ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
14 (1996) 871–880

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

Electrochemical detection in bioanalysis[1]

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Received for review 13 September 1995

Abstract

This review article presents an overview of current research on the use of amperometric electrochemical detectors in bioanalytical chemistry. Topics covered include microdialysis and ultrafiltration membranes for in-vivo sampling; microbore liquid chromatography; capillary electrophoresis; enzyme, photochemical, and chemical post-column reactions; electrochemiluminescence; and thin-film electrode materials. Selected references are cited.

Keywords: Biosensors; Electrochemical detection; Liquid chromatography; Microdialysis; Post-column reactions

1. Introduction

Liquid chromatography/electrochemistry (LCEC) is now almost 25 years old [1]. In recent years, an emphasis has been placed on miniaturizing the technology to accommodate the study of smaller biological samples, often with a total available volume of only a few microliters. Both liquid chromatography and electrochemistry are largely controlled by surface science. Considering this fact, both technologies benefit from reducing the distance from the bulk of the solution phase to the surface. In LC this is accomplished by using smaller diameter stationary phase particles. In electrochemistry it is accomplished by using packed bed or porous electrodes and/or thin-layer cells with greatly restricted diffusion pathways. For analytical purposes, there is no loss in concentration detection limit by reducing the total surface area available in both methodologies. In LC this reduction is accomplished by using smaller diameter columns and in EC by using smaller electrodes. With LC column diameters of 0.1–1.0 mm and radial flow thin-layer cells with dead volumes of a few tens of nanoliters, it is possible to build analytical instruments capable of routine use by neuroscientists, drug metabolism groups, and pharmacokinetics experts. Recent developments in LCEC emphasize coupling this technique to microdialysis sampling. Examples include the use of post-column enzymatic reactors and polymer modified electrodes as on-line biosensors. Automation of the entire process from dialysis probe to chromatography to electrochemical detection and pharmacokinetic analysis is now quite feasible. Awake animal experiments as well as in-vitro examples (microsomes) are considered to be routine in some laboratories. This

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[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

PII S0731-7085(96)01781-5

paper will selectively review several of these developments in capsule form.

2. Overview of current research

2.1. Microdialysis/ultrafiltration sampling

The evolution of membrane-based sampling devices has provided a major impetus for advances in electrochemical detection and vice versa. Hydrophilic membrane fibers can be implanted in living tissue and used to isolate low molecular weight substances from the extracellular space. Such fibers are typically constructed of regenerated cellulose, cupraphane, polycarbonate, or polyacrylonitrile hydrogels with controlled size-exclusion properties. They are normally used in a dialysis mode whereby a physiological saline is pumped through the lumen of the membrane capillary while substances traverse the capillary wall by diffusion down their concentration gradient. For example, drug within the lumen can diffuse outward into tissue, and metabolite in the tissue can diffuse inward to the perfusate stream. By analyzing the effluent, one can determine how much drug was delivered to the tissue and/or how much metabolite was recovered. The process is schematically illustrated in Fig. 1.

This methodology was developed specifically for neuroscience applications in the 1970s, whereby adrenergic neurotransmitters could be isolated from living brain tissue and determined using liquid chromatography/electrochemistry [2]. The technique was primarily popularized by Ungerstedt and his group at the Karolinska Institute in Stockholm. A number of procedures and accessory devices were necessary to make the microdialysis technique practical. Today it is possible to carry out some totally automated experiments completely unattended. While there is room for improvement, much of Ungerstedt's dream has been realized.

Similar membranes can also be used in an ultrafiltration mode whereby a slight vacuum is applied to the lumen of the membrane fiber and low molecular weight substances are swept across the membrane with the flow of extracellular water and electrolytes. This approach was originally developed by Janle, Ash and co-workers [3] and later by Linhares and Kissinger [4, 5]. It is an excellent approach to sampling electrolytes, lactate, glucose, amino acids, and many drug substances in subcutaneous tissue.

