Some clinical applications of the electrochemical biosensors

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Abstract: Electrochemical biosensing, due to its sensitivity and specificity, combined with the low-cost and operation convenience of the equipment, is considered as a promising point-of-care approach in clinical analysis. This review presents the basic principles of operation, the current status, and the trends in the development and the clinical implementation of some selected electrochemical biosensors. These include: electrochemical glucose biosensors successfully applied in diabetes management, and electrochemical biosensors for cholinesterases and trypsin activities determination. The latter, although less common, demonstrate the potential of improving the existing clinical methods in the diagnostics and the treatment of neurotoxic, neurological, and pancreatic diseases.

Keywords: Biosensors, clinical analysis, cholinesterases, glucose, trypsin.

INTRODUCTION

The improvement of the analytical techniques for the detection of physiologically important species for the purposes of the medical diagnosis and therapy has always been a major defy to medical science. The progress in clinical analysis, remembering the beginning, 100 years ago, when interferences and errors accompanied the determinations because of the use of lab-produced reagents and a lack of knowledge, is spectacular [1]. A significant shift was experienced during the 1970s due to two major innovations: instruments automation and reagents preparation industrialization. These contributions served as a starting point to answer the growing demand of clinical analysis, in concert with the enhancement of the understanding of the diseases provoking factors. The industrial production of a large quantity of reagents ensured their standardization, as well as the quality improvement. On the other hand, the automation allowed analyzing a considerable amount of samples and drastically decreasing the error factors, although the first automated systems used high volumes of substances and were difficult to operate. Nowadays, vital benefits are obtained from the application of test kits and transportable, portable, and handheld instruments, thus promoting the implementation of Point-of-Care Testing (POCT) systems [2]. POCT, performed conveniently and immediately to the patient, allows a rapid clinical management decisions to be made. The role of point-of-care diagnostics in developing world in particular, the appropriate diagnostic technologies already in distribution, as well as the emerging technologies, and the related technical needs and technical barriers are extensively reviewed by Yager *et al.* [3].

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POCT approach created new challenging problems, involving the development of cheaper, smaller, faster, and smarter POCT devices with improved performance characteristics.

The electrochemical biosensors are considered as a new generation of POCT systems able to supply specific, sensitive, accurate and cost-effective *in situ* and *on line* measurements in a real time, without or with a minimum sample preparation. Because of the requests of the clinical analysis, governing the biosensors application market, it is expected that the registered in 2010 growth of the world biosensor industry of US$ 7 billion will reach US$12 billion by 2015 [4].

The present work is intended to provide an overview on the basic principles of operation, the current status, and the trends in the development and the clinical application of some electrochemical biosensors of crucial importance for the diagnostics and treatment of diabetes, neurotoxic, neurological and pancreatic disorders, such as the biosensors for glucose quantification and for cholinesterases and trypsin activities determination.

ELECTROCHEMICAL BIOSENSORS

The electrochemical biosensor is an analytical device designed by coupling a biological recognition element and an electrochemical transducer [5]. The transducer converts the analytical signal produced as a result of the biochemical and electrochemical interactions into measurable electrical one. The biorecognition component of the biosensor is typically biocatalytic (enzymes, cells, cell organelles, tissues) or biocomplexing (antibodies, biomimetic materials, cell receptors, nucleic acids), according to the biological specificity-confering event. It is immobilized or retained in direct spatial contact with the electrochemical transducer. The biorecognition element being often an enzyme, the term "enzyme immobilization" was defined at the First Enzyme

1875-5607/12 $58.00+.00

Engineering Conference held at Hennicker, NH, USA, in 1971. It describes "enzymes physically confined at or located in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" [6]. Immobilization methods are classified as physical and chemical and include [5]: adsorption, entrapment behind a membrane, within a polymeric matrix, or within self-assembled monolayers, covalent bonding, and bulk modification of entire electrode material (carbon paste or graphite epoxy-resin), among other.

The applied electrochemical transduction mode is commonly potentiometric or amperometric one [5]. The potentiometric determinations are based on the measurement of the potential of an electrochemical cell comprising an indicator and a reference electrode. The potential of the reference electrode remains constant, while the potential of the indicator electrode and consequently the cell potential vary as a function of the analyte concentration, according to the Nernst equation. The logarithmic character of the response of the indicator electrode determines the wide linear concentration range of the calibration plot (3-4 decades), but also the unsatisfactory accuracy of the analysis. In terms of accuracy, the amperometric detection suits better the analytical requirements. The amperometry involves the measurement, at a constant potential, of the current response of an indicator electrode, as a function of the concentration of the present electroactive specie. The technique is sensitive and allows in addition controlling the process through the applied electrode potential.

CLINICAL APPLICATIONS OF THE ELECTROCHEMICAL BIOSENSORS

Electrochemical Glucose Biosensors

Glucose is the chief energy supplier for almost all of the bodies' cells and a starting point for a number of important metabolic pathways [7]. Thus, glucose concentration monitoring in biological fluids is of primary importance in clinical diagnostics and a biochemical test for the assessment of the carbohydrate metabolism impairment, namely diabetes, hypoglycemia, and various endocrine disorders. In 2000, according to the World Health Organization (WHO), 2.8% of the population of the world suffered from diabetes, and this number will double by 2030 [8]. Diabetes ranks among the leading 10 causes of death in Mexico affecting 3.7 millions of people [9]. The WHO criteria for the diagnosis of diabetes, based on the establishment of the glucose levels in whole blood and plasma are presented in Table **1** [10].

