

Biosensing Devices: Conjugated Polymer Based Scaffolds

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Abstract

Conjugated polymer (CP)-based biosensors are recognized to be a next generation building architecture for highly sensitive and fast biosensing systems. This entry highlights an overview on the recent expansion of research in the field of CP-based biosensors. We start by first introducing some of the important knowledge about the CPs and biosensors. This is followed by emphasizing the importance of CPs in biosensor construction. Lastly, an overall survey of CP-based biosensor studies reported between 2010 and 2015 is discussed. Several reviews in literature mostly either refer to a specific enzyme or focus on the techniques of biosensor construction together with general information on conducting polymers. Hence, we tend to emphasize on a number of enzymes studied for the past 5 years.

Keywords: Amperometric biosensor; Conjugated polymer; Conjugated polymer-based biosensor; Enzyme-based amperometric biosensor.

INTRODUCTION

The main concern for public health and environment has given rise to a significant interest in the analysis of some important analytes. The accurate determination of such important analytes is of great importance in environmental and human health analysis. Undesirable accumulation of such analytes is an indicator in the diagnosis and prevention of several diseases. To date, various methods have been proposed for their analysis including spectrophotometric, chromatographic, and electrochemical methods. However, some methods have certain disadvantages such as lack of selectivity, high cost, long analysis time, and requirement of pretreatment of the samples. On the contrary, for developing fast, reliable, and sensitive methods, a large number of studies have devoted to develop biosensors. Biosensors have been considered to be one of the most popular devices due to their fast response, miniature size, reliability, and high sensitivity. In literature, different types of materials can be used for designing biosensors. In this review, we will focus on conjugated polymer (CP)-based biosensors and concentrate on their major importance in biosensor design by considering the recent studies.

Biosensors based on CPs and their derivatives attract a great deal of attention. A number of CPs such as polyaniline (PANI), polythiophene (PTh), and polypyrrole (PPy) have been studied for the development of biosensors to be used in clinical and environmental diagnostics. CPs open a new perspective in the field of biosensors due to their fascinating properties and are called “synthetic metals.” This has been attributed to their excellent conductivity and high mechanical strength that provide easy processability for many types of applications. Taking the advantages of ability to conduct electricity in a desired level, low cost, and easy preparation techniques make them fascinating materials in large areas. CPs were used for technological applications in sensors,^[1] light-emitting diodes,^[2] field effect transistors,^[3] electrochromic devices,^[4] rechargeable materials,^[5] photovoltaic devices,^[6] drug delivery,^[7] and artificial muscles.^[8] Considering all the abovementioned awesome properties, this review points the importance of CPs in biosensor design and up-to-date literature results concerning their use as biosensors. In this review, we will concentrate our attention on the development of CP-based amperometric biosensors. This field presently refers to a considerably increasing number of publications.

However, this is the first CP-based electrochemical biosensor report summarizing the recent work between 2010 and 2015.

CONJUGATED POLYMERS

CPs have emerged as feasible semiconducting electronic materials for numerous applications since a new age began at the end of the 1970s with the investigation of high electrical conductivity in doped poly(acetylene) (PA).^[9] This discovery brought the 2,000 Nobel Prize in Chemistry award to Alan Heeger, Alan MacDiarmid, and Hideki Shirakawa.^[10] These three scientists advanced the field of “plastic electronics.” A polymer plastic contains alternating single and double bonds along the polymer chain. Due to the localized position of the bonds, the electrons on the polymer backbone cannot carry the electric current. However, in case the material is doped with strong electron acceptors, electrons move freely and plastics turn into conductive materials. Since PA is air sensitive, new CPs were designed and synthesized over the past decades. This newly synthesized CPs led to new uses as supporting materials in modern science.

Alternating single and double bonds provide conjugation that result in charge mobility. Each bond contains sp- or sp²-hybridized atoms in the backbone. This *p*-orbital overlap provides conductivity in chemically doped CPs, and electrons can freely move. However, conjugation alone is not sufficient to make the material conductive. The electrical conductivity results from the presence of dopant stimulated charge carriers which allow transmitting electrical current along the CP backbone.^[11] Chemical and electrochemical polymerizations are alternative ways to synthesize CPs. There are also several techniques such as photochemical polymerization, solid-state polymerization, and pyrolysis for their synthesis.^[12] In chemical polymerization procedure, monomers are oxidized in the presence of oxidizing agents. For heterocyclic monomers, FeCl₃ is generally suggested as an oxidant chemical, although other chemicals can be used for oxidizing agents during chemical polymerization.^[13] Although chemical polymerization provides low-cost polymerization, the use of strong oxidizing agents may cause over oxidation and decomposition of the polymer and side reactions as well.^[14] Electrochemical polymerization technique overcomes these disadvantages and offers many advantages over chemical polymerization. This technique allows to control the thickness of the CP-coated surface in terms of charge passing through the cell. Moreover, the method is a simple, reproducible, and obvious process for growth of the CPs on the electrode surface allows well-defined and finely controlled deposition. Yet, the major drawback is that the synthesized polymer is insoluble; hence, it is difficult to characterize via traditional methods.

BIOSENSORS

Biosensors are promising analytical devices which lead to provide specific quantitative or semiquantitative analytical information using a biological recognition element that is sustained in direct contact with a transducer. In recent years, biosensors have attracted great attention throughout the world. With the emerging technology, specific, rapid, and simple-to-operate sensors are needed.^[15] Hence, at present, numerous biosensors have been developed for simultaneous determination of number of analytes in our daily life such as in diagnosis, food technology, biotechnology, genetic engineering, and environmental monitoring.^[16] A biological detection element and a transducer are mainly the two parts of the biosensors. In the construction, catalytic or non-catalytic biological components can be used. Enzymes, tissues, and microorganisms are examples of catalytic groups, whereas antibodies, nucleic acids, and receptors are examples of non-catalytic groups. Transducers convert the biological signal into an understandable signal. There are several transducers for the fabrication of biosensor, namely, electrochemical, optical, colorimetric, and acoustic ones.^[17] As shown in Fig. 1, biosensors can be categorized according to the types of transducer and biological detection element. In the construction of electrochemical biosensors, the transducer transforms the biochemical information to an understandable signal, designating selectivity of the sensor. Especially, electrochemical transducers are preferred for construction of a biosensor, which can be classified as conducting, semiconducting, and ionic conducting material. Electrochemical species are consumed or produced during the biological reaction, and the electrochemical signal is recorded by an electrochemical detector.^[18] Electrochemical techniques are divided into subgroups depending on the type of transducer: conductometric, potentiometric, and amperometric biosensors. Among the diversity of sensing methods applied, amperometric detection is our main focus in this review.