The dialysis and ultrafiltration approaches both have specific advantages and liabilities. The dialysis technique is easier to miniaturize and operate with a well-controlled flow, but the concentration recovery of substances in the extracellular space can be low. Dialysis is bidirectional and this is a primary virtue. Ultrafiltration probes generally are larger, but ions and low molecular weight hydrophilic substances are quantitatively recovered. Ultrafiltration pulls fluid from the tissue

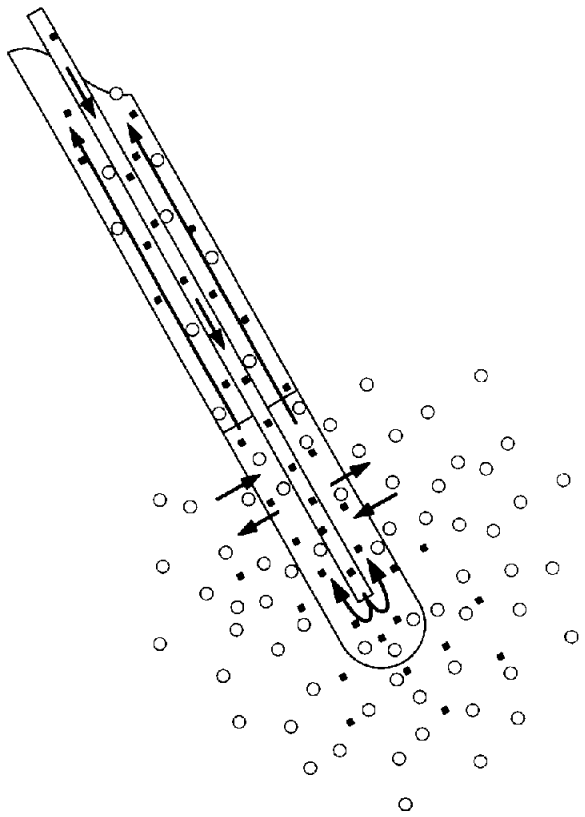

Fig. 1. Schematic diagram of the dialysis process using a concentric tube microdialysis probe. A drug in the perfusate (squares) can dialyze outward into tissue while small molecules in the tissue (circles) diffuse inward and are collected with the dialysate.

