic chambers, fast analysis times, complete automation in sample handling and data treatment and requires extremely small sample volumes. Although only a few applications of CE-based biosensors have been described up to the present, it is anticipated that this hyphenated technique could have a considerable expansion in the coming years.

Keywords: Reviews; Capillary electrophoresis–biosensors detection; Biosensors; Transducers

Contents

1. Introduction

Over the last two decades the analytical potential of biosensors has been widely recognised. Clear evidence of the importance of biosensors comes from the interest of the scientific community expressed in

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terms of number of patents, reviews and publications. An even more significant proof comes from the market, where commercial biosensors have been successfully sold for applications in healthcare (e.g. near-patient monitoring of diabetes and real-time measurements in drug development), the food industry (e.g. sensors for sugars and amino acids including on-line systems), or for environmental applications (e.g. BOD measurement).

Biosensors are analytical systems comprising an immobilised biological sensing element and a physical transducer. The nature of the transducer (electrochemical, thermal, piezoelectric, optical or magnetic) has commonly been used as a way of classifying the various devices. A wide range of different biological sensors have been developed, ranging from classical amperometric electrodes with immobilised oxidoreductases [1] to optical sensors, based on fluorescent-labelled proteins, designed to sense and report data concerning the dynamic distribution of specific reactions or reaction kinetics [2]. The general trend in the overall development of biosensors is evident: the features required for sensors are high selectivity, high sensitivity, short times of analysis, flexibility, reusability of the devices and, in some cases, remote control.

Since the demand for analysis of complex media is increasing, selective chemical recognition elements have been used for the development of arrays biosensors. These devices are assemblies of linked biosensors enabling a multidimensional analysis of the signals, giving information about the analyte composition. Different techniques could be integrated into a single instrument in different ways. Often a separation tool and a sensor element are brought together, as demonstrated by liquid chromatography equipped with electrochemical detection [3], and, sometimes, additionally coupled with microdialysis sampling [4]. Even more complex assemblies, including the combination of liquid chromatography with a post-column enzymatic reactor, reflect the current trend in designing analytical instrumentation intended for the routine use in neuroscience and pharmacokinetics [5].

Chromatography is far from being unique as a separation technique that can be usefully combined with biosensors; it was logical that efforts should be devoted to the integration of specific biosensor elements with other separation technologies such as capillary electrophoresis (CE). Nowadays CE has become an important tool for the determination of analytes in complex samples and has been successfully applied to the separation of chemically different substances, including ions, drug metabolites, peptides, proteins and DNA [6]. CE allows the use of different working conditions: free solution, capillaries filled with sieving polymers or analyte-micelle complexes. Separations can be performed in aqueous media or in organic solvents, proving that CE is a versatile technique. Relevant features of CE are the high separation selectivity, the low amount of sample required (nl), the short time of analysis, the high efficiency (typically 10^4 plates/cm), versatility, low reagent consumption and full automation. The advantages provided by CE are highly compatible with the main features required by the sensor technology. The resulting associated biosensors could represent a new and interesting tool for the analytical chemist. Therefore the principles, advantages and limitations of CE-based biosensors are here discussed and an overview of the current applications is proposed.

2. Biological sensors

Chemical sensors, as reviewed by Janata et al. [7], are analytically powerful alternatives to conventional technologies that allow the identification and the quantitation of the target molecule. The detection of the target compound, among the various interfering species present in the analyte, is highly specific, since it is based on a host–guest-like recognition mechanism. At the molecular level, the specific chemical interaction between the sensor and the target compound gives rise to a detectable change of some chemical or physical properties of at least one of the molecules involved in the recognition. The presence of an appropriate transducer element permits the chemical event to be translated into a quantifiable output signal. Finally the signal is correlated to the concentration of the analyte. The concept of a biological sensor is shown in Fig. 1; it implies the presence of a biological component, such as enzymes, antibodies, receptors, or their synthetic analogues, devoted to the selective identification of analytes [8–11].