Glucose determination in whole blood, serum and plasma is performed currently applying spectrophotometric enzymatic methods, such as the standard glucose oxidase-peroxidase method of Saifer and Gerstenfeld [11], and the reference hexokinase one [12, 13]. The electrochemical glucose biosensors preserve the specificity of the enzymatic analysis, but they take also advantage of the biological component immobilization and of the sensitivity and rapidity of the electrochemical detections. In addition, they are very suitable for continuous and *in-vivo* monitoring of blood glucose, in contrast to the mentioned spectrophotometric techniques.

Three generations of electrochemical biosensors for glucose quantification are developed until now [14]. The principle of their operation is illustrated by the following reaction scheme:

$$\beta\text{-D-glucose} + E_{ox} = E_{red} + \text{gluconic acid} \quad (1)$$

$$E_{red} \rightarrow E_{ox} \quad (2)$$

where E_{ox} and E_{red} are the oxidized and the reduced forms of the enzyme glucose oxidase.

E_{red} conversion into E_{ox} is achieved in various ways: by E_{red} oxidation with O_2 (Eq. 3) in the first generation glucose biosensors, by E_{red} oxidation with a redox mediator M (Eq. 4) in the second generation glucose biosensors, and by direct E_{red} oxidation on the electrode surface (Eq.5) in the third generation glucose biosensors:

$$E_{red} + O_2 = E_{ox} + H_2O_2 \quad (3)$$

$$E_{red} + M_{ox} = E_{ox} + M_{red} \quad (4)$$

$$E_{red} \rightarrow E_{ox} \quad (5)$$

The analytical signal of the biosensors is respectively the current of H_2O_2, M_{red}, and E_{red} oxidation (Eqs. 6, 7 and 5).

$$H_2O_2 \xrightarrow{\text{electrode}} 2H^+ + O_2 + 2e^- \quad (6)$$

$$M_{red} \xrightarrow{\text{electrode}} M_{ox} + ne^- \quad (7)$$

The first generation electrochemical biosensors suffer from two main drawbacks [15, 16]: the fluctuations of the oxygen concentration influencing the output signal and the presence in the biological fluids of electroactive species such

Table 1. WHO Criteria for the Diagnosis of Diabetes

Diagnosis	Sampling Time	Glucose Concentration (mmol/L)		
		Whole Blood, Venous	Whole Blood, Capillary	Plasma/Serum
Normal	Fasting		< 5.6	< 6.4
Diabetes mellitus	Fasting	> 6.7	> 6-7	> 7.8
	At 2 hours of glucose load	> 10.0	> 11.1	> 11.1
Impaired glucose tolerance	Fasting	< 6.7	< 6.7	< 7.8
	At 2 hours of glucose load	6.7 – 10.0	7.8 - 11.1	7.8 - 11.1

as ascorbic acid, uric acid, and gluthathione, interfering with the electrochemical H_2O_2 oxidation. In spite of these problems, the first generation electrochemical biosensors for glucose analysis were commercialized [17, 18].

In second generation electrochemical biosensors for glucose determination the natural glucose oxidase substrate (oxygen) was substituted by an artificial electron acceptor (mediator), allowing the electrochemical measurements to be performed at low electrode potential, thus avoiding the interferences and the O_2 dependence. As mediators were successfully used ferrocene, ferricyanide, quinones, tetrathialfulvalene, tetracyanoquinodimethane, methyl viologen, and so forth [14]. The standard potentials (vs. Ag/AgCl) of some commonly used mediators in glucose oxidase electrochemical sensors of second generation are as low as: −0.370 V for benzyl viologen, -0.188 V for indigo disulfonate, +0.137 V for 2,5-dihydroxybenzoquinone, +0.216 V for ferrocenemethanol, +0.217 V for methylene blue, etc [19]. In 1987 MediSense launched the pen-sized ExactechTM glucose sensors of second generation.

Promising results have also been obtained combining the artificial mediators with glucose dehydrogenase, e.g. the highly specific and stable FAD-dependent glucose dehydrogenase [20-23]. The latter came to substitute the water soluble quinoprotein glucose dehydrogenase, which exhibits low substrate specificity and lack of thermal stability.

The approach applied in the third generation electrochemical biosensors for glucose analysis seems to be the most efficient. As known, glucose oxidase could be directly oxidized at the surface of Hg, Au, Ag, and glassy carbon electrodes in the potential range of -0.30 to -0.70 V/SCE. Nevertheless, the oxidation rate is low and the results are not enough reproducible. Although the issue is questionable [14, 24-33], it could be assumed that the electrode material modification with conducting organic salts favors the heterogeneous electron transfer between the glucose oxidase and the electrode surface. The first used for this purpose was the *N*-methylphenazinium salt of the tetracyanoquinodimethane [24, 33]. Of particular interest is the complex tetrathiafulvalene-tetracyanoquinodimethane in 1:1 ratio, because of its higher conductivity [29, 30]. The commercialization of this technology is still not available.

The review of the recent research activities on glucose biosensing demonstrates that current efforts are focused on the development of nanomaterials-based electrochemical glucose biosensors with improved selectivity and sensitivity and enhanced direct electrochemistry of glucose oxidase.

The nanotechnological approach in electrochemical biosensors development [34-49] takes advantage of the electrocatalytical properties of the nanostructures, their action as electron transfer mediators or electrical wires, large surface to volume ratio, structural robustness, and biocompatibility. Therefore, it yields the following chief issues: electrode potential lowering, enhancement of the electron transfer rate with no electrode surface fouling, sensitivity increase, stability improvement, and interface functionalization. Nevertheless, only few publications comment on the development, in the last years, of electrochemical glucose biosensors using single type nanomaterials. Some of them are those incorporating Pt [50], Au [51] or iron [52] nanoparticles, nano-ZnO particles and nanotubes [53-55], mesoporous silica particles [56], or modified with redox mediators graphene oxide [57], carbon fibers [58-60], carbon nanotubes [61-64], and ZnO [57, 65]. The majority of the electrochemical glucose biosensors designed recently incorporates nanocomposites with improved nanomaterials dispersity, and new or enhanced mechanic, catalytic, electric or magnetic properties. These include:

- Natural and synthetic polymer-matrix nanocomposites, such as: chitosan-Prussian blue-multiwall carbon nanotubes-hollow PtCo nanochains films [66], palladium nanoparticles-chitosan-grafted graphene [67], chitosan dispersed Pt nanoparticles supported on carbon nanotubes [68], thulium(III) hexacyanoferrate(II) nanoparticles within a chitosan film [69], Ag nanoprisms-chitosane [70], poly-ciclodextrin-carbon nanotubes [71], and Au nanoparticles modified synthetic polymers [72];
- Sol-gel composites, integrating: carbon nanotubes [73], carbon nanotubes/polyacrilonitrile [74], Pt nanoparticles [75], or Au nanoparticles [76];
- Metal, metal oxides, and carbon nanotubes hybrid materials derived from: Au nanoparticles and carbon nanotubes [77, 78], Au nanocrystals growing on ZnO nanorods [79], Au nanoparticles deposited on TiO_2 nanotubes [80], Ni^{2+}/MgFe layered double hydroxide [81], and Pt nanoparticles electrodeposited on iron oxide-carbon nanotubes [82], among other.

Investigating direct glucose oxidase electrochemistry and developing a third generation biosensors is the other challenging problem in glucose biosensing. It has been demonstrated that the carbon-based nanomaterials, such as the carbon nanotubes [83, 84] the boron-doped carbon nanotubes [85], the functionalized TiO_2 [86] coated carbon nanotubes, the graphene [87, 88], and the graphene composites [89] show excellent electron-transfer capabilities. The reported apparent heterogeneous electron transfer rate constants range between 1.08 s^{-1} and 5.9 s^{-1} [83, 89], thus confirming nanomaterials efficiency in promoting the direct electron exchange between the electrode surface and the glucose oxidase molecules. Special attention has to be paid to graphene and its composites. Graphene consists in a one atom thick carbon sheet (sp^2 hybridized), with a surface area nearly twice as large as that of single walled carbon nanotubes [47], good electrical conductivity and electrocatalytic ability, and capability to form charge-transfer complexes. Nanocomposites integrating graphene and metal nanoparticles or nano-sized CdS exhibit enhanced electron transfer properties, due to synergy effects [89].

Latest trends in electrochemical glucose biosensors development have been extensively reviewed by a number of authors [15, 17, 41, 89-94]. The analytical performances of some relevant recently developed electrochemical glucose biosensors are summarized in Table **2**.

Table 2. Analytical Performances of Some Relevant Recent Electrochemical Glucose Biosensors