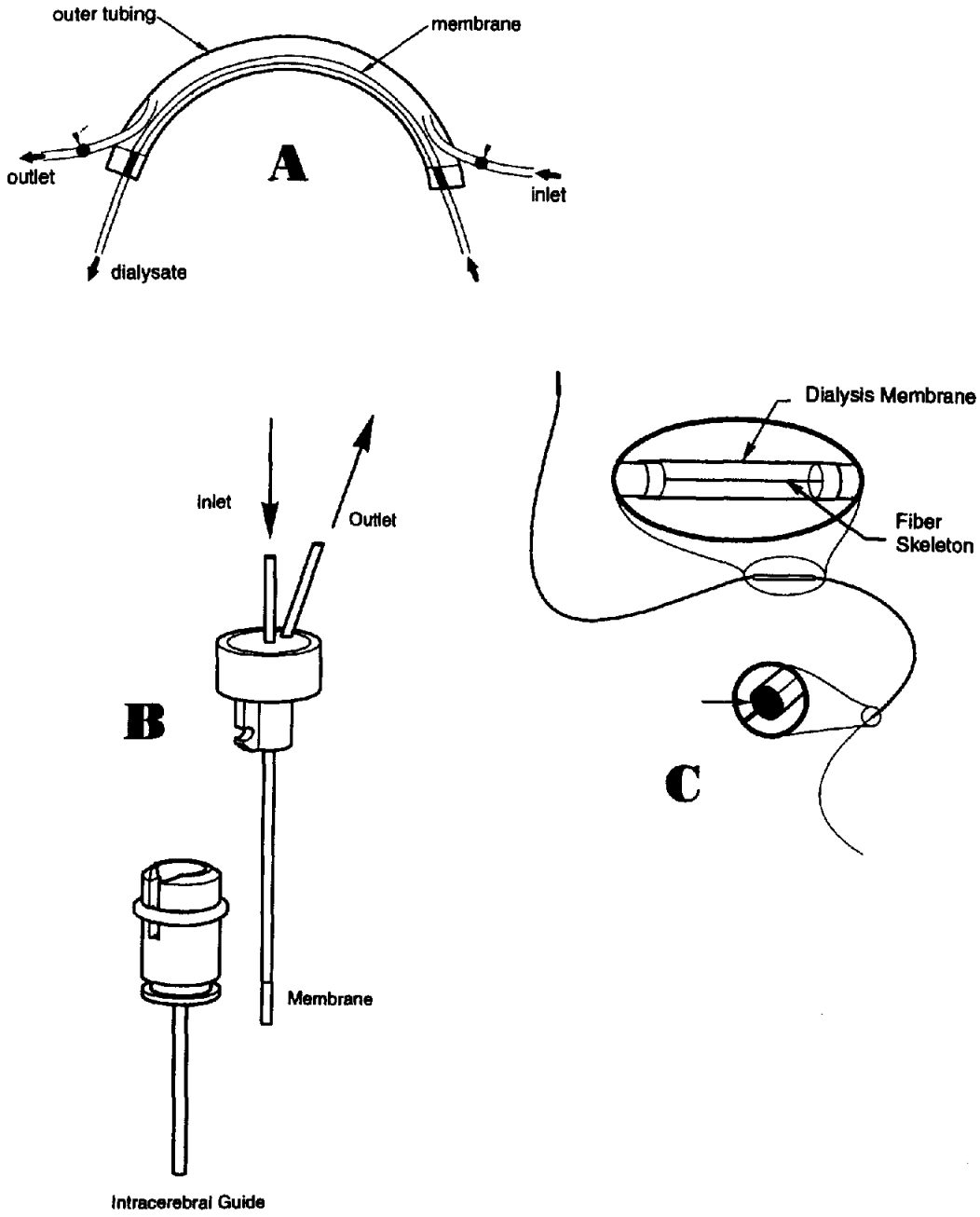

Fig. 2. Several dialysis probe geometries: (A) a shunt or bypass probe for bile; (B) a rat brain probe with guide cannula; (C) a linear probe for soft tissue studies.

(albeit at a very low rate) while dialysis does not. Both modalities isolate low molecular weight substances from metabolizing enzymes. Both are well suited to hydrophilic substances and are not very promising for lipophilic compounds. Both result in microliter volumes of protein-free solutions which are well suited to liquid chromatography, capillary electrophoresis and immunoassays.

Fig. 2 illustrates several geometries for both in-vivo and in-vitro dialysis sampling probes. While this area began with in-vivo experiments in the central nervous system, it has evolved to include a wide range of target organs and analytes. Fig. 3 illustrates typical ultrafiltration probes with an evacuated sample collection tube or miniature peristaltic pump to apply suction to the membrane.

There is considerable promise for using membrane sampling for drug metabolism and pharmacokinetics work [4–8]. Toxicokinetic studies are also a potentially attractive application since tissue-specific data can be correlated with traditional blood concentration studies. In-vitro enzymatic reactions (e.g. microsome incubations) can be followed by using membrane sampling to isolate product and determine substrate consumption online. Fermentations can be followed. Protein binding can be examined.

One of the more exciting features of membrane sampling is that it can be carried out with awake, freely-moving animals. In many cases data could previously be obtained only by sacrificing an animal, taking periodic blood samples, or by studying animals under anesthesia. Now detailed longitudinal studies are possible in awake subjects and several applications to humans have been explored. Fig. 4 shows a typical system for carrying out microdialysis experiments in the rat. A multichannel syringe pump coupled in a zero dead volume switching valve makes it convenient to use pharmacological tools or to carry out dose-response curves. A two-channel liquid swivel enables fluid lines to be brought to the animal and back out of the cage while avoiding twisting the low dead volume tubing. A balance beam minimizes mechanical pressure on the animal or the implant. Using a round-bottom bowl for animal containment (introduced by Ungerstedt) provides an optimum centrosymmetric system. The outlet fluid is either sampled periodically with a microfraction collector or (as shown in Fig. 4) with a valve for on-line injection on a liquid chromatography system. It is relatively straightforward to automate the entire process from control of the syringe pump to processing pharmacokinetic data.