In the biosensor construction, considerable attention has been paid for developing the amperometric electrochemical biosensors. They detect substrate with the advantages of high sensitivity and reliability, fast response, good selectivity, and low detection limit.^[19] This method employs the differences in the current on the working electrode upon applied constant potential. The reaction brings a change in the current, and the change is associated with the amount of analytes in the reaction. The transducer surface can be modified with mediators and CPs for such purposes.^[15] During the amperometric measurements, a reference electrode keeps the applied potential stable which improves the repeatability of the electrochemical reaction. Clark and Lyons developed the first amperometric type enzyme electrode in 1962.^[20] Their construction mechanism is based on recording either the production of H₂O₂ or the consumption of O₂ during enzymatic reaction. Platinum is used as the counter electrode and Ag/AgCl electrode is used as the reference electrode. Upon

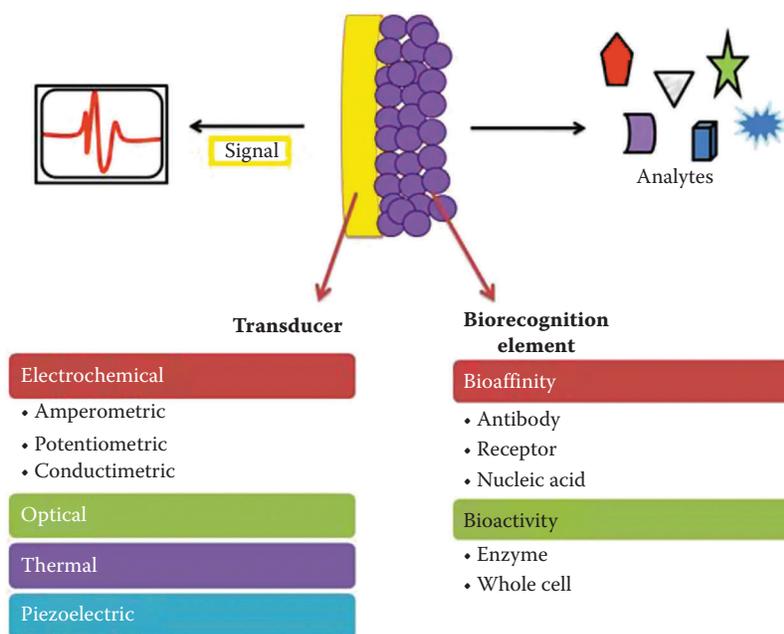


Fig. 1 Simple representation of a biosensor

application of -0.68 V to the cathode, a current proportional to the oxygen concentration was obtained. Hence, the reduction of the diffused oxygen concentration is detected by following the current change. In addition to this approach, amperometric biosensor can be followed by the current change upon applied $+0.68\text{ V}$ potential. During this method, the production of hydrogen peroxide is measured. Since the applied potential is much higher than the oxygen electrode, electroactive species in the reaction medium may cause a decrease in the biosensor performance. Preferring the lower working potential method can overcome this limitation.^[21]

CPS IN BIOSENSING DESIGN

CP interfaces offer a suitable platform for localizing biomolecules onto the electrode surfaces. They can be utilized to improve sensitivity, stability, and selectivity of the biosensors fabricated for different purposes such as medical diagnostics and environmental monitoring.^[22] Use of CPs in biosensor construction enhances the electrocatalytic properties of the biomolecules since the electrons can move freely on the conjugated π electron backbones.^[16] Hence, this property improves rapid electron transfer and satisfies direct communication between the transducer and the biomolecule.^[23] In addition, CP-modified surfaces give extensive stability to enzymes on the electrode surface.^[24] In enzyme immobilization strategy, one important factor is holding the biomolecule onto the substrate for long-term stability without losing enzyme activity. Thus, CPs are excellent candidates for their superior properties. CPs can be deposited on the electrode surface using

the electropolymerization technique by arranging the thickness of the film upon demand.^[25]

Besides, CPs can be electrochemically produced at room temperature which is very important for the stability of biomolecules.^[26] They can be modified by incorporating specific functional moieties that can link to biomolecules of interest. This attachment leads retaining the enzyme activity for a long time^[27,28] via creating a suitable three-dimensional matrix on the electrode surface. The polymer structure may include carboxyl group, amino group, and so on which are open to covalent bond to achieve a robust biosensor. In medical diagnostics and detection of environmental pollutants, convenient, highly precise, and rapid detection sensing methods are of great importance. In this regard, as a sensing platform, CPs are very attractive materials since their use gives variable binding interactions with biomolecules that may offer sensitive and long-term stability during measurements.

CP CONTAINING BIOSENSOR PLATFORMS

Glucose Biosensors

Glucose is an essential carbohydrate circulating in the physiological system. The beta cells of the pancreas produce the hormone insulin which converts the sugar into the skeletal muscle and adipose tissue. Improper function of pancreas results in failure to abnormal blood glucose concentration. Therefore, the accurate determination of glucose level in any physiological fluid is crucial in diagnostics and in the long-term treatment of diabetes. Glucose oxidase (GOx) was often used as a model enzyme to

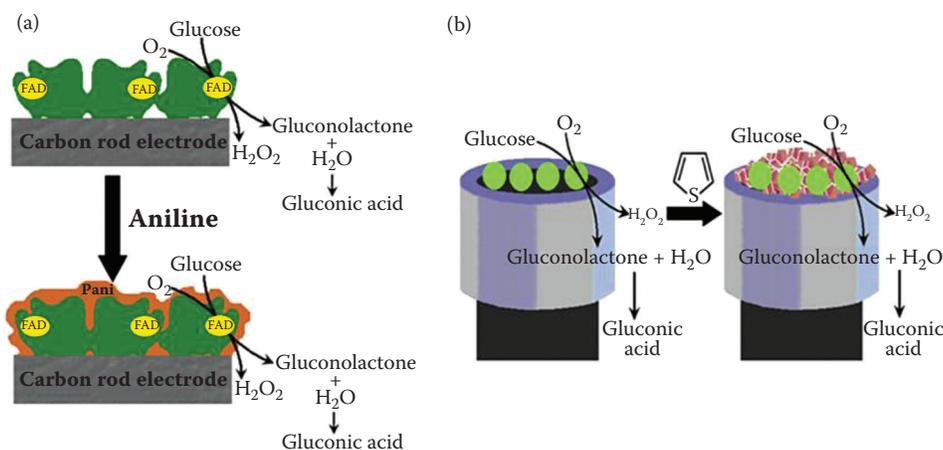


Fig. 2 Schematic illustration of (a) modification of GOx-coated electrode by PANI. (b) PTP layer deposition on the GRE/GOx-modified electrode

Source: (a) Adapted with permission from Elsevier Science Inc., © 2010.^[30] (b) Adapted with permission from Elsevier Science Inc., © 2014.^[31]

analyze various biosensor configurations. It is a flavoprotein, containing two tightly bounded flavine adenine dinucleotide redox centers. The redox centers lead electron transfer during the enzymatic reaction. GOx catalyzes the oxidation of β -glucose to *d*-glucono- δ -lactone in the presence of molecular oxygen, which is subsequently nonenzymatically hydrolyzed into gluconic acid and hydrogen peroxide.^[29]

Among other CPs, PANI, PPy, and PTh are often used as the immobilization matrices for GOx. Apart from high conductivity, electrochemical stability, and thermal stability, PANI creates a favorable environment since it can be synthesized in an aqueous medium. Kausaite-Minkstiniene et al.^[30] reported amperometric glucose sensors to investigate the effect of PANI layer on biosensor responses (Fig. 2a). The GOx was self-encapsulated within PANI matrix and an increase in the upper detection limit, and sensor stability was detected compared to those of unmodified GOx electrode. The same fabrication process as seen in Fig. 2b was followed by Krikstolaityte and coworkers using PTh.^[31] Polymerization of PTh was defined as “green chemistry” since no hazardous chemicals were involved during the reaction. Changes in enzymatic PTh polymerization period led tuning of K_M^{app} and other kinetic parameters. Poly(1,10-phenanthroline-5,6-dione) (PPD) was also used as the immobilization matrix for GOx conducted by Ciftci et al.^[32] Taking advantages of a mediator like PPD, the proposed sensing design exhibited satisfactory selectivity toward glucose together with good storage stability.

One of the important reasons of preferring CPs as the support matrices in biosensor design is that CPs allow the structural and electronic modifications of various surfaces to be used as immobilization matrices for biomolecules.^[33] The polymer backbone can be tuned by according to desired properties. For instance, presence

of hydrophobic alkyl chain in the structure of poly((2-dodecyl-4,7-di(thiophen-2-yl)-2*H*-benzo[d][1,2,3]triazole (PTBT) improved the interaction between the enzyme solution (GOx and isoleucine) and polymer coated support electrode.^[34] In our previous study, two different CPs containing benzotriazole and benzoselenadiazole units were used for the fabrication of glucose biosensor.^[35] The effect of selenium- and sulfur heteroatom-containing polymer backbones on biosensor performance was investigated. Due to better interaction ability of Se with NH, C=O, COO⁻, and C–N groups as well as higher affinity for Se to protein, Se-containing polymer showed high biosensor responses and stability. Moreover, it provides an enzyme mimic environment for the biosensor since Se has also the role of an antioxidant preventing the cell degeneration in tissues.^[36]

CPs can be functionalized with several groups such as carboxyl (–COOH), amine (–NH₂), and hydroxyl (–OH) that are reactive toward biomolecules and used in covalent immobilization with the help of specific cross-linking agents to achieve the lifetime stability of the enzyme electrode.^[16] It involves the formation of covalent attachment using pendant functional groups on the immobilization matrix with the enzyme. Carboxyl group containing poly(2-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl) acetic acid (Fig. 3a) was utilized to immobilize GOx successfully via *N*'-ethylcarbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS).^[37] Electrodeposited poly([2,2';5',2'']-terthiophene-3'-carbaldehyde) (Fig. 3b) film serves as an excellent host matrix for immobilization of biomolecule via covalent immobilization due to the free aldehyde groups of the polymeric structure.^[38] Azak et al. synthesized a CP, poly(5-(4*H*-dithieno[3,2-*b*:2',9'-*d*] pyrrol-4-yl)naphthalen-1-amine) (Fig. 3c) where GOx was deposited onto the free amino groups containing polymeric support matrix using glutaraldehyde as the crosslinking

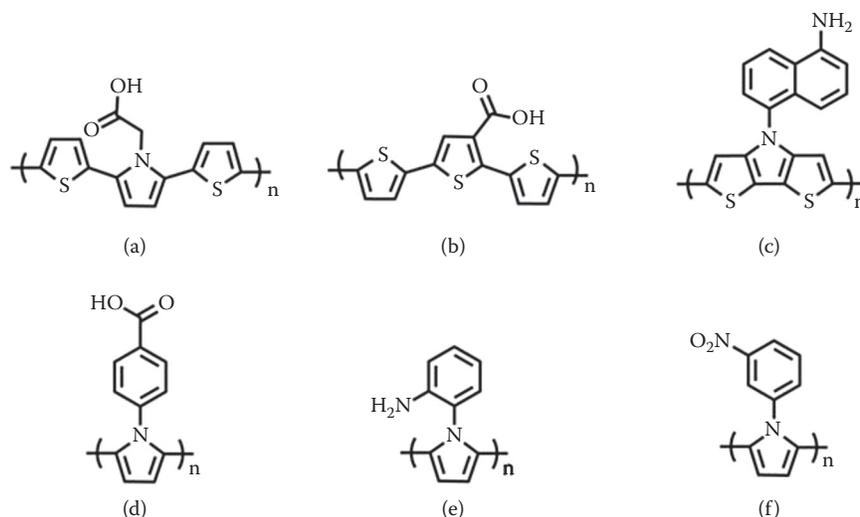


Fig. 3 Structures of the functional CPs

agent.^[39] Poly(*N*-phenylglycine) (PPG) was also used to fabricate an enzyme electrode to immobilize GOx covalently on the PPG-modified transducer by the condensation reaction of amino groups of GOx and the carboxyl groups present on the film.^[40] *N*-Substituted pyrrole polymers were used to investigate their matrix properties in biosensor applications.^[41] Amperometric biosensors were fabricated through immobilizing GOx onto the synthesized polymers, *N*-(*p*-benzoic acid)polypyrrole (NpbPPy), *N*-(*o*-aminophenyl)polypyrrole (NoaPPy), and *N*-(*m*-nitrophenyl) polypyrrole (NmnPPy) (Fig. 3d–f). Since the amount of immobilized GOx was enhanced with the help of condensation reaction of –COOH groups on NpbPPy matrix, the best biosensor performance was succeeded for NpbPPy matrix.

Also, comparative investigation of isomeric derivatives of several units was performed to examine the effect of position of pendant functional groups on the ability as a biosensing platform. Zhou et al. studied three phenylenediamine isomers (including ortho, meta, and para derivatives) which were electrodeposited on platinized glassy carbon electrodes.^[42] Amperometric glucose sensors were constructed using these polymer films. Ortho and para derivatives of the polymer matrices exhibited similar sensitivities and anti-interference ability, whereas meta derivative of the poly(phenylenediamine) revealed enhanced substrate sensitivity and lower permeability to the interfering species. On the other hand, Ayranci et al.^[43] considered biosensor application of para and meta derivatives of thienylpyrrole. For this purpose, spectroelectrochemical studies of the two polymers were achieved. Accordingly, since the introduction of paraamine in the polymeric scaffold generated more conjugated and more electroactive structure compared to the polymeric form of its meta isomer, para isomer provided more favorable interaction with the enzyme molecules to obtain a stable immobilization.

Copolymer films based on the integration of different polymeric structures in the same scaffold have the properties of individual components. These copolymers are expected to embody the superiority of both parent polymers; thus, tailor-made design of such copolymers allows obtaining innumerable kinds of materials with desired functionalities.^[44] Moreover, electrochemical copolymerization technique provides opportunities to deposit the copolymer at the electrode surface by controlling the several parameters as well as to obtain the desired polymer with no need of purification. Teodorescu et al.^[45] generated a copolymer film of poly(azulene-*co*-3-thiophene acetic acid) which was tested as the host matrix for the construction of a glucose biosensor. The pendant carboxyl groups in the copolymer chain are open to form covalent attachments with the enzyme molecules. Such strong attachments provided to determine wide substrate concentrations ranging from 40 to 200 μM with a sensitivity of 0.7 $\text{nAcm}^{-1}\mu\text{M}^{-1}$ at the -0.07 V versus Ag/AgCl. Co-electrodeposition of poly(*N*-acetylene)-Prussian blue (PNAANI-PB) film exhibited remarkable synergistic effects compared to PB films.^[46] The sensitivity was improved and the working range of the glucose sensor was expanded (Fig. 4a).

Another approach to merge various polymers onto the electrode surface is the mixing of polymers or their solutions onto the electrode surface without any treatment. Karagollu et al.^[47] synthesized a copolymer of glycidyl methacrylate with 3-thienyl methacrylate (poly(GMA-*co*-MTM)); then the polymer solution was drop-casted onto the electrode surface to construct glucose biosensor. Kakhki et al.^[48] developed a PEDOT/poly(methylene blue) (PMB)-modified electrode for a GOx-based biosensor. PEDOT films generated on top of the PMB-modified bare electrode was used to enhance the stability of PMB-modified electrode. The proposed sensing architecture (GOx/PEDOT/PMB/GCE) showed superior biosensor performance compared to GOx/GCE and GOx/PEDOT/GCE biosensors. It

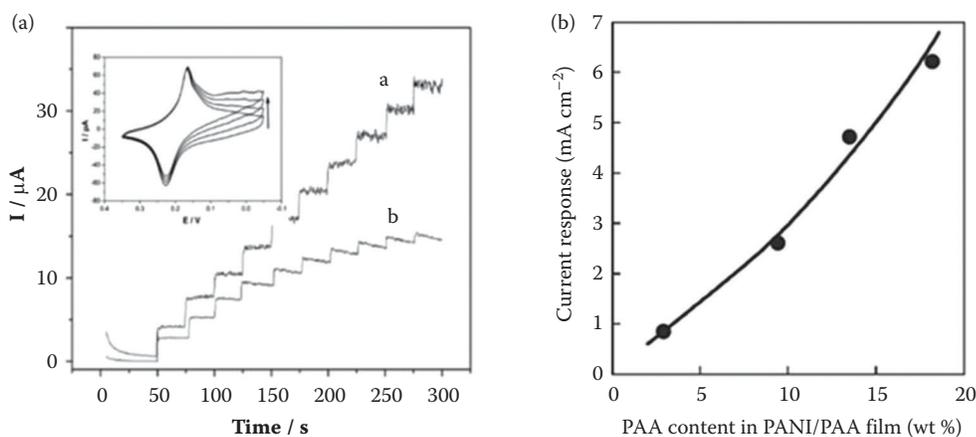


Fig. 4 (a) Amperometric response of (a) PNAANI–PB and (b) PB films. (b) Relation between the PAA content in the PANI/PAA film and the current response of the PANI/PAA-GOx film to 90 mM of glucose

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also maintained the enzyme activity after 5 weeks of storage, which is a critical issue for a practical application of the prepared glucose biosensors. A conducting film composed of PANI and poly(acrylic acid) (PAA) were prepared by electropolymerization of aniline in the presence of various concentrations of PAA.^[49] The biosensor response to a given concentration increased as PAA content in PANI/PAA film increased (Fig. 4b). This behavior was related to conductivity of the PANI/PAA film since carboxyl groups within PAA chain caused protonation of nitrogen atoms on PANI.

Immobilization of mediators in the sensing matrices generates enhanced the electron transfer efficiency of redox enzymes since active sites of the biomolecules are deeply embedded inside the protein.^[50] Among other mediators, ferrocene and its derivatives are one of the most popular choices in biosensor design due to their ability to generate stable redox species.^[51] The incorporation of ferrocene moiety into polymeric matrices allows direct attachment to the enzyme electrode. By this way, leakage of the mediator from the surface, which is the main problem of reagentless devices, can be prevented.^[52] Electrodeposited copolymer of pyrrole and ferrocene carboxylate-modified pyrrole p(Py-FcPy);^[53] copolymer of pristine pyrrole, carboxylate-modified pyrrole, and ferrocene-modified pyrrole;^[54] co-deposition of 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)aniline (SNS-NH₂) and 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)amidoferrocenyldithiophosphonate (SNS-NH₂-Fc);^[55] and copolymer of O-4-(1H-pyrrol-1-yl)-ferrocenyldithiophosphonate with 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)butane-1-amine^[56] were employed as electron-mediating support materials for developing GOx-immobilized electrodes. Furthermore, Antiochia et al.^[57] proposed a GOx biosensor based on [Os(bpy)₂(4-aminomethylpyridine)Cl]PF₆-modified nanostructured macroporous material (m-PANI)

on polystyrene nanoparticles. Enzyme molecules are covalently bound to functional groups of amine terminated osmium complex on the electrode. Low redox potentials and high efficiency of electron transfer rate are counted as the important benefits of osmium mediators compared to other transition metal analogs.^[58] Therefore, immobilization within the PANI of an osmium complex as electrochemical mediator led to a sensitive and stable glucose biosensor.

In recent years, the combination of nanomaterials with analytical and biological sciences for the development of ultrasensitive detection methods has attracted great attention in modern science.^[59] Particularly, the use of gold nanoparticles (AuNPs) in the field of biotechnology is attractive since they have localized surface plasmon resonance, high light absorption capability, electron transport ability, and excellent conductivity which are useful in a broad range of applications.^[60] Mazeiko et al.^[61] proposed the use of AuNPs with CP (PANI) with different surface concentrations in the glucose sensor architecture (GOx/AuNPs/PANI) as well as the comparison of biosensor characteristics with other types of GOx and GOx/PANI-based sensors. The influence of diameter of AuNPs was also investigated using this electrode design. Thanks to their ability to preserve the catalytic activity of biomolecules, the successive addition of AuNPs to the biosensor design influenced amperometric signals positively and resulted in a significant increase in K_M^{app} (Fig. 5). It was found that the highest electron transfer rate was obtained for 3.5 nm AuNPs. Moreover, nanoparticles functionalized with different kind of groups provide affinity sites for binding the biomolecules.^[62] For example, AuNPs were modified with mercaptopropionic acid (MPA) yielding Au–S bonds.^[63] The carboxyl groups react covalently with the enzyme molecules and the amino groups of functional conducting polymer (BEDOA-6) which was synthesized to use as the immobilization matrix for the

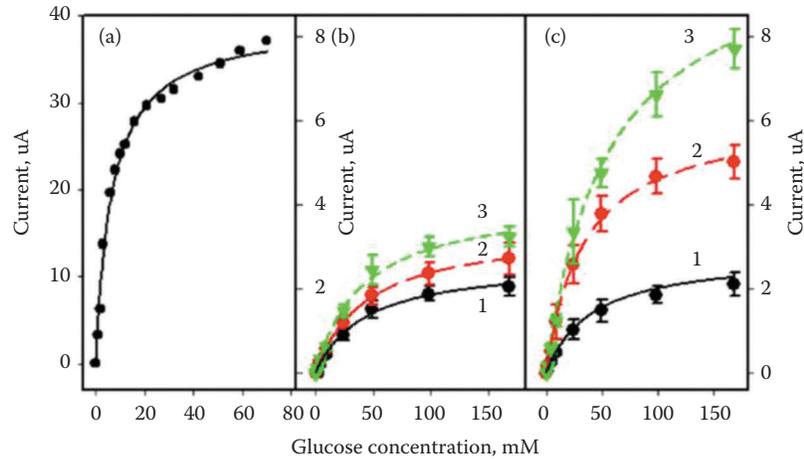


Fig. 5 Current dependence on the amount of the synthesized nanocomposite immobilized on working electrode surface: (a) GOx control electrode; (b) GOx/PANI electrode (particles: 1—40 mg/cm^2 , 2—120 mg/cm^2 , 3—360 mg/cm^2); (c) GOx/Au-NPs/PANI electrode (particles: 1—40 mg/cm^2 , 2—120 mg/cm^2 , 3—360 mg/cm^2)

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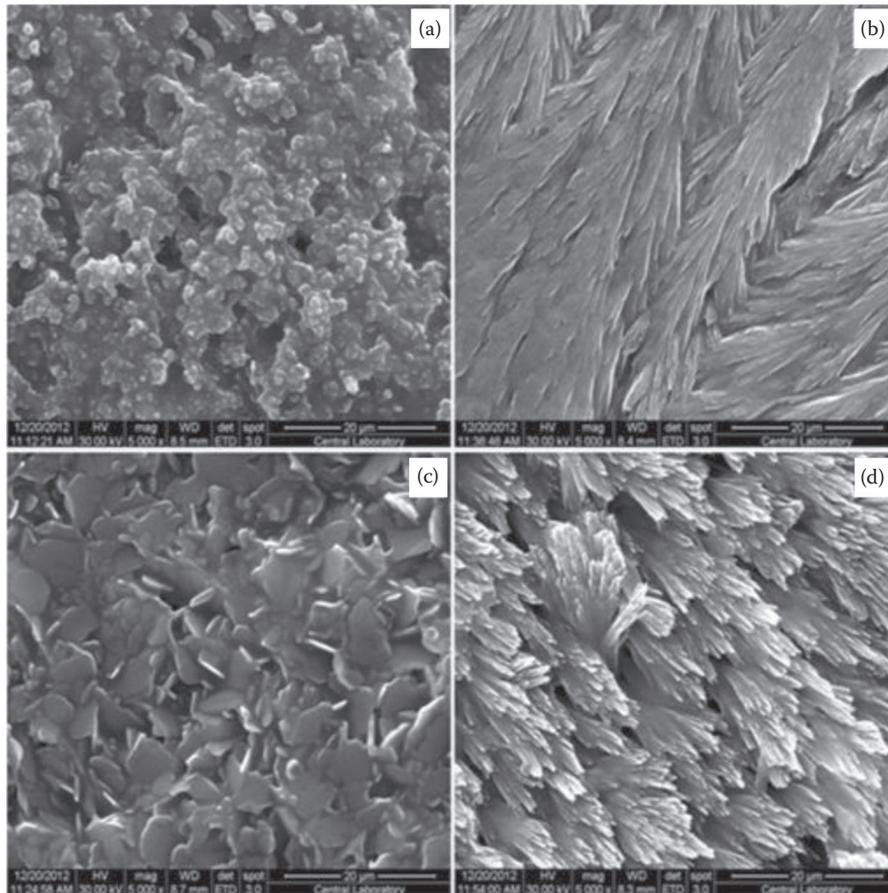


Fig. 6 SEM images of (a) poly(BEDO A-6), (b) poly(BEDO A-6)/GOx, (c) poly(BEDO A-6)/Au NPs/MPA and (D) poly(BEDO A-6)/Au NPs/MPA/GOx under optimized conditions

Source: Adapted with permission from Elsevier Science Inc., © 2013.^[63]

GOx. In this way, the proposed biosensor was fabricated with self-assembled monolayer techniques as illustrated in Fig. 6. This modification leads to an increase in the

surface area of the three-dimensional electrode surface.^[64] SEM images suggest that π -conjugated conductive film stimulates the self-assembly of the modified nanoparticles

resulting in a three-dimensional wiring system that stabilizes the immobilized enzyme with enhanced biosensor performance.

In biosensor design, the wrapping of carbon nanotubes (CNTs) with CPs leads to produce new composite materials with combined properties of each component. The synergistic effect gave rise to a remarkable improvement in the biosensor efficiency.^[65] High-performance glucose biosensors were constructed by immobilization of GOx onto a poly(2,6-diaminopyridine)/multi-walled CNT (MWCNT) transducer^[66] or 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine (SNS-NH₂) modified with CNT surface.^[67] The sensors were envisaged for the determination of glucose level in various real samples which could make them a promising bio-probe for precise detection of glucose. Adronov group^[68] used a water-soluble CP, poly[3-(3-*N,N*-diethylaminopropoxy) thiophene] (PDAOT) in supramolecular functionalization of single-walled CNTs (SWCNT). The PDAOT-SWCNT combination offers a universal platform for enzyme deposition within coated films revealing good film quality, improved electron transfer kinetics, and high electronic conductivity. A Nafion overcoat results in improved selectivity for this sensor design. This interface effectively suppressed the response to possible interferents such as ascorbic acid and uric acid while maintaining much of the sensor sensitivity to target substrate such as glucose. Furthermore, our group incorporated nylon 6,6 nanofibers and MWCNT with an aldehyde-functionalized conducting polymer (PBIBA) to investigate the sensor properties for detection of glucose for the first time.^[69] Nylon nanofibers provide excellent reproducibility and high enzyme loading with their highly porous structure.^[70] Remarkable compatibility between MWCNTs and nylon, which improves the dispersion and the interfacial adhesion, is a reason to select nylon nanofibers as the host matrix for the MWCNTs. Also, the use of the PBIBA and nanofibers onto the same platform provides long-term stability due to the robust covalent bonds between GOx and nanofibrous-coated surfaces.

Furthermore, the two-dimensional nanocarbons such as graphenes (G) have attracted growing interests in the field of biosensor technology. They are assumed to have superior properties compared to CNT counterparts owing to their larger surface area, easy production with no hazardous by-products, and higher intensity of active edge planes per unit mass.^[71] Highly sensitive GOx biosensors based on G-PEDOT:PSS-modified electrode^[72] and G-PANI-chitosan (CS)-modified electrodes^[73] were developed for electrochemical detection of glucose.

Apart from these, there are numerous nanomaterials used in the fabrication of glucose sensors. Xiao et al.^[74] used PMB-doped silica nanocomposite on glassy carbon electrode to construct third-generation GOx biosensors. As seen in Fig. 7, the grape like aggregation proved linkage of PMB and SiO_{2(nano)} and fixing nanoscale SiO₂ cages on the electrode surface during electropolymerization of MB. This compact morphology demonstrated large effective surface area for enzyme loading. In another work, a PANI-TiO₂ nanotube composite was prepared for the development of electrochemical biosensor. Compared to individual components, the suggested matrix sustained their individual characteristic features while restricting the shortcomings of each component. Furthermore, a number of works suggest that the combinational use of clay containing polymer nanocomposites and conducting polymers or thermoplastics enhances physical and chemical properties as well as mechanical properties, solvent resistance, and anti-corrosion capacities.^[75] Therefore, we proposed two different biosensor architectures: an amino-functionalized CP incorporating PMMA/clay nanocomposite^[76] and a benzimidazole-based CP with PMMA/clay nanocomposite for glucose sensing purpose.^[77] The modified surface revealed well permeability of the matrix, providing a better microenvironment for any biomolecule.

As an immobilization platform, the use of supramolecular compounds for biosensing systems creates one of the most remarkable topics. Calixarenes are the host molecules which have a cavity composed of several cyclic

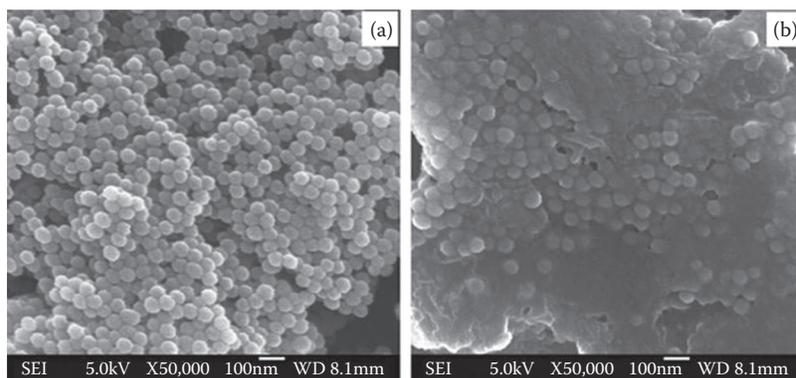


Fig. 7 FESEM images of (a) PMB@SiO₂(nano) film and (b) GOx-PMB@SiO₂(nano) film on GCE surface
Source: Adapted with permission from Elsevier Science Inc., © 2012.^[74]

phenolic units.^[78] They can be modified according to the desired properties. Also, their cavity properties enable easy deposition of the guest molecules on any surface.^[79] Thus, these macrocyclic molecules are promising materials for enzyme immobilization. Various types of sensors combined with a conducting polymer and calixarene have been designed and tested for biological components in any test solution. Safarnavadeh et al.^[80] reported the capability of calixarene containing parasulfonate as the anion dopant for loading GOx on PPy film in biosensor construction. They emphasized that leaching of enzyme was prevented by calixarene dopant anions. This fact enhanced the stability of the suggested biosensor. In another work, the sensor matrix included AuNPs apart from a calixarene derivative and selenium-containing CP (poly(SBTz)).^[81] The proposed calixarene compound contained free thiol and carboxyl groups. By taking the advantage of high affinity between AuNPs and thiols and covalent binding between calixarene and GOx using carbodiimide chemistry, the design resulted in improved sensor stability together with a superior analytical performance.

Poly(amidoamine) (PAMAM) dendrimers with different generations are also strong candidates for the design of immobilization matrix in biosensor construction. Şenel and Nergiz^[82] synthesized different generations of amidoamine-pyrrole dendrimers with branched amine periphery and focal pyrrole functionality. GOx was covalently immobilized onto the copolymer of amidoamine-pyrrole dendrimers. Different biosensors were constructed using PAMAM G1, PAMAM G2, and PAMAM G3 and without any PAMAM dendritic wedges. As seen in Fig. 8, the biosensor with PAMAM G2 exhibited a broader linear range with a lower detection limit than the other electrodes. Also, the biosensor fabricated with pyrrole linked to any PAMAM dendron showed better biosensor signal than the one with pyrrole alone concerning the steady-state current response curves of the electrodes. The reason is that PAMAM dendrons enable covalent linkage with biomolecules, serving higher enzyme loading capacity. This conclusion is also confirmed by our group.^[83] A carboxyl group containing CP film was functionalized with PAMAM dendrimers: G2 and G4 and their matrix properties were investigated for glucose biosensor applications. The PAMAM G2- and G4-modified biosensors illustrate wider linear range with higher current response. Also, it was emphasized that the crowded microenvironment of G4 around enzyme molecules had a higher K_M^{app} value than that of the G2 modified one while achieving higher I_{max} .

Owing to its biocompatibility, biodegradability to harmless products, nontoxicity, physiological inertness, hydrophilicity, and remarkable affinity for proteins, CS as a functional material is widely exploited in biological applications. CS can be generally used with other immobilization platforms for glucose sensing. For instance, substituted PANI/CS composites,^[84] CS pyrrole

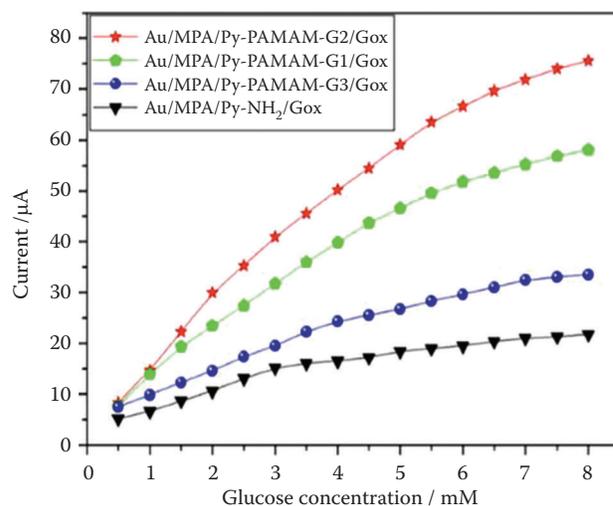


Fig. 8 Dependence of current on glucose concentration for enzyme electrode

Source: Adapted with permission from Elsevier Science Inc., © 2012.^[82]

composite,^[85] and CS-coupled CNT on PANI^[86] are several biosensor architectures reported previously in the literature. Divergent immobilization support material has been used for the development of glucose biosensors. Luo et al.^[87] studied that palygorskite (Pal) combined with a CP, poly(*o*-phenylenediamine) was examined as a platform for immobilization of GOx. This design combines the characteristic features of Pal: large specific surface area, environmental friendliness and nontoxicity, and high conductivity and stability.^[88] In another study, aldehyde-bearing phosphazene polymer (PPA) was coupled on a GOx support matrix with an amino functionalized polymer poly(SNS-NH₂).^[89] Polymers with inorganic backbone like PPA derivatives allow to fine-tune physical and chemical properties.^[90] Thus, such scaffolds are compelling materials which are used in a number of biosensors via using suitable co-support materials. Akbulut et al. developed a biosensor based on a peptide-modified PTh for glucose analysis.^[91] This work was the first demonstration of glucose sensing utilizing a conjugated graft copolymer by taking advantages of the fluorescence features and reactivity of the amino side chains of polymer.

Apart from the glucose analyses which were generally performed with GOx as the target biomolecule, pyranose oxidase (PyOx) can be an alternative enzyme for construction of a glucose biosensor. Although PyOx and GOx have similar mechanisms, PyOx has high affinity for D-glucose and ability to oxidize various sugars.^[92] Gokoglan et al.^[93] developed selenium-containing CP-based PyOx biosensor for glucose detection. The proposed sensing system was applied to determine glucose levels in beverages, soda, ice tea, and milk successfully. Guler et al.^[94] presented the fabrication of a functional conducting polymer (4-amino-*N*-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzamide (HKNC))-based PyOx biosensor for the detection of glucose.

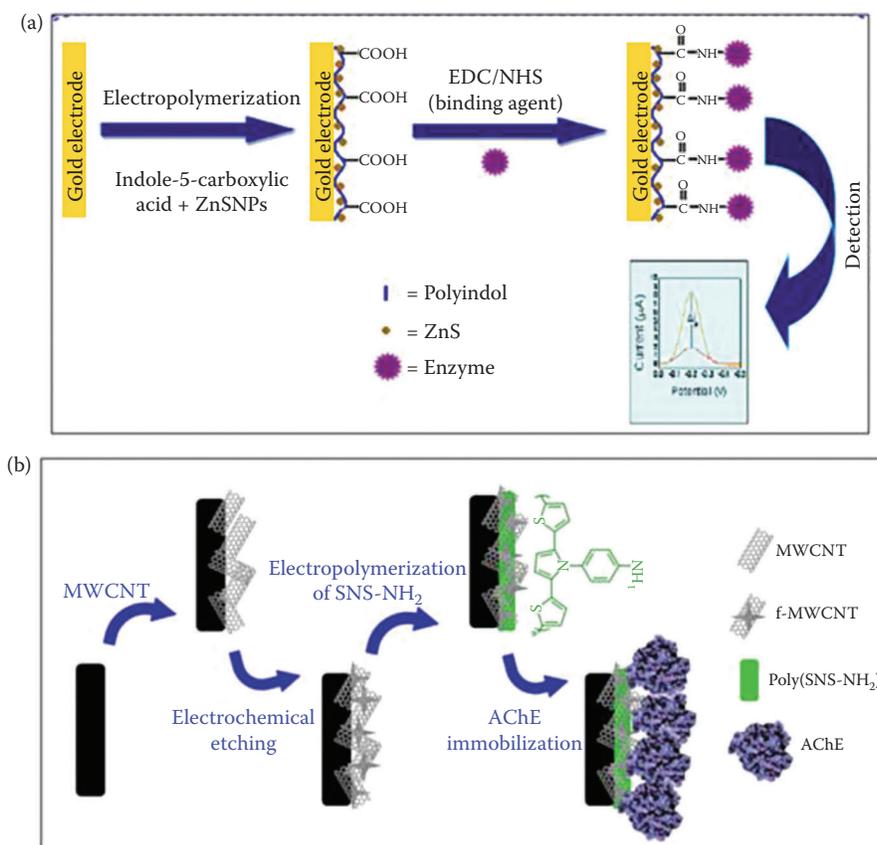


Fig. 9 (a) Schematic illustration of the stepwise biosensor fabrication process. (b) Schematic representation of the proposed biosensor
 Source: (a) Adapted with permission from Elsevier Science Inc., © 2011.^[106] (b) Adapted with permission from Elsevier Science Inc., © 2014.^[107]

Pesticide Sensors

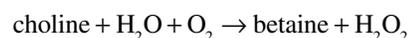
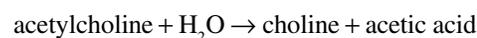
The detection and monitoring of environmental pollutants are some of the fundamental requirements of our well-being. Environmental security remains still a major global challenge. Numerous monitoring techniques have been developed for this purpose. Although the common methods based on chromatographic separation such as gas and liquid chromatography are highly selective and reliable, they are time consuming, require expensive equipment and high-qualified personnel, and are not adapted for in situ detection.^[95] In other words, there is need for simple, rapid, and portable methods to analyze environmental pollutants in soil, water, or air. Thus, the development of inhibition-based enzyme biosensors appears as a promising alternative to such classical methods.

Acetylcholinesterase (AChE) is a member of the family of hydrolases playing a key role in cholinergic transmission of the central nervous system. AChE catalyzes the hydrolysis of neurotransmitter acetylcholine (ACh) and terminates the chemical impulse at cholinergic synapses.^[96] Inhibition of cholinesterase by the pesticides was observed through the formation of covalent phosphoryl-enzyme complexes. Both organophosphorus and carbamate pesticides can react with AChE in the same manner since the acetylation

of the serine residue at the catalytic center is analogous to phosphorylation and carbamylation. Carbamated enzyme can restore its catalytic activity more rapidly than phosphorylated enzyme.^[97] Cholinesterase inhibitors interfere with this process while inhibiting the catalytic activity of AChE. Since ACh level depends on the availability of active AChE, abnormal production of ACh leads to severe impairment of nerve function or even death.^[98]

In recent years, CP-modified electrochemical biosensors have been constructed for the detection of pesticides using different types of enzymes: (1) AChE—choline oxidase (ChOx) bienzyme and (2) AChE.

Since the redox potential of ACh is too high for direct determination using electrochemical reaction, AChE and ChOx were used together on the electrode surface to promote the following enzymatic reactions:

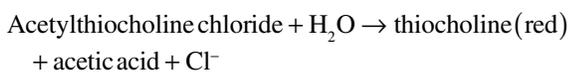


The crucial point during fabrication of high-performance biosensors in the bienzymatic system is that the ratio of catalytic activity of AChE to ChOx should be suitable.

Usually, an excess amount of ChOx was mixed with AChE and immobilized onto the modified transducer. Overall reaction rate can be determined by the rate of AChE-catalyzed reaction since the following electrochemical oxidation of H_2O_2 is sufficiently fast.^[99]

Aynaci et al.^[100] used polypyrrole-polyvinyl sulfonate (PPy-PVS) film as the enzyme support for the detection of ACh. No interference effect was observed for ascorbic acid, uric acid, ethanol, methanol, paracetamol, cystine, cysteine, and glucose. Biocompatibility of the composite, PPy-PVS, makes the material a good candidate for the development of selective ACh sensor. In another study, an amperometric ACh biosensor based on an amino-functionalized CP (poly(SNS-NH₂)) was reported.^[101] For paraoxon-ethyl detection as a model pesticide, AChE and ChOx were co-immobilized onto the polymer-coated electrode via the covalent binding technique. The results of proposed biosensor for the determination of paraoxon in spiked tap water samples were compared with a conventional quantification method using high-performance liquid chromatography (HPLC) with a diode-array detector (DAD). Considering the results, the sensing architecture can practically be used as a promising complementary method for detection and quantification in tap water samples to replace HPLC detector.

On the other hand, the oxidation potential of choline is too high to be electrochemically oxidized. Thus, ChOx is used to oxidize choline. There is no need to use a second enzyme, ChOx, if reaction products can be oxidized readily on the electrode.^[99,102] Considering this, AChE-modified electrode was developed in the presence of acetylthiocholine instead of ACh as the substrate of AChE. The reaction mechanism is as follows:^[103,104]



Dutta et al.^[105] described PPy-entrapped AChE electrode for detection of paraoxon as the model organophosphate and carbofuran as the model organocarbamate pesticide. Its ease of fabrication, reusability, and stability were comparable with other studies based on different matrices which do not include any derivative of a CP. