

Flow Injection Analysis Based Enzymic Methods and Biosensors

Mamas I. Prodromidis and Miltiades I. Karayannis*

Department of Chemistry, University of Ioannina, 45 110 Ioannina, Greece

Abstract

This review introduces a number of flow injection analysis based enzymic methods and biosensors for the determination of various analytes with interest in food, environmental and cosmetics analysis. Particular attention has been paid on the selectivity of the reviewed works as well as on their practicality in terms of flow injection manifold assembly, the fabrication of the enzyme electrodes and the performance of the measurements. A flexible and easy way to achieve upgrading of a typical flow injection manifold to a fully automated one, by means of resident software, is presented. Enzymatic methods based on packed-bed reactors bearing the immobilized enzyme(s) are discussed. The reviewed biosensors are based on a multi-membrane architecture consisted: i) of a cellulose acetate membrane in order to keep away possible interferants from the electrode surface, ii) the enzymic membrane and iii) an outer one, which protects immobilized enzyme(s) from leaching and microbial attack. Examples of various enzyme electrodes, published in the literature during the last decade, along with some flow amperometric cells are also presented.

Keywords Flow Injection Analysis; Amperometric biosensors; Enzyme electrodes; Food analysis; Commercial biosensors; Review

1. Introduction

As is shown in Fig.1, a biosensor is a system of two transducers, one biochemical and one physical, in intimate contact or in close proximity with each other that relates the concentration of an analyte to a measurable signal.

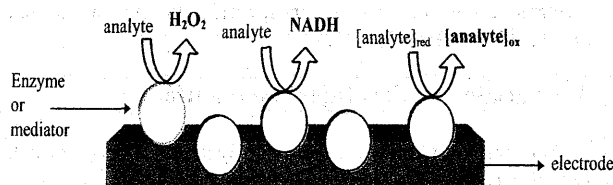
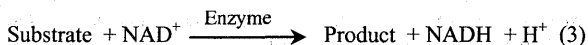
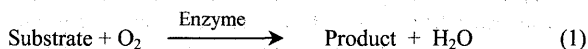
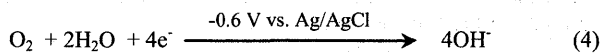


Figure 1. Schematic representation of various types of amperometric biosensors.

Amperometric biosensors are based on enzymes that, either consume oxygen or produce hydrogen peroxide, or produce (indirectly) the reduced form of β -nicotinamide adenine dinucleotide (phosphate), NAD(P)H, during the course of the catalytic reaction on the substrate of interest. The general equations of the fore mentioned types of amperometric biosensors are [1-2]:



The consumption of oxygen according to reactions described with the Eqs. 1 and 2 can be measured by with a Clark-type electrode at a platinum cathode at 0.6-0.9 V versus a Ag/AgCl reference electrode, according to the following equation.

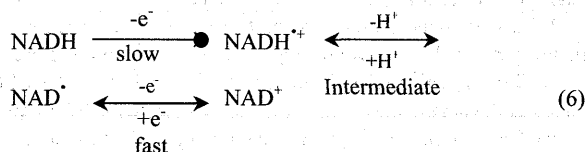


*Corresponding author
E-mail: mkaragia@cc.uoi.gr

Hydrogen peroxide generated according to Eq.2 can be measured amperometrically by oxidation at the anode of a solid (platinum, glassy carbon) electrode, polarized at +0.65V, according to the Eq.5.



Although there is still considerable uncertainty about the detailed nature of the electrochemical oxidation of NADH the following (tentative) reaction scheme is generally accepted [8]:



2. Mediators

In many cases the redox reactions taking place on a plain electrode show a low kinetic rate transfer mechanism of the participating electrons, resulting the increase of the applied potential over the thermodynamic one. The attachment on the electrode surface of an appropriate mediator, can support the transfer of the electric charge, and can catalyze this kind of reactions. The active form of the mediator is regenerated electrochemically on the surface of the electrode and creates an electron shuttling. So, the applied potential can be decreased to the value of the standard potential of the mediator resulting avoidance of interferences and the increase of current density. The efficiency of this electrocatalytic shuttling depends on the real distance of the mediator from the electrode surface and the morphology of the surface. The decrease of the overvoltage is mainly dictated by the formal potential E^0 of the mediator. Low potentials minimize the electrode fouling and also prevent interfering reactions. Nevertheless one of the commonest problems encountered is the poor stability of the modified electrodes. For example the oxidation of NADH at bare electrodes takes place at high overvoltage ranging from 450 to 1100 mV depending on the material of the electrode.

