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BIOMIMETIC NANOSENSORS FOR DETERMINATION OF TOXIC COMPOUNDS IN FOOD AND AGRICULTURAL PRODUCTS (REVIEW)

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ABSTRACT

In the recent years many types of biosensors have been developed and used in a wide variety of analytical settings with applications in biomedicine, health care, drug design, environmental monitoring, detection of biological, chemical and toxic agents etc.

In the field of sensor research and development, bionanotechnology is poised to make significant contributions and has the potential to radically alter the way sensors are designed, constructed and implemented.

The most recent unrestrictive techniques used in biosensor design are surface plasmon resonance (SPR) and quartz crystal microbalance (QCM), combined with a biological receptor. On that basis several enzymes and microbial cells have been immobilized onto newly synthesized hybrid membranes. They contain particles from SiO_2, TiO_2, polymers and dendrimers and are attached to the sensor's surface. They are applied for determination of xenobiotics in crops, milk, nuts and other food and agricultural materials.

The aim of this review is to summarize some of the most significant research achievements in the field of biomimetic nanosensors design based on immobilized tyrosinase for determination of toxic compounds and smart biosensors for determination of Mycotoxines.

Keywords: optical biosensors, tyrosinase, peroxidase, multy enzime systems.

INTRODUCTION

Tyrosinases (monophenol, o-diphenol:oxygen oxidoreductase, EC 1.14.18.1) belong to a larger group of proteins named "type-3 copper proteins", which includes the catecholoxidases from plants and the oxygen-carrier haemocyanins from molluscs and arthropods [1, 2].

Although tyrosinases are widely distributed in microorganisms, plants and animals, much of the current interest in the development of their biotechnological applications has focused on the use of mushroom tyrosinases. Several aspects of mushroom tyrosinases, such as their biochemical characteristics, their roles in the metabolism of the producing organism and some of their potential biotechnological applications have been extensively reviewed [3-6].

Phenols, due to their toxicity, persistence and common occurrence in the biosphere, are one of the most important groups of ecotoxins. These compounds, as ingredients (components) and precursors of other chemicals including polymers, solvents, dyes (aminophenols), explosives (nitrophenols), surfactants (alkylphenols) or drugs are in common use [7]. Wastewaters containing phenols and phenolic derivatives are generated by the textile, coal, chemical, petrochemical, mining and paper industries, amongst others [8-10]. Increasingly strict environmental laws are providing an impetus for the development of analytical techniques for fast monitoring of these compounds.

Traditionally, analysis has been based on spectro-

photopmetric [11] or chromatographic [12] methods. New techniques that are currently being developed include capillary electrophoresis [13], immunoassays [14] and biosensors [15-18]. They potentially provide better specificity, lower costs, faster and simpler sample processing. Biosensors for the detection of phenolic compounds, based on the reaction of these compounds with immobilized mushroom tyrosinase are currently being developed.

In food quality control, biosensors have already confirmed their potential usefulness as tools for the detection of several types of compounds of interest: carbohydrates, alcohols, phenols, carboxylic acids, amino acids, biogenic amines, heterocyclic and inorganic compounds, additives or contaminants. The most common enzyme used for this purpose is tyrosinase. When this enzyme is entrapped in different supports, it is able to react with polyphenols to measure their concentration in samples such as olive extracts, tea [19], wine [20] and beer [21].

Mycotoxins are biological pollutants. They are toxic metabolites produced by several fungi in foods and feeds, and are probably the best known and most intensively examined mycotoxins in the world.

Mycotoxins are non volatile, relatively low-molecular mass secondary metabolic products that may affect exposed persons in a variety of ways. These compounds are considered secondary metabolites because they are not necessary for fungal growth and are simply a product of the primary metabolic processes. The functions of mycotoxins have not been totally studied, but it is established that they play a key role in the antagonistic processes concerning microorganisms from the same environment. They are also believed to help parasitic fungi invade host tissues. The amount of toxins needed to produce adverse health effects varies widely among toxins, as well as to each person's immune system.

Some mycotoxins are carcinogenic, some are vasoactive, and some cause damage to the central nerve system. Often, a single mycotoxin can cause more than one kind of toxic effect. More than 240 fungi produce 100 toxic compounds, which cause lancination and chronic diseases called mycotoxicoses [22].

The latest experiments with environmental biosensors are concentrated on molecular affinity reactions such as photosynthetic systems, antibody actions. The antibody-antigen interactions are preferred in low concentration measurements. There are immunochemical biosensors applied for mycotoxin assays. Gaag at al. developed a method based on surface plasmon resonance for measuring of four different mycotoxins – aflatoxin B_1, zearalenone, ochratoxin A and fumarotoxin B_1 [23]. The scheme below (Fig. 1) presents the main components in biomimetic nanosensors design and application.