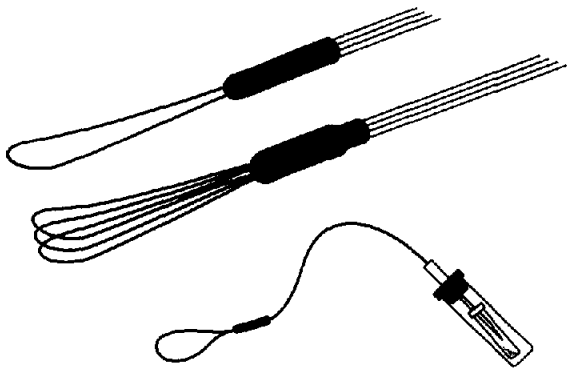

Fig. 3. Ultrafiltration probes are normally of a loop design and use either an evacuated chamber or a miniature peristaltic pump to pull extracellular fluid across the hydrophilic membrane fiber (reproduced by permission of Bioanalytical Systems, Inc.).

There are many variations on this theme. Some substances (e.g. amino acids) require derivatization prior to the separation step. Some workers have used mass spectrometry (although high salt content remains a problem). S. Lunte and co-workers at Kansas University have cleverly coupled microdialysis directly to capillary electrophoresis [9].

The workhorse tool remains microbore liquid chromatography/electrochemistry. Fig. 5 illustrates a system in which the radial-flow thin-layer electrochemical detector is configured as the end fitting on a microbore column. The chromatograms in Fig. 6 demonstrate the performance achievable with such a system coupled to the brain of a fully functioning rat.

2.2. Carbon thin-film electrodes and other innovations

The most common electrode material used in LCEC is carbon, either as solid "glassy carbon" disks in thin-layer cells, or as a high surface area porous matrix through which the mobile phase can flow. Gold electrodes are useful to support a mercury film and these are primarily used to determine thiols and disulfides [10]. Platinum electrodes are occasionally useful for specific analytes, but are most frequently employed to determine hydrogen peroxide following an oxidase immobilized enzyme reactor (IMER). Quite recently, cop-

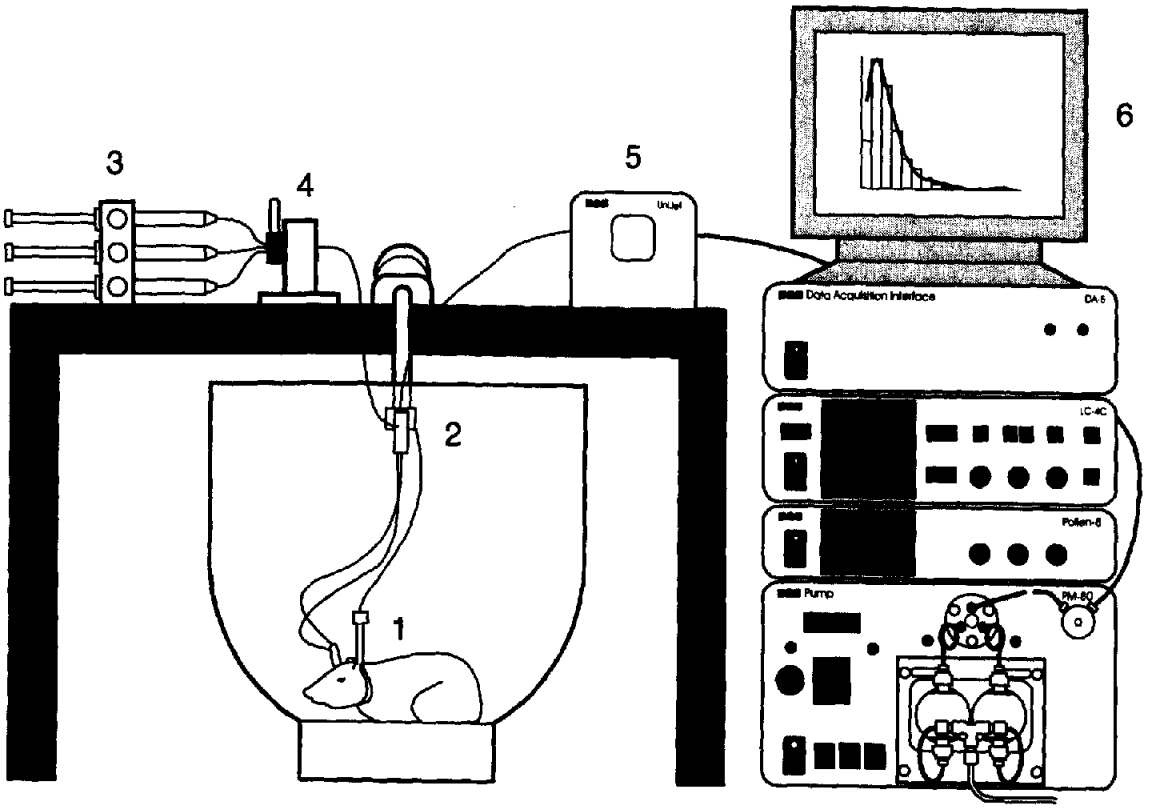

Fig. 4. A complete apparatus for study of metabolism in the rat using an in-vivo membrane probe to sample extracellular fluid: (1) animal with probe implanted; (2) dual-channel liquid swivel on balanced beam; (3) multichannel-processor-controlled syringe pump; (4) liquid switch; (5) automated injection valve with microbore LCEC; (6) computer-controlled chromatograph with pharmacokinetics output.

per electrodes have begun to attract serious interest for determination of carbohydrates in basic mobile phases [11,12]. Glassy carbon is the overwhelming favorite choice due to its wide range of applicable potentials and its rugged convenience. Bulk glassy carbon is difficult to use in geometries other than disks and plates. There are a number of other geometries which have practical interest for multiple electrode detectors. One of the more valuable recent contributions to LCEC derives from the ability to deposit conducting vitreous carbon films on silicon or quartz substrates using lithography techniques. This innovation originated at Nippon Telephone and Telegraph Company (NTT) [13–15] and makes practical a number of ideas which were developed only when gold films were available. Gold simply does not afford the potential range needed for electrochemical detection of most substances of biological interest. The lithography technology makes it possible to lay down a variety of electrode geometries which could not possibly be manufactured in small sizes by traditional machining. While such electrodes are still at the research stage, they show considerable promise. The interdigitated array (IDA) shown in Fig. 7 provides for redox cycling as schematically shown in Fig. 8. Using this approach enhances the total current for reversible analytes while minimizing the response from electrochemically irreversible reactions. Detection limits for catecholamines using 1 mm microbore LC columns as low as 0.02 pg have been achieved [16].

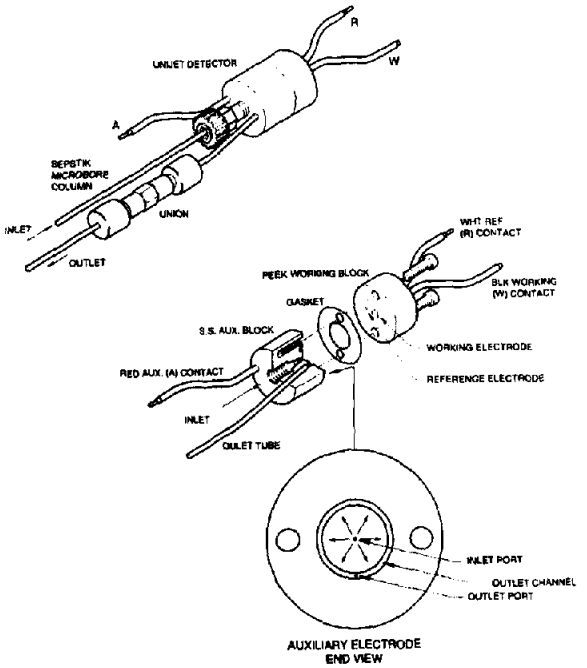

Fig. 5. Radial flow thin-layer electrochemical detector cell as an end-fitting on a microbore column.

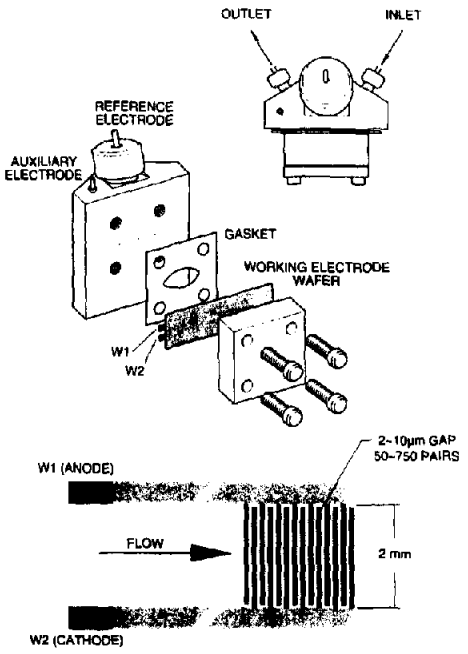

Fig. 7. A cross-flow thin-layer electrochemical detector using an IDA carbon film electrode.

2.3. Pulsed electrochemical detection

There are many substances which would appear to be good candidates for LCEC from a thermodynamic point of view, but which do not behave well due to kinetic limitations. The next few sections address ways in which LCEC applications have been dramatically extended. Johnson and co-workers at Iowa State University used some fundamental ideas about electrocatalysis to revolutionize the determination of carbohydrates, nearly intractable substances which do not readily lend themselves to ultraviolet absorption, fluorescence, or traditional DC amperometry. At the time this work began, the LC of carbohydrates was more or less relegated to refractive index detection of microgram amounts. The importance of polysaccharides and glycoproteins, as well as

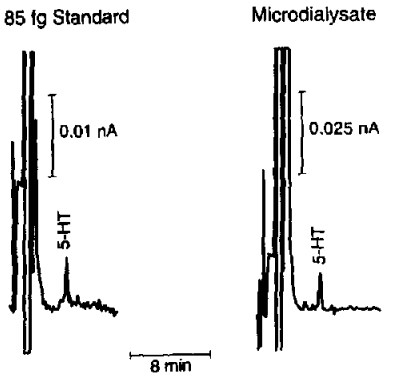

Fig. 6. Determination of basal serotonin in rat brain microdialysate (0.5 μl) using a 0.32 mm × 150 mm 3 μm C_{18} column with a 3 mm radial flow glassy carbon thin-layer electrode at 650 mV vs. Ag/AgCl.

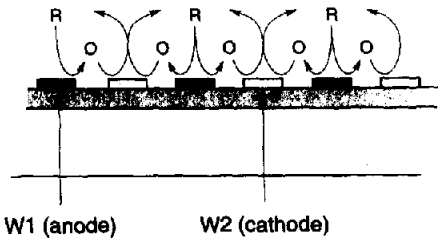

Fig. 8. Using an IDA electrode makes it possible to amplify the number of electrons exchanged for a given analyte as it passes through the thin-layer cell. This is accomplished by redox cycling reversible electrochemical reactions.

traditional sugars, has focused a lot of attention on pulsed electrochemical detection methodology [17–19]. The detection limits are not competitive with DC amperometry of more easily oxidized substances such as phenols and aromatic amines; however, they are far superior to optical detection approaches.

2.4. Post-column photochemical reactions

Photochemical reactions have been used to convert substances to products which exhibit an improved response to ultraviolet absorbance, fluorescence, or electrochemical detection. This area was clearly given an enormous boost by the work of Krull's group at Northeastern University. In the most practical implementation, a UV germicidal lamp is wrapped with Teflon tubing to form a post-column reactor prior to the detector. The photochemical process can enhance the electrochemical detection process by converting substances into a more easily reacted form. For example, phenols and aromatic amines can be photohydroxylated to hydroquinones or aminophenols, nitro compounds can be converted into nitrite, and proteins can be detected and utilized in peptide mapping [20]. A number of heterocyclic drugs have been successfully determined using post-column photochemical methodology. The convenient diagnostic of comparing "light on" and "light off" chromatograms has been very useful. This fascinating area has been reviewed [21]. Several commercial instruments have been available for a number of years.