Surface Modification	Detected Specie	Sensitivity	LOD	Reference
Au/PtNPs/oPD	H_2O_2 at 0.3 V/Ag, AgCl	1.2 mA mM^{-1} cm^{-2}	3.0 μM	[50]
Nano-ZnO (transferred)	H_2O_2 at 0.8 V/Ag, AgCl	15.46 μA mM^{-1} cm^{-2}	0.05 mM	[53]
Nano-ZnO (grown)	H_2O_2 at 0.8 V/Ag, AgCl	23.43 μA mM^{-1} cm^{-2}	0.01 mM	[53]
CNT/felt/CuHCNFe/Ppy	H_2O_2 at 0.0 V/Ag, AgCl	194 μA mM^{-1}	10 μM	[61]
SWCNT/PVI-Os	0.3 V/Ag, AgCl	32 μA mM^{-1} cm^{-2}	0.07 μM	[62]
SWCNT/polyBCB	polyBC at -0.25 V/SCE	N	1.0 μM	[63]
CS/PB/MWNT/H-Pt(Co)	PB at -0.1 V/SCE	23.4 μA mM^{-1} cm^{-2}	0.47 μM	[66]
CS-GR/PdNPs	H_2O_2 at 0.7 V/SCE	31.2 μA mM^{-1} cm^{-2}	0.2 μM	[67]
CS/Pt/CNT	GOD at 0.3 V/SCE	41.9 μA mM^{-1} cm^{-2}	0.4 μM	[68]
CS/TmHCF NPs	SWVA	2.35 μA mM^{-1}	6.0 μM	[69]
AgTNPs/CS	H_2O_2 at 0.6 V/Ag, AgCl	67.67 μA mM^{-1} cm^{-2}	1 μM	[70]
AuNPs/SNS-NH_2	O_2 at -0.7 V/Ag, AgCl	1.597 μA mM^{-1} cm^{-2}	2.1 μM	[72]
CS/PB/MWNT	PB at -0.1 V/SCE	15.2 μA mM^{-1} cm^{-2}	7.5 μM	[73]
AuNPs/MWCNT	Ferrocenmethanol at 0.3 V/SCE	19.27 μA mM^{-1} cm^{-2}	2.3 μM	[77]
AuNPs/TiO_2NT	GOD at -0.25 V/Ag, AgCl	N	0.31 mM	[80]
CNT/TiO_2-NH_2	GOD at -0.35 V/Ag, AgCl	7.0 μA mM^{-1}	0.44 μM	[86]
GR	GOD at -0.47 V/SCE	110 μA mM^{-1} cm^{-2}	0.01 mM	[87]
Poly(ViBulm$^+$Br$^-$)/GR	GOD by CV: -0.1 to -0.7 V/Ag, AgCl	0.77 μA mM^{-1}	0.267 mM	[88]
GR/CdS	GOD by CV: -0.1 to -0.7 V/Ag, AgCl	1.76 μA mM^{-1} cm^{-2}	0.7 mM	[89]
CS/BCNi	GOD at -0.2 V/SCE	0.25 μA mM^{-1}	8.33 μM	[94]
PB-Au/Pt-NCs	PB at -0.15 V/SCE	2.77 mA M^{-1}	1.0 μM	[95]
PtNPs/OMC	H_2O_2 at -0.1 V/Ag, AgCl	0.38 μA mM^{-1}	0.05 μM	[96]
AuNPs	O_2 at -0.7 V/Ag, AgCl	9.42 mg L^{-1} O_2 depletion	3.5 μM	[97]
OMC/AuNPs	DPV at -0.1 to -0.7 V/Ag, AgCl	4.34 μA mM^{-1}	N	[98]
PPMH	GOD at 0.2 V/Ag, AgCl	N	0.05 mM	[99]

AgTNPs: triangular silver nanoprisms; AuNPs: Au nanoparticles; BCB: brilliant cresyl blue; CNT: carbon nanotubes; CS: chitosan; CS/BCNi: chitosan boron doped nickel nanoparticles; CS-GR: chitosane grafted graphene; CV: cyclic voltammetry; DPV: differential pulse voltammetry; GOD: glucose oxidase; MWCNT: multiwalled carbon nanotubes; MWNT: multiwalled nanotubes; OMC: ordered mesoporous carbon; oPD: o-phenylenediamine; PB: Prussian blue; PdNPs: Pd nanoparticles; Poly(ViBulm$^+$Br$^-$)/GR: poly(1-vinyl-3-butylimidazolium bromide)-graphene; PPMH: poly-phenantroline monohydrate; PPy: polypyrrol; Pt-NCs: Pt nanoclusters; PVI: Poly(1-vinylimidazole); SWCNT: single walled carbon nanotubes; SWVA: square wave voltammetry; TmHCF: thulium(III) hexacyanoferrate(II).

Electrochemical biosenSors for Cholinesterases Activity Determination

The cholinesterases are a family of enzymes, belonging to the class of the hydrolases [100]. The acetylcholinesterase AChE (EC 3.1.1.7) or "true" cholinesterase is present in the red cells and the neural synapses. It is involved in the transmission of the nerve impulses, catalyzing the hydrolysis of the neurotransmitter acetylcholine to choline. The reduction in the activity of the cholinergic neurons is known as a feature of Alzheimer's disease [101]. The butyrylcholinesterase BuChE (EC 3.1.1.8) or "pseudocholinesterase" is synthesized in the liver and is present in the serum. Pseudocholinesterase activity increase is associated with nephritis syndrome and myocardial infarction, while the decrease is related to lesions in the liver's parenchyma (cirrhosis, carcinomas, and acute forms of infectious and toxic hepatitis) [102]. Both acetylcholinesterase and butyrylcholinesterase are inhibited by neurotoxic substances such as organophosphorus and carbamate pesticides and warfare agents. Acetylcholinesterase inhibition results in impairing of the

transduction of the nerve impulses and induces decreased intraocular pressure, bradycardia, hypotension, hypersecretion, bronchoconstriction, prolonged muscle contraction, and death. Acetylcholinesterase inhibition is also applied for the management of Alzheimer's disease symptoms, by reducing the rate of acetylcholine break down, thus balancing the loss of acetylcholine due to the failure of cholinergic neurons. Thus, the accurate assessment of the cholinesterases activity and cholinesterases inhibition is of vital importance for the diagnostics and the treatment of neurotoxic and neurological disorders, among other.

The evaluation of the cholinesterases activity is performed using various analytical methods: spectrophotometric [103, 104], pH-metric [105, 106], conductometric [107], radiometric [108, 109], fluorimetric [109] and so forth, revised comprehensively in the recent work of Miau *et al.* [108]. The electrochemical approach to cholinesterases activity determination exploiting the unique analytical performances of the electrochemical biosensors was introduced in 1960s.