Immobilization is essential because it ensures intimate contact between the enzyme and the underlying signal detector and also prevents the enzyme from being washed off the electrode when readings are made in aqueous samples. One of the biggest challenges in

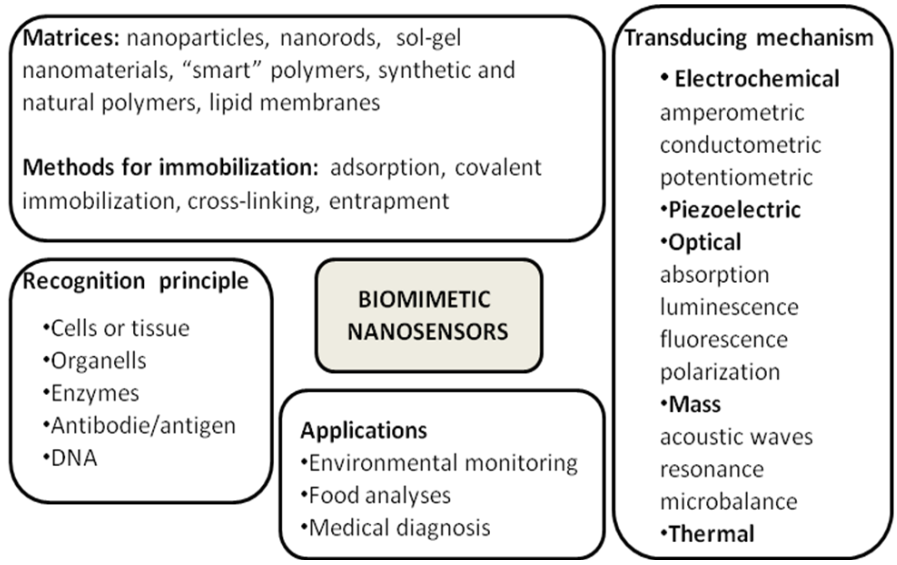

Fig. 1. A schematic view of the basics in biomimetic nanosensors design and application.

designing a new enzyme-based biosensor is to find the optimal balance between stability and activity of the enzyme [24]. Surfaces play an important role in biology and medical research, since most biological reactions occur on surfaces and interfaces [25]. Tailored surface properties such as tunable reactivity, biocompatibility or wettability could be obtained by different approaches of surface modification, so that the design of biofunctional surfaces is of great interest in bioanalysis research [26]. Immobilization methods such as cross-link bonding [27], covalent attachment [28], polymer inclusion [29] and sample adsorption [30], have different effectiveness with regard to the stability of the signal generated, because a higher or lower percentage of enzyme immobilized is lost during the measurement. A good combination of support material and immobilization methods is of a fundamental importance to achieve the desired performances from the sensing system [31].

1. Gold nanoparticles for biosensors design

Bionanoconjugates of the enzyme tyrosinase (TYR) and gold nanoparticles (AuNPs) functionalized with a peptide (Cys-Ala-Leu-Asn-Asn) CALNN were produced in solution and characterized. The conjugation of enzymes with AuNPs can lead to the retention or even - to an increase of their biological stability/ activity. Electron transfer between the catalytic sites of immobilized enzymes and the electrode materials is facilitated, improving the analytical sensitivity and selectivity of the biosensors and often overcoming the need for enzyme mediators. These properties have led to an intensive study of AuNPs in the construction of electrochemical biosensors with enhanced analytical performance in respect to other biosensor designs. The enzyme activity of the BNCs was stable for 6 weeks, with a loss of activity towards 4-methyl catechol of only 10 %. This indicates that these conjugates present a good level of storage stability for preparation and assembly of the biosensor at a later stage, suggesting that they will also be stable after immobilization onto the electrode surface [32].

An indirect colorimetric method is available for detection of trace amounts of hydroquinone, catechol and pyrogallol. The reduction of $AuCl_4^-$ to gold nanoparticles (Au-NPs) by these phenolic compounds in the presence of cetyltrimethylammonium chloride (CTAC) produces very intense surface plasmon resonance peak of Au-NPs. The plasmon absorbance of Au-NPs allows the quantitative colorimetric detection of the phenolic compounds. The calibration curves, derived from the changes in absorbance at $\lambda = 568$ nm, were linear with concentration of hydroquinone, catechol and pyrogallol in the range of 7.0×10^{-7} to 1.0×10^{-4}M, 6.0×10^{-6} to 2.0×10^{-4} M and 6.0×10^{-7} to 1.0×10^{-4} M, respectively [33].

A. Carralero and co-authors report for the development of a new tyrosinase biosensor which is based on a construction of graphite–Teflon composite electrode matrix in which the enzyme and colloidal gold nanoparticles are incorporated by simple physical inclusion. The Tyr–Au_{coll}–graphite–Teflon biosensor exhibited suitable amperometric responses at −0.10 V for the different phenolic compounds tested (catechol; phenol; 3,4-dimethylphenol; 4-chloro-3-methylphenol; 4-chlorophenol; 4-chloro-2-methylphenol; 3-methylphenol and 4-methylphenol). The limits of detection obtained were 3 nM for catechol, 3.3 μM for 4-chloro-2-methylphenol, and approximately 20 nM for the rest of phenolic compounds [34]. A sequential competitive configuration between the analyte and progesterone, labeled with alkaline phosphatase (AP), was used. Phenyl phosphate was employed as the AP-substrate and the enzyme reaction product, phenol, was oxidized by tyrosinase to o-quinone, which is subsequently reduced at −0.1 V at the biocomposite electrode. The response shows a good linearity for low progesterone concentrations (it should be noted that usual progesterone concentration in cow`s milk is around 5 ng mL^{-1}). Conversely, some of the progesterone immunosensors described previously exhibited a hook effect, i.e. a curvature in the calibration graph, for low antigen concentrations. Although this hook effect was not observed for the calibration graph constructed with the immunosensor using a colloidal gold-graphite-Teflon composite electrode, the monitoring of the affinity reaction was accomplished in this case at +0.30 V. On the contrary, the use of a Tyr-Au_{coll} – graphite – Teflon biosensor as transducer allows a detection potential such as – 0.1 V to be applied, thus minimizing the impact of potential interferences from electrochemically active compounds that may be present in a real sample [35].

Sanz and co-workers report for immobilization of the tyrosinase onto a glassy carbon electrode modified with electrodeposited gold nanoparticles (Tyr-nAu-GCE). The enzyme immobilized by cross-linking with glutaraldehyde retains a high bioactivity on this

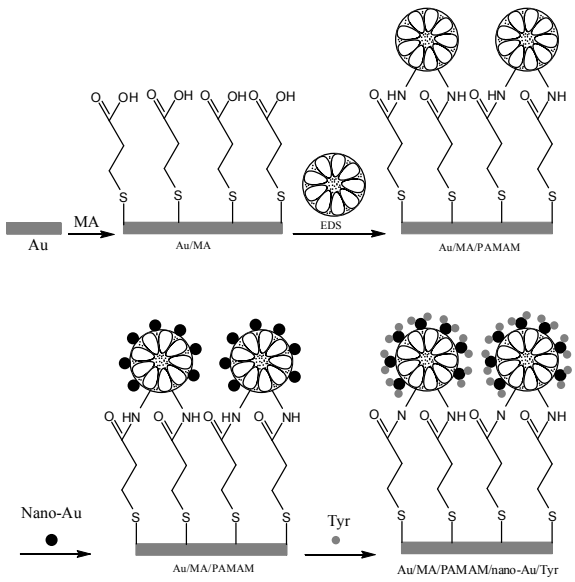

Fig. 2. A schematic representation of the surface modification process and fabrication of a tyrosinase according to Li and Kwak [37].

electrode material. The Tyr-nAu-GCE was applied for the estimation of the phenolic compounds content in red and white wines [36].

Penicillamine (PCA) is a sulfhydryl amino acid with a hydrogen ion in the beta-carbon of cysteine replaced by a methyl group. In addition to chelation of heavy metals, such as copper, it suppresses the cross-linking of collagen by formation of a thiazolidine bond with the aldehyde group of collagen. Gold electrodes were modified with mono layers of 3-mercaptopropionic acid and further reacted with poly-(amidoamine) (PAMAM) dendrimers to obtain thin films. The high affinity of PAMAM dendrimer for nano-Au due to its amine groups is used to ensure the role of nano-Au as an intermediator to immobilize the enzyme of tyrosinase. When penicillamine was added to the solution, it reacted with o-benzoquinone to form the corresponding thioquinone derivatives, which resulted in decrease of the reduction current of o-benzoquinone. Based on this, a new electrochemical sensor for determination of penicillamine was developed. Fig. 2 describes the basic strategy for the preparation of a tyrosinase-modified electrode.