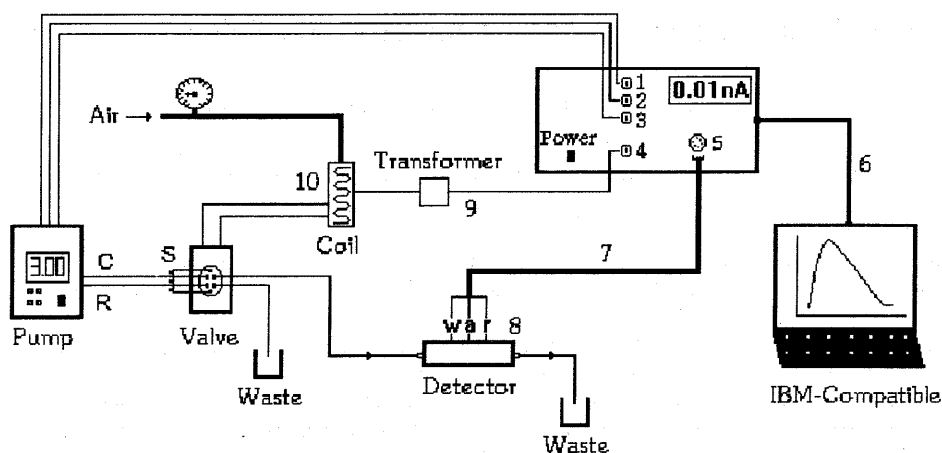


Figure 2. FIA manifold: 1,2,3,4: Digital/Analog (D/A) converters with a resolution of 16 bits. 5: DIN connector. 6: Interface cable. 7: the cell cable with four connectors for the working (w), reference (r), auxiliary (a) electrodes and the analog ground. 8: Electrochemical detector, with wall-jet type cell (volume <math>< 1\mu\text{l}</math>). 9: DC transformer. 10: 12 Volt coil. C: carrier, R: reagent, S: sample.

A series of papers published from our group intended to exploit the application of different mediators, specifically the excellent electrochemistry of DCPI (2,6-dichlororophenol indophenol) and other redox couples synthesized in our laboratory and to describe the surface attachment of the redox mediators on the surface of graphite. The electrochemical behavior of the plain and modified electrodes was also investigated [3-7]. As an example, lanthanum- or thorium-DCPI modified porous graphite electrodes were investigated and the electrocatalytic properties of the sensor were studied. The following results were received [3]:

i) The electrochemical redox reaction of the DCPI was found to be fairly reversible at low coverage with $E^0 = +55$ mV at pH 6.5.

ii) The pK_a value of 5.8 ± 0.1 for the reduced form of DCPI is calculated from the intersection of the lines in the plot E^0 vs. pH.

iii) The current I_p has a linear relationship with the scan rate up to 1200 mVs^{-1} , which is indicative for very fast electron transfer kinetics. iv) The value of the standard rate constant was found to be $k^0 = 18 \pm 4$ s^{-1} .

v) The DCPI-modified electrodes mounted in a flow injection manifold, were poised at $+60$ mV reducing thus the oxidation overpotential of NADH for about 400 mV.

vi) Comparison of the formal potentials shows that interactions between the immobilized molecule and the surface of the electrode, to which it is attached, do not strongly perturb the electronic structure of the immobilized molecule or its electron transfer products.

vii) The peak potential separation ΔE_p is moderate as expected for surface-immobilized redox species. Values of 9-30 mV were obtained for surface coverage Γ between 0.52 and 3.12 nmolcm^{-2} and scan rate 50 mVs^{-1} .

A lot of work has been done on this direction and the advantages from the use of mediators have been documented in comprehensive reviews [8-10]. However some drawbacks are introduced since the mediators may facilitate charge transfer between possible interferants and the electrode, increasing the interference problem and leaching from the system, resulting thus a progressively diminishing response to the analyte. A lot of work has been focused on the combination of the advantages of

working at low redox potential of the mediator and/or the perm-selective properties of a polymeric membrane employed in the probe. Different transport mechanisms based on size exclusion (cellulose acetate), charge exclusion (Nafion), mixed permselectivity/electrocatalysis (cellulose acetate/ 2, - dichlorophenolindophenol) mixed control (cellulose Acetate/Nafion), or polarity (phosphatidylcholine), have been extensively reviewed [11-12].

3. Upgrade of a flow injection manifold

Prodromidis et al. [13], presented a resident program (which can be run simultaneously and complementary to any other program), able to fully control (on/off, speed, direction) a pump as well as a pneumatic valve (emptying and filling position). A schematic representation of the FI manifold is given in Fig. 2.

Since the new aspect of technology creates automated instruments, in the majority of cases coupled with powerful software systems, they require the complete use of a personal computer as a set of switchers to control it. The concept of resident (TSR) programs in order to improve the performance of an instrument provides an excellent solution. A TSR program easily automates an additional control necessary for an experiment, using the existing computer and optimally synchronising to the software for data acquisition (e.g. time depending temperature and humidity control of a gas-sensing amperometric cell, sampler in a FI manifold e.t.c.). The resident program shown here is a very versatile, cheap and easy to operate software. It offers data acquisition and evaluation and can be upgraded to an automatic one. The software offers high immobilization efficiencies, which could not be realized manually. Valve control is also very powerful for any kind of FIA experiment like routine, relative standard deviation, Simplex Optimisation etc., since it allows the selection of different sample volumes with high accuracy and reproducibility, without replacing the loop.

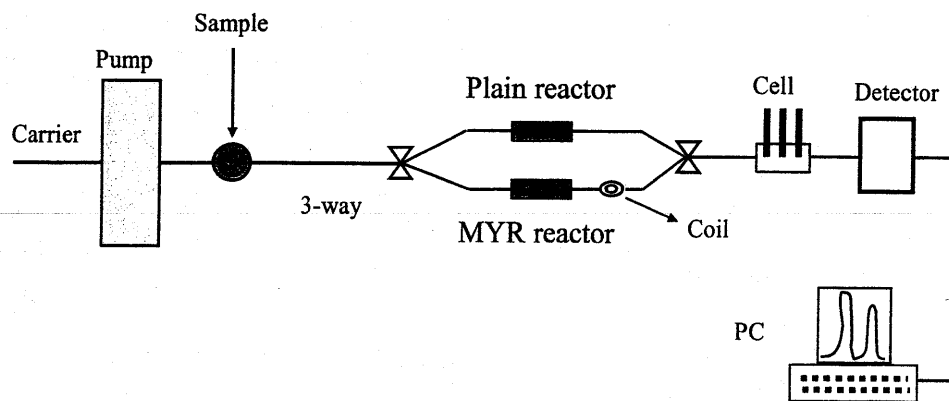


Figure 3. Schematic representation of FI manifold employed for the determination of myrosinase.

More specifically the TSR has the full control of the pump and the injection valve gives to the user the ability:

- 1) to work without manual manipulations,
- 2) to repeat an experiment many times in order to improve statistical parameters (average, relative standard deviation)
- 3) to measure different sample volumes with high accuracy and reproducibility, needed during a "Simplex Optimisation" experiment, without replacement of the loop.

The full pump control allows also a good enzyme immobilization, by circulating the enzyme solution through a reactor bearing the support, for any desired circles and periods on both flow directions.

4. Worked enzymic methods based on packed-bed reactors

4.1. Malic acid

Malic acid is predominant acid in many fruits and vegetables and the second highest in citrus fruits. Except in fruit industry, malic acid determination is of great importance in the wine industry. Prodromidis *et al.* [14], immobilized the enzymes malate dehydrogenase and diaphorase in isothiocyanate-CPG and have proposed the flow determination of malate in fruits and vegetables by monitoring the oxidation of NADH at +0.3 vs. Ag/AgCl in the presence of ferricyanide as mediator. A relative error of 4% and 2% was respectively calculated, compared with a reference photometric method. Enzyme reactors have shown an extended operational and storage stability reducing thus the cost per analysis. Using the same enzymic pathway a reagentless enzyme electrode for malate was proposed by Maines *et al.*, based on an unplasticised spin coated PVC/polycarbonate resin, by adding ascorbate oxidase in order to eliminate ascorbates interference [15]. The proposed electrode is able to determine malate in undiluted neutral or acidic media. The device responds linear up to 20 mM (pH 3.3) or 40 mM (pH 7.8) of malates.

4.2. Glycerol

Glycerol is the most important secondary product of alcoholic fermentation contributing to the smoothness and viscosity of a wine with a favorable effect on the taste [10]. A FIA manifold, incorporating amperometric detection and enzyme reactor for glycerol determination in alcoholic beverages, has been proposed

by Prodromidis *et al.* [16]. The reactor is based on the glycerol dehydrogenase system, and the enzyme was immobilized through chemical modification onto aminopropyl and isothiocyanate CPGs, aminopolystyrene resin and *m*-aminobenzylxymethyl cellulose. NADH, the product of the enzymatic reaction, was monitored at + 0.5 V vs. Ag/AgCl. The reactors are stable for a period over of 3 months or after about 2500 injections, which comes first. The successive application of the method was certified by comparison with a reference method. The mean relative error was 2.2% and the recovery 95-102%.

4.3. Total glucosinolates

Tsafoulis *et al.* described for the first time an amperometric flow analyzer, based on the biosensor concept, capable of determining total glucosinolates in real samples [17]. Myrosinase was immobilized on aminopropyl-modified CPG, which was then used for the construction of a packed bed reactor (Figure 3).

Myrosinase catalyses the hydrolysis of glucosinolates (sinigrin) to glucose, which by the action of glucose oxidase is then oxidized to hydrogen peroxide (Fig.4); The reaction might lead also to some other potentially interfering products depending on the experimental conditions. The glucose enzyme-electrode is based on a multi-membrane architecture, and was mounted on an amperometric flow cell (hydrogen peroxide detection at a platinum anode poised at +0.65 V vs. Ag/AgCl/3KCl). Different membrane types and different activation procedures were tested. The system was optimized for various working parameters, either as a glucose electrode or as a glucosinolate analyzer. The interference effect of various compounds was also investigated.

Application of the method to real samples was carried out using glucose/glucose, hydrolyzed sinigrin and glucose/sinigrin solution as calibrators of the glucose electrode and the glucosinolate analyzer. Deviations were observed due to the enantio-selectivity of glucose oxidase against the β -glucose anomer, which represent only 64% in a glucose solution in equilibrium after mutarotation, whereas myrosinase produces *in situ* only the β -anomer of glucose. A detailed data elaboration protocol is also proposed. The possibility of the simultaneous determination of glucose and glucosinolates is also demonstrated.

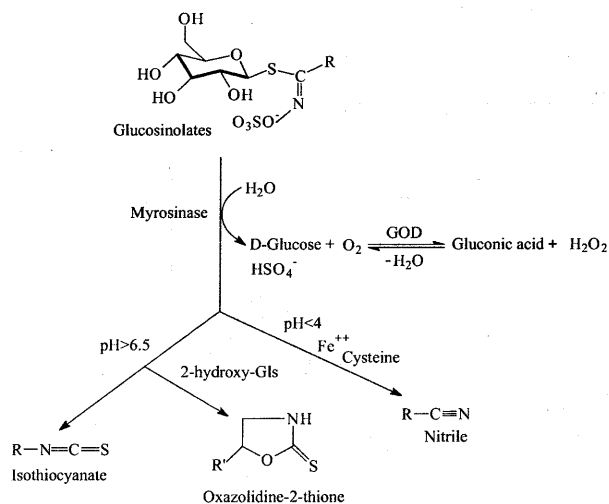


Figure 4. Enzymic pathways of the hydrolysis of glucosinolates and the oxidation of glucose.

5. Multi membrane based biosensors

Polymeric membranes are the most commonly reported matrix for the immobilization of enzymes. Different types of polymeric films were implemented in various capacities in amperometric biosensor designs. They are ordered as conductive, non conductive and composite. Casting, spin coating or electropolymerisation can be applied for the preparation of the films. Their use in amperometric biosensors is very frequently reviewed [10,18,19].

5.1. Ascorbic acid (Vitamin C)

The determination of ascorbic acid is of great value in food industry as an important nutrition compound since it is widely used as preservative because of its redox properties. The majority of the proposed methods are based on voltammetric chemical sensors rather than biosensors, since various selective approaches, based on perm-selective membranes and specific compounds, have been successfully tested in natural samples.

Karayannis and co-workers [20] developed an ascorbate sensor based on a glassy carbon electrode modified with a controlled porosity cellulose acetate polymeric film bearing 2,6-dichlorophenolindophenol. The modified electrodes were mounted in a flow injection (FI) manifold, poised at +100 mV vs. Ag/AgCl at pH 6.5. This was utilized for the determination of ascorbic acid in beverages and juices, where good correlation with a reference method was attained. The sensors showed very good repeatability and operational stability with a recovery of 92-110%.

Controlled porosity cellulose acetate (CA) membranes are found to be a powerful tool for developing chemical or biosensors with high selectivity over the analyte of interest [11]. Of course, its usefulness is restricted to low molecular weight compounds, such as sulfide, ascorbic acid, dopamine, paracetamol, phenols etc. The effect of the hydrolysing procedure on the porosity of the cellulose acetate membrane was recorded on-line utilizing three electroactive species representing a range of molecular weights from 32 to 290. By using untreated glassy carbon electrodes,

covered with cellulose acetate membrane, different intensities of currents were recorded, due to the oxidation of the sodium sulphide, uric acid and DCPI, dependent on the hydrolysis time. This is well illustrated in Fig.5.

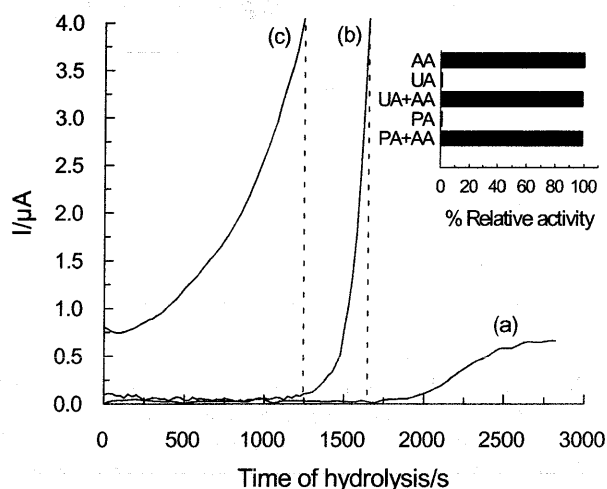


Figure 5: Permeability study of the CA membranes. Glassy carbon electrodes covered with unmodified CA were immersed in a 0.07 M KOH solution (pH 12) in the presence: (a) 0.1 mM DCPI at 0.3 V, (b) 3 mM uric acid at 0.6 V and (c) 3 mM sulphide at 0.9 V. Inset: Interference effect of 4mM uric acid and 4mM paracetamol on the flow assay of 0.4 mM ascorbic acid with a CA/DCPI-CME hydrolysed for 25 min in 0.07 M KOH. Buffer: 0.05 M phosphate in 0.05 M KCl, pH 6.5. Flow rate 0.32 ml min⁻¹. Sample volume: 130 μl. Applied potential: +0.1 V.

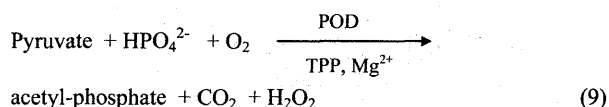
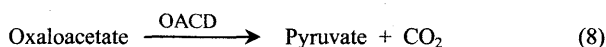
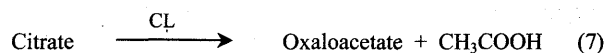
As it can be seen from Fig.5, at 1200 s (after 20 min of hydrolysis) sulphide have passed through the membrane, while no uric acid has reached the electrode surface. At 1600 s (after about 25 min of hydrolysis) uric acid was almost quantitative oxidized, while at this extent of hydrolysis DCPI penetration is still blocked by the membrane.

Improvement of the selectivity can be further achieved by making use of the enhanced relative reactivity of DCPI against specific compounds, which although they have similar molecular weights their reactivity is different. For example in a sample containing ascorbic acid, uric acid or paracetamol, the reactivity of DCPI with these compounds is different. This is shown in the inset of Fig.5 where the response of 0.4 mM ascorbic acid (taken as 100%) is compared with those of pure solutions of 4 mM uric acid and 4mM paracetamol. The interference effect of these compounds was also evaluated with the method of mixed solutions in the presence of 0.4 mM ascorbic acid (Fig.5, inset). All these compounds have access to the electrode surface after a hydrolysis period of 25 min. However no interference from uric acid or paracetamol was occurred since DCPI does not react with uric acid and paracetamol and therefore they cannot be oxidized at 0.1 V.

5.2. Citric acid

Citric acid is present in numerous natural products. Several fresh fruits such as lemons and limes owe their sharp taste to the presence of the citrate anion. Citric acid is also an additive

in the industry, mainly as a preservative and an acidulant. Due to the instability of the citrate lyase (CL) a few papers have been published dealing with biosensors based on immobilized CL. The proposed membrane architecture is based on a sequence of the enzymes, CL, oxaloacetate decarboxylase (OACD) and pyruvate oxidase (POD) in the presence of its co-factor, thiamine pyrophosphate (TPP), according to the following scheme:



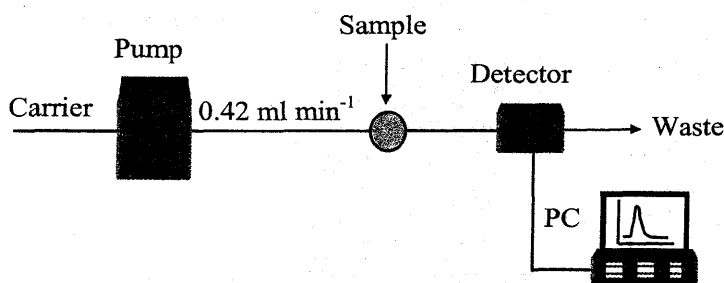
Prodromidis *et al.* [21] proposed an enzymic method for the determination of citric acid in fruits, juices and sport drinks. The method is based on the action of the enzymes citrate lyase in soluble form and oxaloacetate decarboxylase and pyruvate oxidase in immobilized form. A multi-membrane system, consisting of a cellulose acetate membrane for the elimination of interferants, an enzymic membrane and finally a protective polycarbonate membrane were placed on a Pt electrode and used with a fully automated flow injection manifold. The mean relative error was 2.4% compared with a standard enzymatic method (Boehringer F-kit). An 8-10% loss of the initial activity of the sensor was observed after 100-120 injections.

Karayannis and colleagues developed an enzyme electrode for extended linearity of citrate measurements based on modified polymeric membranes [22]. Co-immobilization of POD/OACD and CL as well as of the co-factors FAD and TPP onto a high protein binding membrane of mixed cellulose ester was proposed. pH independent rejection of ascorbate was achieved by the use of a novel cellulose acetate membrane incorporating isopropyl myristate. Extended linearity up to 20 or 100 mM citrate was achieved utilizing outer membranes of unplasticized PVC/polycarbonate resin or PVC/Pluronic F-68, respectively.

5.3. Glycolic acid

The first enzyme-based biosensors capable of determining glycolic acid in various complex matrices such as cosmetics, instant coffee and urine was proposed by Tsiafoulis *et al.* [23].

(A)



(B)

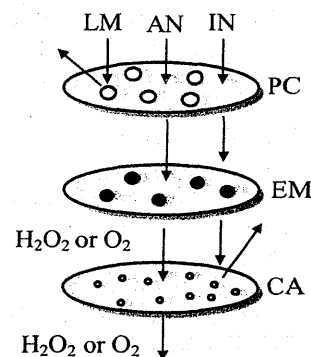


Figure 6: (A) Schematic representation of FI manifold employed for the determination of glycolic acid. (B) Assembly of the biosensors; LM, large molecules, AN, analyte; IN, interferants; CA, cellulose acetate membrane, EM, enzyme membrane (Ultradbind/GIOD for flow method, MF/GIOD/CAT for oxygen electrode; PC, polycarbonate membrane.

Two separate designs, both based on a three-membrane configuration consisting of an inner cellulose acetate membrane (CA) and an outer polycarbonate membrane (PC), which sandwich a third membrane bearing the bio-molecule, are proposed. Glycolate oxidase was immobilized onto a modified polyethersulfonate membrane by means of chemical bonding and glycolate oxidase/catalase enzyme mixture was immobilized into a mixed-ester cellulose acetate membrane through physical adsorption (Fig.6). The membrane assemblies were mounted on an amperometric flow cell (hydrogen peroxide detection at a platinum anode poised at +0.65 V vs. Ag/AgCl/3KCl) or on an oxygen electrode, respectively. Both configurations were optimized in respect of various working parameters. The proposed biosensors are interference-free to common electroactive species and were successfully applied for the determination of glycolic acid in various samples showing an excellent agreement with a reference photometric method. The validity of the proposed method in samples, where the reference method was not applicable, was tested with recovery studies. Values of 102±1% were obtained. Inherent interference of oxalic acid was manipulated by using a primary amine-containing buffer and the enzyme catalase. Both systems were designed so that they are compatible with the current technology of the most widely used commercial analyzers.

6. Instrumentation and cell design

A need exists for inexpensive and fast analyses of materials in various routine procedures. These requirements have created opportunities for measuring analytes in flowing liquids. It is well established that Flow Injection Analysis (FIA) is the most commonly used technique to achieve these tasks. Schematic diagrams of some commercially available flow cells that have been extensively used in flow applications are shown in Fig. 7. Recently a new electrochemical cell is provided by TraceBiotech AG (Braunschweig, Germany) under the brand name TRACE Flow cell. This cell is available in different geometries and has been equipped with dual-channel sensor-chips, allowing thus multiple and difference measurements [10].

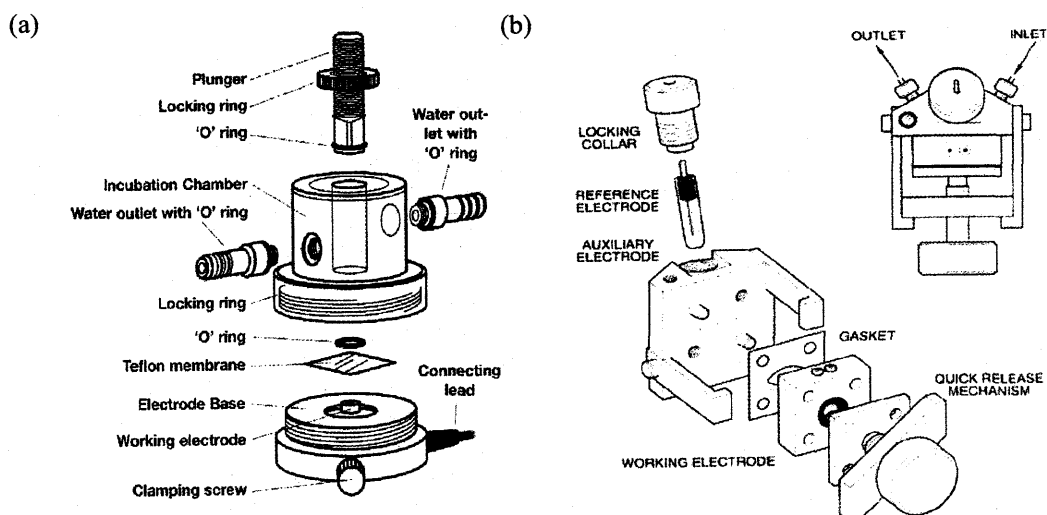
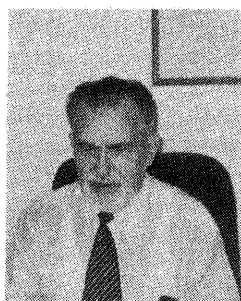


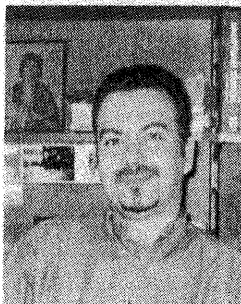
Figure 7. Commercial flow and batch cells: (a) Oxygen electrode assembly by Rank Brothers Ltd. (Cambridge, UK), (b) Thin layer flow-through cell by BAS (IN, USA).

References

- [1] F. Scheller, F.Schubert, *Biosensors*, Elsevier, Amsterdam, 1992.
- [2] A.P.F. Turner, I. Karube, G.S. Wilson., *Biosensors, Fundamentals and Applications*, Oxford University Press, Oxford, 1987.
- [3] A.B.Florou, M.I.Prodromidis, M.I.Karayannis and S.M. Tzouwara-Karayanni, *Electroanalysis*, **10**, 1261 (1998).
- [4] A.B.Florou, M.I.Prodromidis, M.I.Karayannis and S.M. Tzouwara-Karayanni, *Talanta*, **52**, 465 (2000).
- [5] M. I. Prodromidis, A. B. Florou, S. Tzouwara-Karayanni and M.I. Karayannis, *Electroanalysis*, **12**, 1498 (2000).
- [6] M.I..Prodromidis, P.G.Veltsistas and M.I.Karayannis, *Anal. Chem.*, **72**, 3995 (2000).
- [7] M.I.Prodromidis, P.G.Veltsistas, C.E.Efstathiou and M.I. Karayannis, *Electroanalysis*, **13**, 960 (2001).
- [8] I. Katakis, E. Dominquez, *Mikrochim. Acta*, **11**, 126 (1997).
- [9] L. Gorton, *Electroanalysis* **7**, 23 (1995).
- [10] M.I. Prodromidis, M.I. Karayannis, *Electroanalysis* **14**, 241 (2002)
- [11] A.B. Florou, M.I. Prodromidis, M.I. Karayannis, S.M. Tzouwara-Karayanni, *Electroanalysis* **12**, 361 (2000).
- [12] J. Wang, *Anal. Chim. Acta* **41**, 234 (1990).
- [13] M.I. Prodromidis, A.B. Tsibiris, M.I. Karayannis, *J. Autom. Methods Manage. Chem.*, (Former: *J. Autom. Chem.*), **17**, 187 (1995).
- [14] M.I. Prodromidis, S.M. Tzouwara-Karayanni, M.I. Karayannis, P. Vadgama, A.Maines, *Analyst* **121**, 435 (1996).
- [15] A. Maines, M.I. Prodromidis, S.M. Tzouwara-Karayanni, M.I. Karayannis, D.Ashworth, P. Vadgama, *Anal. Chim. Acta* **408**, 217 (2000).
- [16] M.I. Prodromidis, C.D. Stalikas, S.M. Tzouwara-Karayanni, M.I. Karayannis, *Talanta* **43**, 27 (1996).
- [17] C.G. Tsiafoulis, M.I. Prodromidis, M.I. Karayannis, *Anal. Chem.*, **75**, 927 (2003).
- [18] S.A. Erm, A.M. Yacynych, *Electroanalysis* **7**, 913 (1995).
- [19] G. Bontempelli, N. Comisso, R. Toniolo, G. Schiavon, *Electroanalysis*, **9**, 433 (1997).
- [20] A.B. Florou, M.I. Prodromidis, M.I. Karayannis, S.M. Tzouwara-Karayanni, *Anal. Chim. Acta*, **409**, 113 (2000).
- [21] M.I. Prodromidis, S.M. Tzouwara-Karayanni, M.I. Karayannis, P. Vadgama, *Analyst* **122**, 1101 (1997).
- [22] A. Maines, M.I. Prodromidis, S.M. Tzouwara-Karayanni, M.I. Karayannis, D.Ashworth, P. Vadgama, *Electroanalysis* **12**, 1118 (2000).
- [23] C.G. Tsiafoulis, M.I. Prodromidis, M.I. Karayannis, *Anal. Chem.*, **74**, 132 (2002).



Prof. M.I. Karayannis



Dr. M.I. Prodromidis

(Received May 6, 2004)