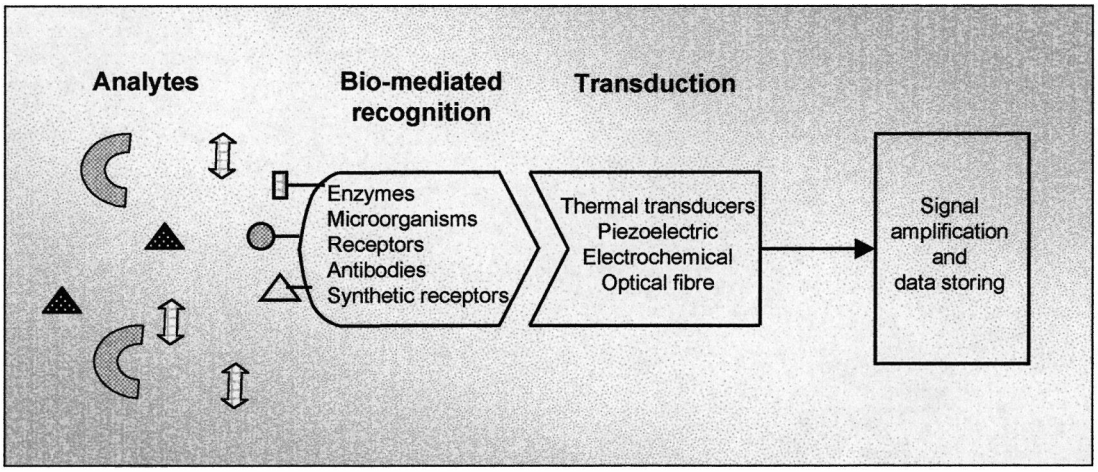

Fig. 1. Schematic diagram of a biological sensor. The various types of analytes will be selectively identified by a biological component (such as an enzyme, antibody, receptor) properly immobilised onto a suitable substrate.

The high potential of biosensor technology versus conventional chemical sensors relies on the wide variety of host–guest mechanisms offered by biological systems. The biological component may either display a catalytic activity, if it belongs to the class of enzymes or micro-organisms (enzyme–substrate recognition event), or it could simply bind the ligand without transformation, if it belongs to the non-catalytic, or affinity class (antigen–antibody, or ligand–receptor interaction). The different recognition in affinity sensors is characterised by different values of dissociation constants. As a result, the choice of the biospecific sensing element is often based on the affinity constant it displays for the target molecule. The recognition episodes could be distinguished into two classes: static or dynamic events. While static recognition is used mainly in immunosensors for monitoring of antigen–antibody complex formation, dynamic recognition is preferably explored in enzyme sensors involving enzyme–substrate interactions. Each recognition event should produce a detectable change of energy, that is subsequently translated into a quantifiable signal by a transducer element.

The possibility of monitoring directly the energy changes associated to each binding event was evaluated by Thomson and Krull [12], who described the most direct cases of biosensor measurements. Unfortunately, the design of such systems was practically impossible due to fundamental limitations in sensitivity, selectivity and response speed set by the thermodynamics of the biosystem. The major problem to overcome, in terms of sensitivity, is the relatively small amount of energy change per binding event. The signal could be increased only if the chemical interaction could modulate the energy derived from an external source. Thus, during the studies aimed at the construction of more efficient biosensors, several kinds of external sources were explored (electrochemical potential, electromagnetic radiation and mechanical motion). These experiments proved to be successful and as a result such external sources of energy are commonly employed in the construction of biosensors.

At present several different types of transducers are used in combinations with biospecific elements, including electrochemical, thermal, acoustic and optical transducers. Table 1 contains a short guide to the principles and common applications of each transduction system. The relative merits and future potential of each transduction system with respect to problems of transition from the laboratory scale to the marketplace are well described in [31]. The optimal transduction element, for a given analyte-biocomponent recognition event, is claimed to be the one able to transform with the highest efficiency the changes arising in the molecular interaction into output signals. High selectivity and efficient trans-

Table 1
Transduction elements

Transducers	Description	Applications and references
Thermal	Small microcalorimeters: biocomponent immobilised near the heat-sensing part	Determination of urea and lactate in whole blood with a miniaturised flow-injection thermal biosensor [13] Semi-continuous monitoring of blood glucose concentration [14] Miniaturised biosensors for the determination of glucose in blood [15]
Acoustic, or piezoelectric	Cut crystals subjected to an alternating electric potential oscillate, generating acoustic waves proportional to the dimensions of the crystals. A selective binding at the surface of the crystal causes mass changes and gives rise to a quantifiable change in the resonance frequency.	Direct, and stable flow injection analysis immunosensor for the detection of African swine fever virus and antibodies [16] Immunosensor for the detection of Salmonella by immobilisation of thiolated antibodies onto the gold surface of piezoelectric quartz crystals [17] Development of an electronic nose by the immobilisation of olfactory receptors onto piezoelectric crystals [18]
Electrochemical transducers	(1) Conductimetric: measure the changes in conductance	An immunoassay technique based on measurement of conductance changes: dosage of human chorionic gonadotropin levels in serum [19]
	(2) Potentiometric: measure the difference of potential between the active electrode and the reference one, under zero current conditions	Development of a disposable urea-sensitive biosensor for the measurement of urea in diluted or undiluted blood and serum samples [20] Direct detection of organophosphorus (OP) neurotoxins by the cryoimmobilisation of recombinant E. coli cells capable of hydrolysing a wide spectrum of OP pesticides and warfare agents [21]
	Direct measure of a natural substrate of the enzymes, or use of a preferential electron-acceptor (mediator molecule)	Miniaturised potentiometric cells with multilayer planar ion-selective sensors for aqueous electrolyte solution [22] Determination of lysine in pharmaceutical samples [23]
	(3) Amperometric: constant potential, measure of the current generated by the oxidation or reduction of an electroactive species.	Phenol biosensor based on a thermostable phenol hydroxylase [24] Initial clinical evaluation of a subcutaneously implanted microsensor-based amperometrically glycemia-monitoring system [25] Bienzyme reactor for glucose and uric acid detection [26]
Optical sensors	The reflection of light beam travelling within an optical fibre generates an electromagnetic evanescent wave, employed to excite fluorescent molecules located at the surface of the waveguide. The energy of the evanescent wave is able to excite: (1) fluorescent molecules (2) a thin metal layer coating the fibre end generating a surface plasmon resonance (SPR) (3) MZI	Rapid analytical detection of toxins [27] Design of a new optochemical sensor based on a gold-adsorbed fluorophore for the detection of nitric oxide [28] Real time analysis of free S protein in human plasma based on surface plasmon resonance [29] Highly selective colorimetric polynucleotide detection method [30]

duction are not the sole features required to a biosensor: sensing devices are judged on the basis of their performance, thus much importance is devoted to the improvement of biological element stability and to the lowering of the detection limits. The stability of the biological sensing element could be increased by using chemical synthetic analogues of receptors or enzymes involved in the recognition [32–34]. Lowering the detection limit could be achieved by using fluorescence detection, which allows the direct analysis of the sample and reduces the need for sample pretreatment. The great versatility of biosensor technology allows the construction of miniaturised devices for the monitoring of clinical parameters [35] with the perspective of the implantation of micro-biosensors in patients, e.g. the implantation of a biosensor coordinating the functions of an artificial pancreas [36]. The clinical applications of biosensors have led to the construction of devices displaying high patient compatibility. Many efforts were directed to the development of novel materials, like phospholipid-based polymer membrane [37] or to the chemical modification of in-use materials, e.g. modification of pacemaker electrodes, for enhancing biocompatibility and to improve cell adhesion properties [38]. The technological and

scientific progresses in the field have greatly enhanced the perspectives of improving biosensors and shortening the time by which biosensors will be routinely used in environmental, industrial and clinical analysis.

3. Capillary electrophoresis

CE was born of the marriage of the powerful separation mechanisms of electrophoresis with the instrumentation and the automation concepts of chromatography [39,40]. CE offers some unique characteristics and advantages, as shown in Fig. 2. Separation is governed by the difference in electrophoretic mobilities of the solutes. High selectivity and efficiency are commonly achieved, in accordance with the charge/mass ratio of each analyte, and strictly dependent on the applied field strength, on the pH, on the conductivity of the separation buffer and on the column features. Time of analysis is short, allowing the run to be completed in a few minutes. Analysis requires only a few nl of sample. CE could work under different separation principles (see Table 2). The choice of one particular methodology is dependent on the nature and composition of the sample to be analysed. CE is widely employed in analytical applications. It has been successfully adopted for the screening of herbicides, pesticides and pollutants in environmental analysis [53–55]. Clinical chemistry and forensic applications of CE methodologies are well known [56,57]. Recently CE has been introduced in food industry for performing quality controls of beverages [58], for checking cheese fabrication against adulteration [59] and for the identification of cereal cultivars [60,61]. Such applications ask for the separation and for the identification of samples made by a high number of components. While separation in CE has proven to be excellent, the identification still suffers from the limitation imposed by the system of detection [usually CE instruments are equipped with UV–Vis absorption or laser-induced fluorescence (LIF) detectors]. A possible way to overcome the problem is to perform a multivariate statistic analysis of the collected data [62]. The statistical analysis introduces a rational approach to peak assignment, allowing the identification of more than 40 different analytes within the same run. Nevertheless, only the coupling between CE separation and sensitive and specific detection is able to impose a dramatic improvement in the analyte identification, thus much attention has been directed to CE equipped with mass spectrometry (CE–MS) [63] and a growing interest is

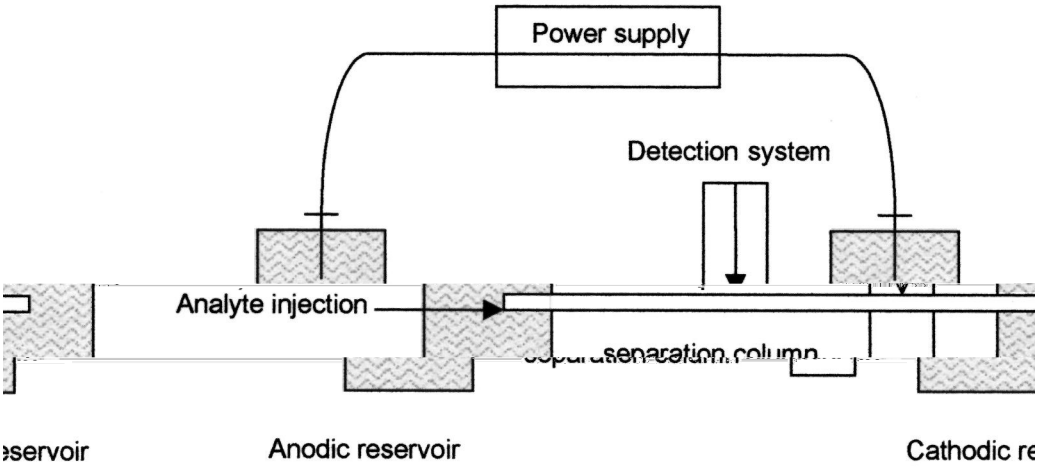

Fig. 2. Schematic diagram of a CE unit. The separation column consists of a fused-silica capillary of 20–100 μm typical inner diameter and 20–100 cm typical length. Runs are usually performed at 100–1000 V/cm, depending on capillary length, diameter and electrical conductivity of the background electrolyte. Standard detectors are based on UV–Vis and on laser-induced fluorescence.

Table 2
CE: methodologies and applications

Methodology	Applications and references
	Protein folding in isoelectric buffers [41]
Capillary zone electrophoresis (CZE)	Detection of mutations in α- and β-human globin chains [42]
	Variety identification in maize lines [60]
Micellar electrokinetic chromatography (MECK)	Determination of biogenic amines [43]
	Separation and determination of nalidixic acid and its metabolites in serum and urine [44]
Capillary electrochromatography (CEC)	Analysis of free fatty acids and fatty acid phenacyl esters in vegetable oils and margarine [45]
	Use of a macrocyclic antibiotic chiral stationary phase for enantiomeric separations [46]
Affinity capillary electrophoresis (ACE)	ACE for the determination of binding constants of ligands to receptors used with a partial filling technique [47]
	Review about ACE for the study of bimolecular interaction [48]
Isoelectric focusing in CE (cIEF)	Analysis of protein phosphorilation by cIEF-electrospray ionisation-mass spectrometry [49]
	Adaptation of cIEF to microchannels on a glass chip [50]
CE in sieving liquid polymers	Temperature-programmed CE for the analysis of high-melting point mutants in thalassemias [51]
	CE of DNA for molecular diagnostics [52]

now addressed to the coupling of CE with biosensor detection.

4. Capillary electrophoresis-based biosensors

CE-based biosensors are analytical devices composed of two distinct elements coupled together: the CE unit and the biological sensor. The CE-unit is the separation tool, able to fractionate the components of the sample on the basis of their charge/mass ratio, while the biological sensor provides the selective detection. The final result is a complex instrumentation. Such an integrated assembly takes advantage of the operational properties of each single unit. In CE-based biosensor development, the analyte selection depends on an event, occurring either before (e.g. the selective derivatisation of the target compound), or immediately after the separation step (e.g. electrochemical detection at the end of the column). The order in which sensor element and capillary column shall be combined together during the construction of CE-based biosensors will not affect the resulting device performance. The coupling of CE with the biosensor takes advantage from the technology developed for CE–MS, in fact CE–MS interfaces [64,65] and liquid junctions [66,67] are well suited for CE-based biosensors. Such sensing devices should take into account the presence of high voltages along the separation column. The biosensor element of CE-based biosensors should display high operational stability and long storage periods also in the presence of the electric field. Maintaining long term stability while withstanding harsh chemical environments and operating at high temperatures and/or pressures is a severe challenge for biological sensors. A new generation of recognition materials, based on molecular imprinted polymers (MIPs) [10,68], has been introduced and has been employed in biosensor technology [11,32–34] and in CE separations [69] with high success. The high selectivity and the chemical resistance of MIPs render them attractive also for the construction of CE-based biosensors. The time of response of the biosensor to the presence of a band eluting from the outlet of the CE separation column is a crucial parameter for the performance of the CE-based biosensor. Since CE gives a time resolution of the analytes and the biosensor is coupled on-line with the separation unit, the analyte recognition-element should be close to the band elution and the ability of sensing an analyte should not be affected by a previously occurred measurement.

The first attempts in building up CE-based biosen-

sors were proposed only four years ago [70]. The approach took advantage of the wide variety of biological sensing elements and transduction systems already described in the literature. The applications of, and the target molecules for, CE-based biosensor systems, are either of clinical or pharmaceutical interest, or they have been developed for quality controls in food industry; therefore CE-based biosensors form a highly inhomogeneous group. A first example of CE-based biosensor is shown in Fig. 3 it displays a bienzyme amperometric electrode coupled to the CE separation column, developed for the detection and quantitation of underivatised carbohydrates [71]. This device performs directly the analysis of carbohydrates, such as glucose and maltose. The specificity of the detection is achieved by immobilising onto the platinum electrode a mixture of two enzymes: glucose oxidase and amyloglucosidase. The selective redox reaction of the substrates generates the response current. Since the best separation conditions were obtained for alkaline buffers (pH 10) and the response of the electrode required rather acidic conditions, a liquid junction was used to lower the pH of the solutes eluting from the separation column, before they would reach the electrode. It was possible to achieve one week of continuous operation. The detection limit for sugars was 0.17 m*M* for maltose and 0.35 m*M* for glucose, sufficient for measuring the levels of glucose and maltose in fruit-juices.

Another example of CE biosensors is microdialysis coupled to CE, as shown in Fig. 4. Microdialysis sampling, followed by on-line derivatisation and coupled to CE separation with LIF detection, was developed for continuous in vivo analysis. Such CE-based biosensors were used to perform near-real-time analysis of the neurotransmitters aspartate and glutamate in rat brain fluids [70]. The levels of aspartate and glutamate, released upon K^+ stimulation, were measured. The on-line fluorescent derivatisation reaction confers selectively the fluorescence to the dialysate components, allowing the correlation between neurotransmitters concentrations and fluorescent signal; the range of linearity of the measurements and the time of response after the stimulation event were determined. Microdialysis coupled to CE has found a wide application in the study of neuroactive drugs, opioids or antineoplastic agents [72–75]. This methodology has been used for continuous in vivo monitoring of chemical reactions induced by pharmacological agents and the determination of drug substances or drug-induced changes of endogenous compounds. The advantage given by the microdialysis CE-based biosensor relies on the possibility of performing the analysis directly on the patient, by inserting the microdialysis system into the living tissues. The continuous sampling allows carrying out the experiment for several hours. The spectrum of compounds which could be analysed could be broadened, due the high flexibility of this CE-based biosensor, by choosing the chemical group of the analyte, towards which

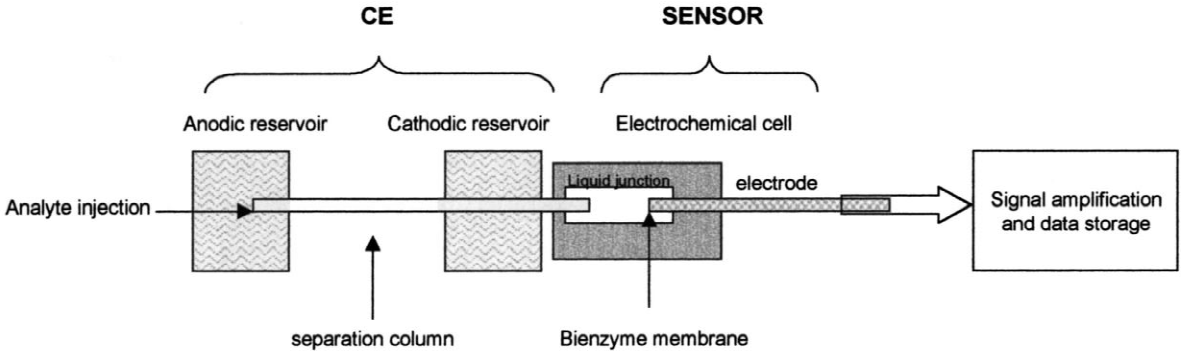

Fig. 3. CE equipped with a bienzyme electrode. Carbohydrate analytes are separated along the CE column by applying the voltage. A fracture/assembly cathode reservoir is used to decouple the high voltages before detection. The capillary outlet and the bienzyme electrode are aligned and fixed onto a plexiglass holder at a distance of about 30 μm (the liquid junction). The electrochemical cell contains the working bienzyme electrode and the Ag/AgCl reference electrode.

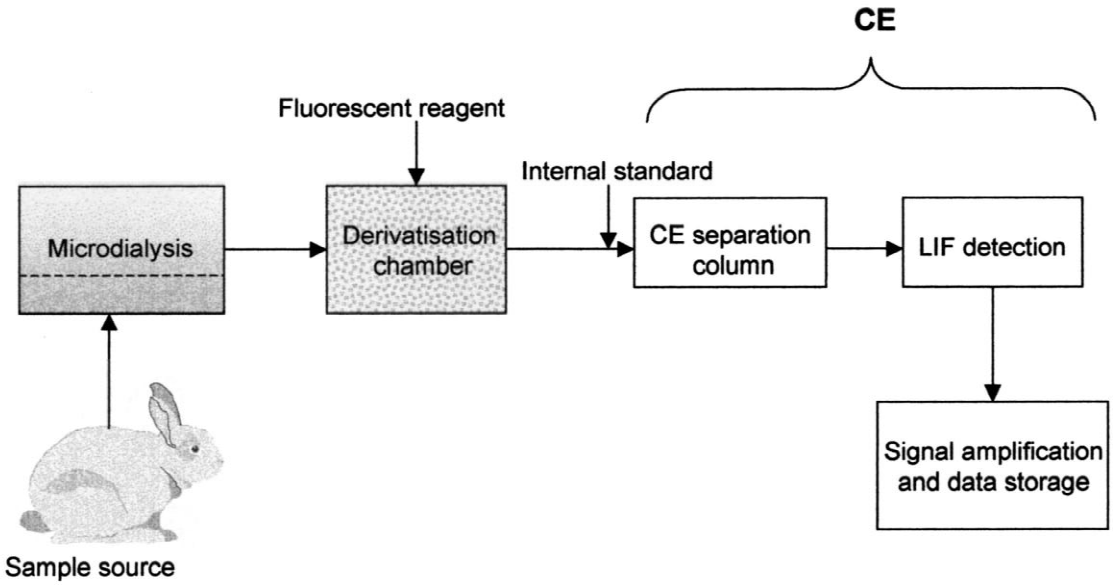

Fig. 4. Microdialysis coupled to CE biosensor. The sample is continuously dialysed from the sample source (the probe or the patient) and pumped into the reaction chamber together with the derivatisation reagents. The derivatisation takes place in the derivatisation chamber. A system of microinjection pumps and an electric valve ensure the injection of the derivatised samples into the separation column. Detection is performed with a LIF detector.

the derivatisation is specifically directed, and the derivatisation reagent.

Finally, a more recent group of CE-based biosensors presents the whole cell as a biological sensing element. These sensors are mainly used for physiological studies and include CE single-cell biosensors and CE patch-clamp biosensors. A single cell biosensor coupled to the end of the CE column, shown in Fig. 5, was firstly proposed for the identification of receptor agonists [76,77]. The putative agonists are separated along the CE column by migration time and are screened for biological activity by the cell-biosensor. The system has taken considerable advantage from the cell-to-cell scanning mode [78], a near-automated scanning, which uses a motorised microscope stage moving along the petri dish from a cell group to another, so that the CE column effluent is directed every time towards ''fresh'' cell groups. The system was validated by using G108-15 cells and bradikinin and kallidin as stimulating agents. The time response of the cells to a band eluting from the CE column was measured, demonstrating that cell-to-cell scanning overcomes the problem of receptor desensitisation (the typical decrement of the cellular response, occurring after a stimulation event), which heavily affects the measurements performed with whole-cell receptors. Recently, patch-clamp technology [79] has been coupled with CE for studying neurotransmitters [80]. Patch-clamp provides detailed microscopic information about receptor-activated ion-channels, such as the kinetics of activation, the conductance and the open and close-times of the ion-channel. The method is helpful in discovering bioactive substances and identifying secreted toxins that promote receptor-mediated neurotoxicity. CE–patch-clamp biosensors introduce the CE fractionation of the compounds that trigger the opening of ligand-gated ion-channels, before the patch-clamp detection. Therefore, the CE–patch-clamp biosensor allows the resolution of the problem of multiple agonists activating the same receptor. The separated components interact with the receptors one at a time, in dependence of their migration time. Agonists are identified by three different parameters: the migration time, the physiological effect (response induction by the agonist and response block when a toxin is added to the receptor-biosensor) and finally the magnitude of response, whose value is often