2.5. Electrochemiluminescence detection

There is clearly some advantage in putting light into the electrochemical detection process. Now, how can one get some light out? Liquid state electrochemiluminescence (ECL) has been of fundamental interest for a number of years. It attracted a lot of attention in the 1960s, but never found any practical use. Recently the situation has changed with the introduction of ECL technology coupled to polymerase chain reaction (PCR) and immunoassay schemes [22–24]. While several approaches to LCECL have been explored, the most promising appears to be the use of ruthenium (II) trisbipyridyl by Nieman's group at the University of Illinois [25]. In one embellishment of this idea, $Ru(bpy)_3^{2+}$ is added to the mobile phase. It reacts at the electrode in the thin-layer ECL flow cell to form $Ru(bpy)_3^{3+}$ which then reacts with eluting substances to form excited $Ru(bpy)_3^{2+}$ which emits into a photomultiplier tube adjacent to the cell. This has proven to be an intriguing approach for amines and also oxalate [26].

2.6. Using the Biuret reaction for peptides

In the view of the author the bioanalytes for which liquid chromatography is most in demand and is least successful are peptides in tissue and biological fluids. For example, there is an enormous demand for determination of peptide hormones and neurochemicals by liquid chromatography, but immunoassay remains the only viable approach in most cases. Neuroscientists often ask the question "Why can't we detect peptides the way we detect catecholamines and serotonin?". The answer is quite obvious: peptides as individual substances do not have clearly distinguishable features for spectroscopy or electrochemistry. Furthermore, there are simply too many of them. Will electrochemistry play any role in solving this problem? One innovative approach has been under development by Weber and his group at the University of Pittsburgh [27–29]. They have coupled the classical Biuret reaction to LCEC. Eluted peptides are complexed with copper(II) in a post-column reaction. The complexes are electrochemically oxidizable to the copper(III) oxidation state. Using a series dual-channel detector, the copper(III) is reduced back to copper(II) at the downstream electrode. The reduction step occurs at favorable potentials on a glassy carbon electrode. While this does not solve all the problems, it is an interesting approach and concentration detection limits as low as 0.1–20 nM have been achieved for some peptides.

2.7. Post-column enzymatic reactions

The use of IMERs in connection with liquid chromatography has been a considerable commer-

cial success, especially for the determination of choline and acetylcholine. This is now routine practice for microbore (1 mm) LCEC. The IMER constants of covalently bound acetylcholine esterase and choline oxidase. The process ultimately reduces oxygen to hydrogen peroxide which can be detected either by direct electrochemical oxidation on platinum or by reduction using a peroxidase catalyst bound to a hydrogel polymer and "wired" to a glassy carbon electrode using Os(II/III) redox centers [30]. The latter approach has made it possible to determine basal acetylcholine in rat brain [31]. This has been very challenging to achieve due to the rapid in-vivo tunover of acetylcholine to choline. Glucose, lactate, glutamate, and polyamines are among those substances where microbore IMERs coupled to a peroxidase hydrogel-modified electrode have considerable promise.

2.8. Capillary electrophoresis (CE)

Since there is LCEC, it is only logical that there should be CEEC. This area was pioneered by Ewing at the Pennsylvania State University. Zare (Stanford University) and S. Lunte (Kansas University) have explored this idea in a number of unique ways. The basic technology has been recently reviewed [32–34]. There are several fundamental problems that do not occur with LCEC. First, the capillaries must be of small diameter to properly dissipate resistive heating. Thus the electrodes used in CEEC are normally carbon fibers or metallic wires placed in or at the capillary end. Second, the electrical current through the capillary which establishes the electroosmotic pumping is much larger than the electrolysis current measured in determining analytes of interest. The ionic and electrolytic currents need to be "decoupled" in some way. A third concern is that the flow rate in CE is not independent of the choice of "mobile phase" or even the sample whereas in LC it is easily predetermined and maintained by a volume displacement pump. In spite of these concerns, CE is very attractive because of its high resolution per unit time and the small sample volumes required. It is especially interesting to take advantage of these two features for high speed coupling of CE to microdialysis, where sample size is very limited. In the case of CEEC, the concentration detection limits are frequently superior to optical detectors for suitable analytes. This is because electrochemical detection is a surface (not volume) dependent technique.

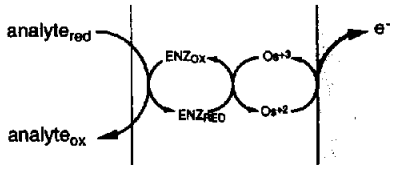

Fig. 9. An oxidase enzyme and an osmium complex are covalently attached to a hydrogel polymer coated on an electrode. Analyte enters the gel phase and is catalytically oxidized by the enzyme. The enzyme is reoxidized by Os(III). The resulting Os(II) is reoxidized by the electrode and the current is therefore related to the original substrate (analyte) concentration.

2.9. The use of electrochemistry without chromatography or electrophoresis

It is rare for finite current electrochemistry to be used without chromatography for bioanalysis, but it is possible in some cases. The classic example is the determination of oxygen in biological materials. This is feasible because oxygen is a hydrophobic gas and it can easily be separated from interferences with a hydrophobic membrane such as Teflon. This is the basis of the Clark oxygen electrode which has been a great commercial success for over 35 years. While biosensors are tough to design for low concentration analytes, they show great promise for substances such as glucose and lactate where the concentrations are favorable and good enzymes are avilable to build selectivity into amperometry. Fig. 9 illustrates an arrangement whereby an amperometric sensor is based on an oxidase enzyme "wired" into a hydrogel polymer containing covalently bound osmium centers. This technology was pioneered by Heller and co-workers at the University of Texas [35,36]. When the substrate is oxidized by the enzyme, Os(III) is reduced to Os(II) in the polymer. This ultimately results in Os(II) near the electrode surface being reoxidized back to Os(III). The resulting anodic current is proportional to the original substrate concentration over a reasonable range.

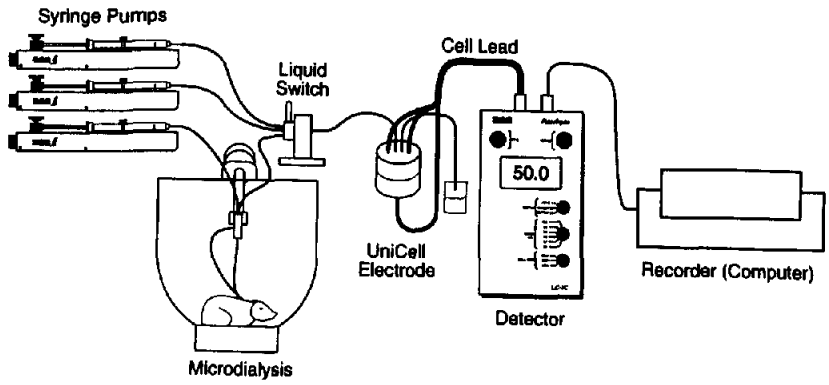

Fig. 10. A system for use of an on-line amperometric biosensor with a microdialysis probe in an awake rat. A zero dead volume liquid switch makes it possible to alternate sensor flow between the animal dialysate, baseline perfusion solution, and perfusion fluid including a standard analyte concentration. Sensor performance can therefore be periodically monitored without stopping the flow.

Fig. 10 depicts the coupling of such a sensor to a microdialysis experiment. Lactate and glucose have both been explored [37,38]. The linear operating ranges are 0.1–20 mM and 0.1–5 mM respectively. The operational half-life is currently 40 h for glucose and 24 h for lactate. This is an exciting alternative to determining these same substances using the LC-oxidase IMER-peroxidase electrode scheme described in an earlier section of this review. The sample is presented to the enzyme quite differently in both approaches. Using LC, the mobile phase can be completely decoupled from the biological system with respect o pH, ionic strength, and ion composition. It is also easy to dilute samples to fit the linear range and an IMER inevitably exhibits a much longer lifetime than current polymer film enzyme electrodes. However, the LC approach is inherently periodic while the on-line sensors are continuous and much less costly.

3. Conclusions

Electrochemical detection has matured considerably over the last 25 years and is routinely used by many laboratories. Nevertheless, there are enhancements possible in both the basic methodology and the way it can be coupled to other techniques to provide more reliable bioanalytical data.

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