According to the transduction mode, the electrochemical sensors for cholinesterases activity determination are mainly potentiometric and amperometric. The potentiometric biosensors detect the pH shift resulting from the acid release during the enzyme catalyzed hydrolysis of the choline esters (Eq. 8), using a variety of pH-sensitive transducers, ranging from the traditional pH glass electrodes [111] to the ion-selective field effect transistors [110]. The equipment is simple, commercially available, and affordable. The detection is performed in a single step, under no current flow conditions. A shortcoming of the method, apart of the non-linearity of the biosensor response and the related error of the determination, represents the increased response time. It varies from 2 to 10 min [112] in dependence of the time needed to reach the equilibrium at the biosensor-solution interface.

The call for analytical devices with higher sensitivity, accuracy, and faster response favored the development of amperometric sensors for cholinesterases activity determination. These of first generation take advantage of the following reactions sequence:

$$\text{R-choline} + H_2O \xrightarrow{\text{ChE}} \text{choline} + \text{R-COOH} \quad (8)$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{ChO}} \text{betaine} + 2H_2O_2 \quad (9)$$

$$2H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^- \quad (10)$$

or

$$O_2 + 4e^- + 2H_2O \longrightarrow 4\ OH^- \quad (11)$$

where R is usually an acetyl or butyryl moiety. Acetylcholinesterase demonstrates a high specificity toward acetylcholine, while butyrylcholinesterase is less specific and hydrolyses a number of choline esters, including acetylcholine.

R-choline hydrolysis catalyzed by the cholinesterases (Eq. 8) does not involve electroactive species. Thus, the process was coupled with the choline oxidase catalyzed choline oxidation (Eq. 9). The current of the oxidation of the produced H_2O_2 (Eq. 10) or the current of the reduction of the consumed O_2 (Eq. 11), is registered as a sensor response. However, the possible interferences at the potential of H_2O_2 oxidation (+0.60 V vs. SCE), and the fluctuations in the oxygen concentration strained the development of the cholinesterases-based sensors of second generation with improved analytical performances.

The sensors for cholinesterases activity determination of second generation involve the enzymatic hydrolysis of acylthiocholine to thiocholine. The enzyme activity is determined by electrochemically monitoring the thiocholine formed. Two alternative routes are explored as response-generating electrochemical reactions:

- Direct electrochemical oxidation of thiocholine at 0.80 V/Ag, AgCl:

$$2(CH_3)_3N^+(CH_2)_2SH \rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2H^+ + 2e^- \quad (12)$$

- Mediated thiocholine oxidation at lower electrode potential (0.1÷0.45 V/Ag, AgCl), using cobalt phtalocyanine [113, 114], tetracyanoquinodimetane [115] or hexacyanoferrate (III) [116] as electron mediators in a heterogeneous or in a homogeneous phase, to avoid interferences:

$$2(CH_3)_3N^+(CH_2)_2SH + 2M_{ox} \rightarrow (CH_3)_3N^+(CH_2)_2S\text{-}S(CH_2)_2N^+(CH_3)_3 + 2M_{red} \quad (13)$$

$$M_{red} \rightarrow M_{ox} + e^- \quad (14)$$

The amperometric biosensors of second generation make use of a simple detection principle and of a single enzyme. The biosensor construction is straightforward, the system being monoenzymatic one. The main problems come from the spontaneous hydrolysis of the thiocholine esters, leading to overestimation of the anodic current response, the passivation of the platinum anodes by the sulfur-containing compounds, and the high potential of thiocholine oxidation (+0.80 V vs. SCE) at conventional metal and graphite transducers [117-121] as a cause of possible interferences.

The various types of cholinesterases amperometric sensors in conformity with the reactions involved are summarized by Turdean *et al.* [122]. The principle of their function is illustrated by (Figs. **1** and **2**).

The electrochemical biosensors for cholinesterases activity determination were primarily designed for toxicity analysis in environmental monitoring, food and quality control. The method is based on the quantification of the cholinesterases activity inhibition, provoked by organophosphorus and carbamate pesticides and other neurotoxic agents. Comprehensive analysis of the current state of the art is provided by a number of authors [111, 123-131]. Recent trends involve nanomaterials transducer modification and genetic engineering of the biological recognition element, to improve electrochemical biosensors performances. Various nanomaterials used as cholinesterases immobilization matrices in electrochemical biosensors for organophosphorus pesticides determination, along with biosensors performance characteristics such as sensitivity, linear dynamic range, and detection limit are evaluated and

summarized in the review work of Periasamy *et al.* [132]. As demonstrated, the nanomaterials transducer modification confers long storage stability of the biosensors, and enables organophosphorus pesticides detection in the nanomole-picomole range. The alternative route leading to biosensors sensitivity, selectivity and stability increase involves the incorporation in the biosensing platform of biorecognition elements with tailor designed properties. These performances are achieved through appropriate site-directed mutagenesis ensuring increased biorecognition element affinity for the target analyte favoring the accessibility of the active site, enhanced electron transfer, and oriented or more stable immobilization [133, 134]. Genetically modified enzymes are extensively used in inhibition based biosensors for organophosphorus pesticides determination [135-139], allowing attaining LOD as low as 10^{-17} M [140].