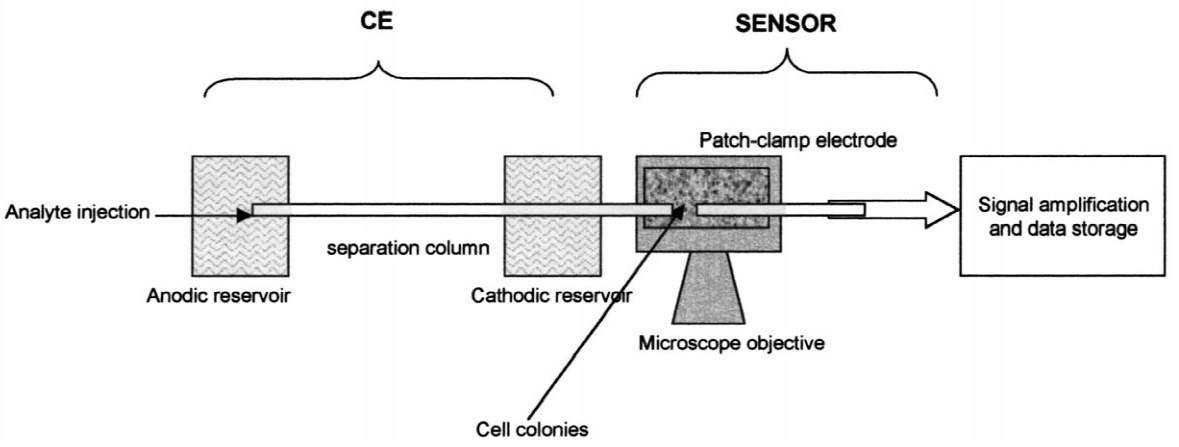

Fig. 5. CE single-cell biosensor and CE patch-clamp biosensor. Analytes (receptor agonists or antagonists, etc.) are separated along the CE column by applying the voltage. A fracture/assembly cathode reservoir is used to decouple the high voltages before detection. The capillary outlet is positioned on a cell-plate mounted on a microscope objective. Single cell detection is performed by monitoring the changes in fluorescence of the single cell colony in proximity of the outlet of the CE column. Cell-to-cell scanning is performed by using a motorised microscope stage, which moves the cell-plate so that the column effluent is directed every time towards fresh cell groups. In patch-clamp detection the tip of the patch-clamp electrode is positioned 5 to 25 μm from the capillary outlet.

compared to the mean values obtained by other techniques. Thus CE–patch-clamp biosensors have been clearly demonstrated to allow a multidimensional identification of the analytes.

5. Conclusions

The coupling of a CE electrophoretic unit with a specific biosensor has led to the development of CE-biosensor technology. CE-based biosensors have demonstrated great versatility, partly due to the characteristics of CE (low amounts of sample, time resolution, speediness in analysis and high efficiency) and partly due to the excellent detector selectivity of biosensors. Such devices are able to fractionate electrophoretically complex mixtures of analytes, which will reach the biological sensor at different times. In principle CE-based biosensors allow a precise detection of each single component of a group of analytes, instead of measuring only the signal composed by the sum of the components of the mixture. A crucial point of the biosensor unit remains the duration of the response time, which should be minimal in order to perform near real-time measurements, to allow a rapid signal evolution consequent to the binding event and to shorten the time of recovery after each measurement. Furthermore, biosensors should be easily manufactured, easily replaceable and inexpensive. From the CE side the major problem rests on column technology [81], where silanol dissociation gives rise to the electroosmotic flow (EOF), which sometimes lowers dramatically the resolution of the separation and acts as a site for the adsorption of peptides and proteins. With further technology maturing, and by overcoming the existing limitations, we foresee an expansion of CE-based biosensors and their commercial success in the near future.

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