When the tyrosinase was immobilized on the nano-Au surface, the AFM image indicates that the enzyme film is even more uniform and smooth than that of the nano-Au film. Cyclic voltammetry (CV) is an effective method for probing the feature of surface-modified electrode and testing the kinetic barrier of the interface because the electron transfer between solution species and the electrode must occur by tunneling either through the barrier, or through the defects in the barrier. Therefore, CV has been chosen as a marker to investigate the changes of electrode behavior after each assembly step [37].

2. Nanoparticles and nanomaterials for tyrosinase immobilization

The application of Quartz Crystal Microbalance (QCM) techniques in the characterization of the successive immobilization steps is involved in the development of bioanalytical platforms. A particular emphasis is placed on the application of these techniques to the characterization of the immobilization of enzymes on different modified and unmodified surfaces as well as on the study of protein interactions, which is a more recent and less spread application [38].

Authors report herein about a biocompatible interface composed by a beta-nanozeolite three-dimensional architecture on an indium tin oxide (ITO) electrode using a layer-by-layer (LbL) assembly technique. The large surface area and unique surface properties of the nanozeolite matrix resulted in a high enzyme adsorption capacity and the enzyme adsorbed in this film retained its activity to a large extent. By adjusting the nanozeolite-assembled layers, thus regulating the amount of the enzyme immobilized, the biocatalytic property of the enzyme electrode could be easily controlled [39].

ZnO nanoparticles, porous film, nanocombs and nanorods have been developed into biosensors to detect phenolic compounds. Notably, the isoelectric point (IEP) of ZnO is as high as about 9.5. This is suitable for immobilization of biomolecules with low IEP, such as enzymes and proteins, assisted by electrostatic attraction in proper pH value. Using zinc powder directly as a source material, ZnO nanorods were fabricated on the surface of gold wire by hydrothermal reaction, without any other surfactant and stabilizing agent. The gold wire was treated to improve the nucleation for growth of ZnO nanostructures and to further improve the performance of the biosensor, constructed by immobilizing tyrosinase on the ZnO nanorods for phenol detection. Electrochemical measurement, Fourier transform infrared (FT-IR)

and scanning electron microscopic (SEM) analyses demonstrated that the TYR was stably adsorbed on the ZnO nanorods surface with bioactivity for phenol oxidation. The film assembly process and enzyme adsorption were monitored by Quartz Crystal Microbalance measurements. The nanozeolite film exhibited an amazing adsorption capacity (about 350 $mg.g^{-1}$) for tyrosinase as a model enzyme. The tyrosinase biosensor showed high sensitivity (400 μAmM^{-1}), short response time (reaching 95 % within 5 s), broad linear response range from 10 nM to 18 μM, very low detection limit (0.5 nM) and high operational and storage stability - more than 2 months [40].

In [41] Dai and co-authors report ed that an electrochemical biosensor for phenol based on immobilization of tyrosinase-peroxidase on mesoporous silica was constructed. An enhanced sensitivity of the tyrosinase-horseradish peroxidase based biosensor to phenol was observed in comparison with tyrosinase or horseradish peroxidase monoenzyme modified electrodes. The two enzymes retained their enzymatic activities for phenol determination without any mediator. The phenol sensor exhibited a fast response of less than 10 seconds. The sensitivity of the biosensor for phenol was 14 mA mM^{-1} cm^2 with a linear range from $2x10^7$ to $2.3x10^4$ M and a detection limit of $4.1x10^9$ M. MCM-41 has a high surface area, controlled porosity and mechanical resistance, which make it more suitable for enzyme loading to get high sensitivity [42]. Authors in [43] reported that the activity of tyrosinase attached to acrylic beads and silica gels was low, despite the large protein content. A carbon nanotube matrix which was easy to prepare was developed by Alarcon and co-workers. It ensures a very good entrapment environment for the enzyme, being simpler and cheaper than other reported strategies. In addition, the proposed matrix allows for a very fast operation of the enzyme, which leads to a response time of 15 s for phenol. The biosensor keeps its activity during continuous flow injection analysis (FIA) measurements at room temperature, showing a stable response (RSD 5 %) within a two week working period at room temperature [44].

3. Sol-gel hybrid materials

The sol-gel process allows for the preparation of porous glasses at low temperature and high purity of the starting materials, for the production of ceramic materials by hydrolysis and polycondensation of alkoxides [45]. TEOS (tetraethyl orthosilicate) was chosen as a precursor of sol–gel silicate for immobilization of a MBTH (3-methyl-2-benzothiazolinone hydrazone) reagent.

The properties of the sol-gel silicate matrix such as surface area, pore size and pore distribution can be affected by various factors. Among them, the pH and the Si to water ratios are the most important parameters for sol–gel silicate preparation. Nafion (sulfonated tetrafluoroethylene) has a hydrophobic fluorocarbon backbone but hydrophilic cation-exchange site. Thus, it exhibits moderate hydrophobicity, which can assist in retaining MBTH in the film and reduce leaching. The lowest response of the biosensor is observed when a pure sol-gel silicate matrix is used. This behavior could be due to the hydrophilic nature of TEOS that could not retain much of the hydrophilic MBTH reagent in the immobilization matrix. The hybrid material with a ratio of 1:1 showed the optimum biosensor response. Further increase in nafion content decreased the sensor response. This may attributed to the decrease of porosity of the sol-gel silicate film, as well as to the hydrophobicity of the matrix when the content of nafion in the hybrid materials increases. Thus, it affects the amount of MBTH trapped in the hybrid nafion/sol-gel silicate network since a decrease in porosity of the hybrid material results in a low amount of MBTH trapped in the hybrid film [46]. Fabrication of a test strip for detection of benzoic acid was implemented by immobilizing tyrosinase, phenol and MBTH onto a filter paper, using polystyrene as a polymeric support. In this fabrication of a test strip, an impregnating method was used. It is one of the possible enzyme immobilization procedures. It has shown a highly reproducible measurement of benzoic acid with a calculated RSD of 0.47 % (n = 10). A linear response of the biosensor was obtained in 100 to 700 ppm of benzoic acid with a detection limit (LOD) of 73.6 ppm. The activity of immobilized tyrosinase, phenol and MBTH in the test strip was fairly sustained during 20 days when stored at 3 °C. The developed test strip was used for detection of benzoic acid in food samples and was observed to have comparable results to the HPLC method [47].

Majidi and co-authors present a sol-gel based biosensor for atrazine determination that has been obtained by introducing the enzyme polyphenol oxidase

from apple tissue in a sol-gel matrix. The biosensor consisted of 10.3 % (mass/mass) of apple tissue. Atrazine is an inactive compound electrochemically and therefore redox coupling of dopamine was used for studying atrazine behavior [48]. Researchers have demonstrated that the silica sol-gel materials can retain the catalytic activities of enzymes to a large extent. The inorganic silica sol-gel material is biocompatible, has a high thermal stability and chemical inertness, and a negligible swelling in nonaqueous solutions. Titanium isopropoxide is much more active to water than tetraethyl orthosilicate. In case of contact with water, a precipitate of titanium dioxide is formed immediately [49]. Compared to the element of silicon, titanium has a larger covalent radius, which results in larger sol-gel pore size. This is also conducive to a fast equilibrium of the substrate between the organic solvent and titania film. Both factors are favorable to create a faster diffusion of the substrate from bulk solution to the enzyme. This results in a faster response of the sensor. Such a short response time further proves that the vapor deposition derived titania sol-gel material is a promising matrix for the construction of organic-phase biosensors. An amperometric biosensor was constructed from Yu and Ju for the determination of phenols in pure organic phase. The biosensor was fabricated by immobilizing tyrosinase in a titania sol-gel membrane which was obtained with a vapor deposition method. This method was facile and avoided the calcination step needed in conventional titania sol-gel process. The titania sol-gel membrane could effectively retain the essential water layer around the enzyme molecule, needed for maintaining its activity in an organic phase [50].

Our research group reported about the creation of new matrices for diverse applications, particularly in the field of biotechnology and food industry. We reported on the synthesis of new matrices based on a mixture of polymer cellulose acetate butyrate/copolymer polyacrylonitrile acrylamide/TiO_2 [51] and silica hybrid membranes [52]. The characterization of the new matrices was performed using IR spectroscopy, QCM technology and SEM. The tests revealed that when polymers were used as carriers, there is a limit of 5 % for the concentration of titanium. Further increase of Ti concentration leads to precipitation processes. The QCM analyses show that a low concentration of $Ti(OBu)_4$ does not influence the viscosity of the obtained sols, but elasticity of matrices changes significantly. The membranes obtained were successfully applied for biofilm formation of yeast strain Saccharomyces cerevisiae [51] and for biosensors construction [52, 53].

The Sonogel-Carbon electrodes show the general good properties of other CCE's (Ceramic Carbon Electrodes). Besides, in comparison with other carbon electrodes, they exhibit especially favorable electrochemical properties [54].

Kaoutit and co-authors report the development of a biosensor based on the bi-immobilization of laccase and tyrosinase phenoloxidase enzymes. This biosensor employs as the electrochemical transducer the Sonogel–Carbon, a novel type of electrode developed by this research group. The immobilization step was accomplished by doping the electrode surface with a mixture of the enzymes, glutaric dialdehyde, and Nafion-ion exchange as protective additive. The response of this biosensor, denoted the dual Trametes versicolor laccase (La) and mushroom tyrosinase based Sonogel–Carbon, was optimized directly in real beer samples and its analytical performance with respect to five individual polyphenols was evaluated. The Lac–Ty/Sonogel–Carbon electrode responds to nanomolar concentrations of flavan-3-ols, hydroxycinnamic acids, and hydroxybenzoic acids. The limit of detection, sensitivity and linear range for caffeic acid, taken as an example, were 26 nM, 167.53 nA M^{-1}, and 0.01–2 μM, respectively [55].

Tyrosinase was covalently immobilized onto amino-functionalized carbon felt surface via glutaraldehyde-coupling under ultrasonic treatment for 10 min. The resulting TYR-immobilized carbon felt was used as a working electrode unit of a bioelectrocatalytic flow-through detector for tyrosinase substrates. Cathodic peak currents based on the electroreduction of enzymatically produced o-quinones were detected at –50 mV vs. Ag/AgCl. Compared with previous work [56], in which the enzyme was immobilized onto amino-functionalized carbon felt for 16 h without the ultrasonic treatment, the researchers in [57] succeeded in shortening the enzyme immobilization time from 16 h to 10 min. In the Table below some biosensors constructed by different kind of carriers, for phenolic compounds detection are presented (Table 1).

Synthetic and natural polymers for biomimetic biosensors construction

Synthetic polymers

One of the most important parameters to be considered in enzyme immobilization is storage stability.

Table 1. Biosensors based on tyrosinase immobilization for phenolic compounds detection.

Method of sensing	Type of membranes	Phenolic compounds	Limit of detection (LOD)	Ref.
Optical detection, surface plasmon resonance (SPR)	Gold nanoparticles	Pyrogallol Hydroquinone Catechol	$3.2.10^{-7}$ (M) $5.3.10^{-7}$ (M) $2.5.10^{-6}$ (M)	[33]
Amperometric	Au_{coll}-graphite-Teflon	Phenol Catechol 3-4-Dimethyl phenol 4-Chloro-3 methylphenol 4- Chlorophenol	0.020 (μM) 0.003 (μM) 0.011 (μM) 0.012 (μM) 0.019 (μM)	[34]
Amperometric	Gold nanoparticles	Phenol Catechol Caffeic acid Callic acid	$2.1.10^{7}$ (M) $1.5.10^{7}$ (M) $6.6.10^{7}$ (M) 70.10^{7} (M)	[36]
Amperometric	Nanozeolite	Phenol	0.5 (nM)	[39]
Amperometric	ZnO nanorods	Phenol	0.623 (μM)	[40]
Amperometric	Mesoporous silica derivatives	Phenol	$4.1.10^{-9}$ (M)	[41]
FIA/amperometric	Carbon nanotubes	Phenol	0.14 (μM)	[44]
Optical	Chitosan/naflon/sol-gel	Catechol Phenol *m*-cresol	0.18 (mg/L) 0.23 (mg/L) 0.43 (mg/L)	[46]
Amperometric	Titania sol-gel	Catechol Phenol *p*-cresol	$6.4.10^{-7}$ (mol L^{-1}) $8.0.10^{-7}$ (mol L^{-1}) $1.6.10^{-6}$ (mol L^{-1})	[50]
Amperometric	Sonogel-carbon	Polyphenols Caffeic acid Ferulic acid (+) catechin (-) epicatechin	19.10^{-2} (μM) $2.6.10^{-2}$ (μM) $6.4.10^{-2}$ (μM) $3.4.10^{-2}$ (μM) $4.3.10^{-2}$ (μM)	[54]
Amperometric	Carbon felt	Catechol Phenol *p*-cresol	$6.5.10^{-9}$ (μmol/L) $3.9.10^{-8}$ (μmol/L) $1.3.10^{-8}$ (μmol/L)	[57]

The stabilities of the free and the immobilized tyrosinase preparations were determined after the preparations were stored in phosphate buffer solution (50 mM, pH 6.5) at 4 ºC for a predetermined period. Under the same storage conditions, the activities of the immobilized tyrosinase preparations decreased slower than that of the free tyrosinase. The free enzyme lost all of its activity within 4 weeks. The immobilized tyrosinase preserved its initial activity during a several months storage period [58], which corresponds to the data reported by other authors [59].

Cross-linked enzyme crystals (CLECs) have several characteristics that confer significant advantages over conventional enzyme immobilization methods, like enhanced temperature stability, absence of an inert support, catalysis under harsh conditions, such as temperature, pH and organic solvents. The insoluble nature of CLECs facilitates easy separation of the enzyme from the reaction mixture, which increases the reuse efficiency of the enzyme. Immobilization/ stabilization of tyrosinase by cross-linking crystallized tyrosinase and a bovine serum albumin complex is a method employed to develop a biocatalyst, which transforms L-tyrosine in the presence of ascorbic acid

to L-DOPA (L-3,4-dihydroxyphenylalanine). Tyrosinase and bovine serum albumin were co-crystallized by saturated ammonium sulfate solution (65 %) using 20 % polyethylene glycol (PEG) 6000 and n-propanol as co-solvents. The developed biocatalyst could be recycled 6 times without further loss of tyrosinase activity. No loss of activity of cross-linked tyrosinase-bovine serum albumin crystals was observed upon storage of the developed CLECs in a refrigerator for six months [60]. A conducting, polymeric film of poly-(indole-5 carboxylic acid) has been prepared by an electrochemical polymerization for covalent immobilization of tyrosinase. As the polymer contains pendant carboxylic groups, an one-step carbodiimide method was used to immobilize tyrosinase on the polymer matrix. The linear dependence was found to be 15 μM of catechol with sensitivity of 250 mA/M cm^2. Biegunski and co-authors describe novel results for immobilization of tyrosinase on the surface of electrochemically polymerized poly-(indole-5 carboxylic acid) (PIn5COOH). The immobilization of tyrosinase on the polymer film was proved by in situ surface enhanced resonance Raman spectra (SERRS) [61].

A research group constructed a novel sensitive amperometric biosensor, based on polyaniline-polyacrylonitrile composite matrix, which was applied for determination of benzoic acid. The inhibiting action of benzoic acid on the polyphenol oxidase electrode was reversible and of the typical competitive type, with an apparent inhibition constant of 38 μM. This biosensor detected levels of benzoic acid as low as $2x10^{-7}$ M in solution. Inhibition studies revealed that the proposed electrochemical biosensor was applicable for monitoring of benzoic acid in real samples such as milk, yoghurt, sprite and cola [62].

Acrylic copolymers are especially versatile as a family of carrier materials for enzyme immobilization that can be prepared with a wide variety of properties. Among these, an epoxy group carrying acrylic copolymer exhibited some significant advantages as a potential carrier matrix, i.e., easy and stable covalent linkages with different groups. The covalent bond formation via amino groups of the immobilized tyrosinase might also reduce the conformational flexibility and may result in a higher activation energy for the molecule to reorganize into the proper conformation required for binding to the substrate. One of the main reasons for enzyme immobilization is the anticipated increase in its stability to various deactivating forces due to restricted conformational mobility of the molecules following immobilization. All immobilization studies published in the literature have been performed under different conditions. Therefore, it is almost impossible to compare immobilization results [63].

Polyamidoamine dendrimers

The synthesis of linear polymers has as a result final products with heterogeneity that could influence the optical performance of the polymer sensor. Poly (amidoamine) dendrimers (PAMAM) are an interesting class of polymers. They have mono dispersive, well defined and developed three-dimensional structures with functional groups in high concentration. In their core they have amidic groups. Structurally modified PAMAM dendrimers with 1,8-naphthalimide derivatives are fluorescent dendrimers and they can be applied as effective and selective sensors for different metal cations and protons in organic solvents. In [64] we present our first results about the possibility for the incorporation of bio-receptors into some new PAMAM dendrimers. Dendrimers under study contain peripherally bonded 1,8-naphthalimide derivatives. The authors present the result of the preparation of fluorescence PAMAM dendrimer – acetyl cellulose membrane by the spin coating method. The fluorescent PAMAM contained a chemically bonded fluorescent dye. Lipoxygenase, peroxidase and aflatoxine antibodies were covalently immobilized and biosensors for mycotoxines detection were constructed [65].

Natural polymers

Recent research performed, using a butyrylcholinesterase and choline oxidase enzyme electrode, suggested the validity of the biosensor approach using enzyme inhibition OPEEs (i.e. enzyme electrodes working in organic phase) in the case of organophosphorus and carbamate pesticides, which were poorly soluble in aqueous solutions. Since these pesticides were generally much more soluble in chloroform than in water, the present research aimed at analysing this class of pesticides using a tyrosinase inhibition OPEE operating in water-saturated chloroform medium. The tyrosinase biosensor was assembled using an oxygen amperometric transducer coupled to the tyrosinase enzyme, immobilized in kappa-carrageenan gel.

The tyrosinase biosensor, in its inhibition OPEE configuration, allows organophosphorus and carbamate pesticides, as well as the triazine or benzotriazine pesticides to be determined. It was possible to achieve a low detection limit of the order 10^{-8} to 10^{-9} mol L^{-1} [66].

Mushroom tyrosinase was immobilized by adsorption onto the totally cinnamoylated derivative of D-sorbitol. The polymerization and cross-linking of the derivative initially obtained was achieved by irradiation in the ultraviolet region, where this prepolymer shows maximum sensitivity. The immobilized enzyme showed an optimum measuring pH of 3.5 and greater activity at acid and neutral pH values than the soluble enzyme. The stability of immobilized tyrosinase enzyme was evaluated by storing it in distilled water at −18 ºC for 7 days. The optimal immobilization reaction time was 3 h and, at longer times, the amount of enzymatic activity retained on the support remained constant [67]. In spite of the fact that p-nitrocatechol is a very bad substrate for tyrosinase (k_{cat} = 0.0241 ± 0.0020 s^{-1}), it has a great affinity for the same enzyme (K_m= 4.27 ± 1.12 μm). Similarly, p-nitrophenol (PNP) is probably a worse substrate than p-nitrocatechol and its affinity for the enzyme is also very high (K_i = 62 ± 6 μm). Maximum enzymatic activity was obtained at 55°C. The optimal reaction temperature was higher than that of the commercial enzymes in their free form or when immobilized on other supports [68,69], which illustrates a substantial degree of enzyme stabilization [70].

The research, described in [71] focuses on the covalent and adsorptive immobilization of tyrosinase from Agaricus bisporus onto cellulose-based carriers using DEAE (diethylaminoethanol) ligands. The effect of carrier anchor groups on the activity and stability of the immobilized tyrosinase was examined by monitoring monophenolase and diphenolase activities using L-tyrosine and L-DOPA as substrates. It should be noted that the two activities can be accurately determined from the amount of dopaquinone spontaneously associating into dark brown pigments. Finally, the stability of the obtained preparation was tested at 55°C to demonstrate the advantages of immobilization. As the immobilized enzyme is about 22 times more stable at 55 ºC, this seems to be a remarkable improvement not often reported before.

The feasibility to build up layer-by-layer (L-b-L) self-assemblies of tyrosinase and quaternized chitosan (CHI+) on a glassy carbon (GC) rotating disk electrode is reviewed in [72]. This work highlights the promising properties of this modified polysaccharide to immobilize PPO in self-assembled structures under conditions that preserve its catalytic activity. Referring to the low amount of enzyme immobilized in the multilayer structure, the amperometric response of the biosensor reached an excellent sensitivity of about 2000 $Amol^{-1}$ cm, proving that an important part of the entrapped enzyme is accessible to the substrate molecule while keeping a good level of activity. The authors show that the many amino groups present in chitosan provide a biocompatible environment for enzyme immobilization, and the enzymes retain the essential feature of their native structure in the chitosan, leading to highly sensitive sensors. The adsorption spectrum of PPO exhibits a broad band centered at 278 nm that was utilized to monitor the efficiency of the L-b-L film growth onto the two faces of quartz cells. Using the absorbance values measured at λ_{max} = 278 nm and the corresponding extinction coefficient of the protein (2.39 mg^{-1} $mLcm^{-1}$), a PPO surface concentration (Γ E, mol cm^{-2}) has been obtained for each layer (considering a PPO molar mass of 128,000 g mol^{-1}). The molecule of PPO is assumed to be spheric with a volume of 219 nm^3 and its estimated projection area is about 67.8 nm^2. The surface concentration of the enzyme which should correspond to the saturation of a monolayer on a 3 mm-diameter disk can be estimated as 1.73×10^{-13} mol.

Research, predicting the 3D structure of tyrosinase from Agaricus bisporus, used a docking algorithm to simulate binding between tyrosinase and oxalic acid, and studied the reversible inhibition of tyrosinase by oxalic acid [73].

CONCLUSIONS

The newly constructed biosensors are characterized by their response time, reproducibility, linear range and operating stability. They demonstrate high stability, shorter response time, high sensitivity and wider linear range than traditional analytical methods. Using nanomaterials allows the construction of biosensors with better parameters like biocompatibility, longer application time and mobility. The results from our research for biosensors design shows good compatibility between membranes and enzymes without a change

of the the conformation of the enzyme molecule and binding always takes place outside the enzyme active centres.

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