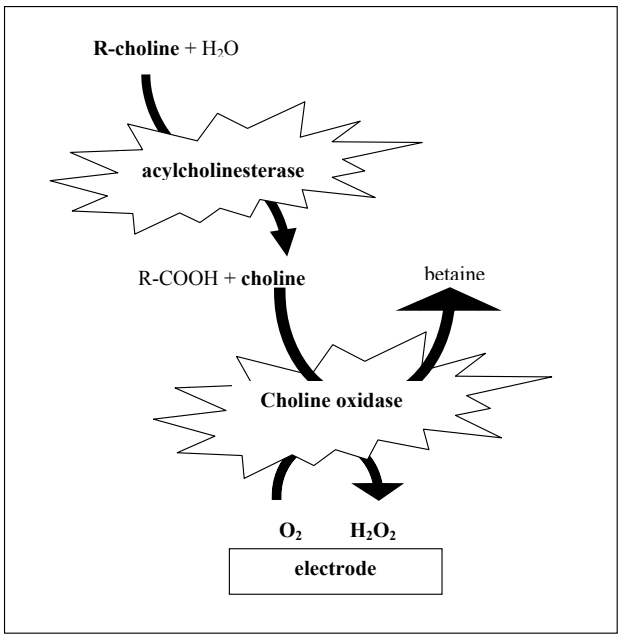

Fig. (1). Amperometric cholinesterase sensor of first generation.

The clinical application of the electrochemical biosensors for cholinesterases activity determination in blood and serum remains still limited. Simple first generation biosensors for cholinesterases activity determination in serum and amniotic fluid involving immobilized choline oxidase associated with the amperometric detection of hydrogen peroxide are reported by Palleschi [141] and Morelis [142] in 1990s.

Sigolaeva *et al.* [143] comment on the development of an electrochemical biosensor of first generation for acetylcholinesterase and butyrylcholinesterase activities measurement in blood hemolysates of mice, rates, and humans. The transducer used is graphite, modified with H_2O_2-sensitive layer of MnO_2 and choline oxidase incorporated into a self-assembled nanostructured polyelectrolyte layer. The amperometric measurements are performed at a potential of 0.35 V/Ag, AgCl and in diluted solutions, to minimize the interferences from extraneous substances in blood. The LOD achieved is of 10-20 nmol min^{-1} ml^{-1} blood. In addition, this sensor in combination with a tyrosinase-based sensor for phenols determination is applied for the simultaneous quantification of acetylcholinesterase and butyrylcholinesterase activities in test mixtures. Phenols are obtained by cholinesterases catalyzed hydrolysis of phenolic esters.

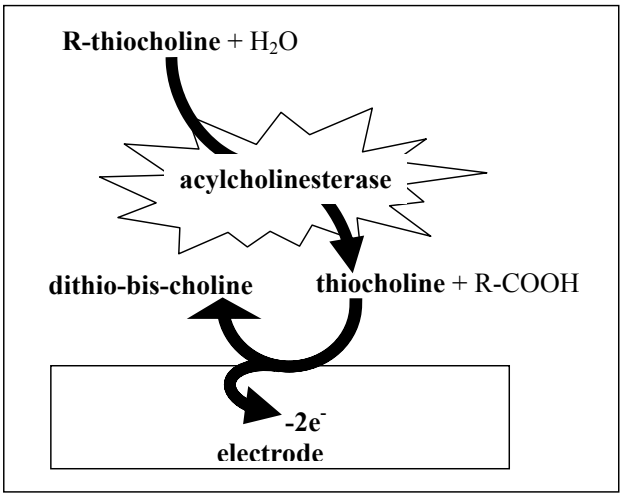

Fig. (2). Amperometric cholinesterase sensor of second generation.

Hsieh *et al.* [144] propose an amperometric flow injection biosensor of first generation for cholinesterase activity determination in human serum. The Pt working electrode is covered with a chitinous membrane, and the enzyme choline oxidase is covalently immobilized onto the membrane surface. It is demonstrated that the dynamic range of the biosensor is sufficient for diagnostic purposes.

An original bacterial electrochemical sensor for cholinesterase activity determination is developed by Stoytcheva *et al.* [145]. It is designed by coupling *Arthrobacter globiformis* and a dissolved oxygen electrode. The natural cells metabolism involves choline oxidation to betaine with oxygen consumption. Hence, current proportional to bacteria respiration is registered as a sensor response. It is correlated to the activity of the cholinesterase, catalyzing the acetylcholine hydrolysis to choline. The measurements are free of interferences: the unique electrochemical reaction taking place is the O_2 reduction, occurring behind the polymer membrane of the oxygen probe, permeable for gases only. The analytical performances of the biosensor are evaluated by cholinesterase activity determination in reconstituted lyophilized serum.

Several works are devoted to the "*in vitro*" study of the kinetics of cholinesterases inhibition and reactivation by using electrochemical biosensors for cholinesterases activity determination both of first, and of second generation [146-154]. Results from such investigations are useful for toxicity effects studies, for the development of appropriate antidotes, and for drug sensitivity evaluation in the treatment of dementia.

Electrochemical Biosensors for Trypsin Activity Determination

Trypsin (EC 3.4.21.4) is an enzyme of the class of the serine proteases, found in the human digestive system [155]. It is produced by the pancreas as trypsinogen (inactive enzyme) and is then activated in the duodenum by the intestinal enterokinase to trypsin (active enzyme) by proteolytic cleavage [156]. Therefore, the determination of trypsin could represent a specific and reliable diagnostic test of pancreatic function and its alteration (pancreatitis, pancreatic cancer, cystic fibrosis, etc) [157, 158]. It seems to be the most sensitive test for the diagnosis of acute pancreatitis (AP). The latter is considered as a major cause of morbidity and mortality worldwide [159, 160], including in Mexico [161].

The laboratory diagnosis of pancreatic disorders is commonly based on the determination of the levels of amylase, lipase, C-reactive protein, or cytokines in serum. Nevertheless, the severity of acute pancreatitis does not correlate well with the level of increase in serum amylase and lipase [162-163]. C-reactive protein is a useful predictor of PA after 48 hours of onset of symptoms, but not at the earlier stage [166-169]. The serum levels of cytokines are early indicators, but certain cytokines have shown low specificity as predictors of the disease severity [170]. Thus, the degradation of gelatin by trypsin is used as a simple semi-quantitative method for pancreatic disorders detection. However, such a test can not be easily calibrated. Therefore, for the quantitative trypsin activity determinations were suggested spectrophotometric and sensitive radioimmunoassay-based methods [171, 172]. Some others make use of Bragg reflector devices for measuring the change of temperature, pressure [173] and humidity [174], due to the action of trypsin on gelatin films. All these techniques are time consuming and require sophisticated laboratory equipment and trained personnel. Hence, faster, precise and simple tools and methods have to be developed. Such an alternative offer the biosensing devices. The holographic sensor of Millington *et al.* [175] is designed to register the change in color (wavelength) or brightness created when trypsin cleaves at peptide bonds adjacent to the arginine and lysine residues of gelatin and causes swelling of the hologram. Another technique to obtain a visually observable response is suggested by Chuang *et al.* [176]. They exploit the surface plasmon resonance wavelength shift of colloidal gold nanoparticles (AuNPs) when they aggregate. The AuNPs are modified with gelatin as a proteinase substrate and subsequently modified with 6-mercaptohexan-1-ol. After proteinase digestion, the AuNPs lose shelter, and gradually move closer to each other, to form aggregates. The AuNPs aggregation is monitored by the red shift of surface plasmon absorption and a visible color change of the AuNPs is from red to blue.

A simple, sensitive, and cost-effective piezoelectric sensor for trypsin activity determination is proposed by Zlatev *et al.* [177]. The change in frequency of an oscillating quartz crystal, due to the proteolytic digestion of the immobilized on its surface gelatin is recorded as a sensor response and is correlated to trypsin activity.

Only few electrochemical biosensors for trypsin activity determination are created until now. For instance, Zaccheo *et al.* [178] report a self-powered sensor for naked-eye detection of serum trypsin. It consists in a galvanic cell containing gelatin and Al barrier layers incorporated between the two half-cells. The degradation of the layers by trypsin and a hydroxide respectively closes the electrical circuit and a light-emitting diode signals the presence of trypsin. Assay time is ~ 3 h, and the limit of detection reached is 0.5 μg mL^{-1}.

More sensitive and faster electrochemical sensors for trypsin activity determination are developed by Ionescu *et al.* [179, 180]. The disposable conductometric one is elaborated via the modification of microfabricated integrated gold electrodes by urease /BSA coating covered by a gelatin film. The proteolytic digestion of the gelatin film results in the increase of the conductometric response of the biosensor to urea, as a function of the trypsin concentration. The achieved detection limit is of 100 pg mL^{-1} (1 mU mL^{-1}) for 70 min of incubation time. In the second one, the proteolytic digestion of gelatin, conjugated with the glucose oxidase catalyzed glucose oxidation, and the registration of the current of oxidation of the produced H_2O_2 is used to construct an amperometric biosensor for trypsin determination. Glucose oxidase is immobilized into an inner polypyrrole layer, covered by a gelatin film. Low trypsin concentrations down to 42 pM (9.38 mU mL^{-1}) are detected with a response time of ~ 10 min.

Another electrochemical method for trypsin activity determination, combined with portable sensing system was recently developed by Baş *et al.* [181]. It is based on the registration of the cyclic voltammetric response of $[Fe(CN)_6]^{3-}$ on gelatin coated ITO electrodes. The gelatin film behaves as a kinetic barrier and decreases the penetration of $[Fe(CN)_6]^{3-}$, causing an increase of the irreversibility of the system. In contrast, gelatin digestion by trypsin results in an increase in the penetration of the electroactive specie, leading to a change in the reduction peak potential. The time based change in the reduction peak potential during the proteolytic digestion is proportional with the enzyme activity. Trypsin activity is determined in less than three minutes. The detection limit was found to be 2.7 U mL^{-1}.

Newly, a simple square wave voltammetric method allowing the rapid trypsin activity determination in the normal and acute pancreatitis range was proposed by Stoytcheva *et al.* [182]. The analysis is based on the 1,2-benzoquinone electrochemical reduction on gelatin coated disposable screen printed electrodes of low cost, suitable for point-of-care testing. The proteolytic digestion of the gelatin film facilitates the transport of the electroactive specie to the electrode surface (Fig. **3**) and results in reduction peak current increase.

Current response as a function of trypsin concentration within 10 min of incubation time is evaluated in the range of 0.1 to1000 μg mL^{-1} trypsin corresponding to enzyme activity in the range from 0.75 U mL^{-1} to 7500 U mL^{-1}, respectively. The limit of detection is determined to be as low as 0.01 μg mL^{-1} (0.075 U mL^{-1}) trypsin after 140 min of incubation

time. The optimal hydrogel permeability defined by the concentration and the volume of the deposited gelatin solution (6% and 8 μL, respectively) is established performing preliminary experiments using a gelatin modified glassy carbon electrode of conventional type and applying factorial design approach.

A reagentless way for real-time detection of trypsin activities is reported by Yan Chen *et al.* [183], using a potentiometric biosensor. The method makes use of the trypsin-catalyzed degradation of protamine, which is released from the inner solution of a protamine-conditioned polycation-sensitive electrode. The hydrolysis catalyzed with trypsin in the sample solution decreases the concentration of free protamine released at the sample-membrane interface. This facilitates the stripping of protamine out of the membrane surface via the ion-exchange process with sodium ions from the sample solution, thus decreasing the membrane potential, by which trypsin is sensed potentiometrically. Under optimum conditions, the proposed protamine-sensitive electrode is useful for continuous and reversible detection of trypsin over the concentration range of 0.5-5 U mL^{-1} with a detection limit of 0.3 U mL^{-1}.

Trypsin activity assaying electrochemical sensors are summarized in Table **3**.

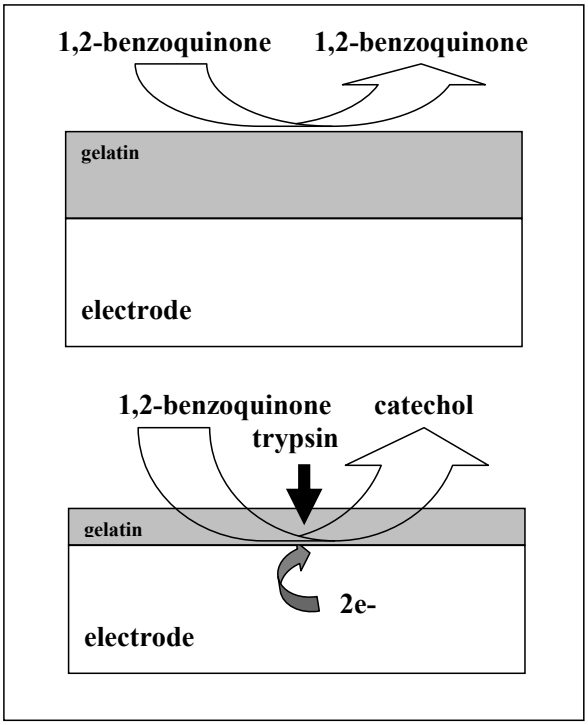

Fig. (3). Electrochemical sensor for trypsin activity determination [182]. The proteolityc gelatin digestion allows the transport of the electroactive specie to the electrode surface.

Table 3. Trypsin Activity Assaying Electrochemical Sensors

Detection Mode	Trypsin Substrat	Involved Reactions	Sensor Response	LOD	Reference
Galvanic cell	Gelatin	Hydroxide etching of an Al membrane $Mg \xrightarrow{anode} Mg^{2+}+2e^-$ $Fe^{3+}+3e^- \xrightarrow{cathode} Fe$	LED illumination	0.5 μg mL^{-1}	[178]
Conductometry	Gelatin	$(NH_2)_2CO+3H_2O \xrightarrow{urease}$ $2NH_4^++HCO_3^-+OH^-$	Conductivity change	$1x10^{-3}$ U mL^{-1}	[179]
Amperometry	Gelatin	β-D-glucose+$O_2 \xrightarrow{glucosoeoxidase}$ D-gluconic acid+H_2O_2 $H_2O_2 \xrightarrow{anode} 2H^++O_2+2e^-$	Anodic current change	$9.38x10^{-3}$ U mL^{-1}	[180]

(Table 3) contd….

Detection Mode	Trypsin Substrat	Involved Reactions	Sensor Response	LOD	Reference
Cyclic voltammetry	Gelatin	$Fe(CN_6)^{3-}+e \xrightarrow{\text{cathode}} Fe(CN_6)^{4-}$	Reduction peak potential change	27 U mL^{-1}	[181]
Square wave voltammetry	Gelatin	1,2-benzoquinone+$2e^-$ +$2H^+$ $\xrightarrow{\text{cathode}}$ catechol	Cathodic current change	$75x10^{-3}$ U mL^{-1}	[182]
Potentiometry	Protamine	reagentless	Membrane potential change	0.3 U mL^{-1}	[183]

CONCLUSIONS

Biosensors research targeting medical diagnostics is stimulated almost exclusively by the glucose-sensing market. Novel biosensors with clinical applications, however, could expand the field significantly. In this work are revised, apart of the current achievement and trends in glucose electrochemical biosensors, those of the less common electrochemical biosensors for cholinesterases and trypsin activity evaluation. It was demonstrated that this new generation analytical devices have the potential to improve routine methods in clinical analysis.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

Declared none.

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Received: December 23, 2011 Revised: April 24, 2012 Accepted: April 30, 2012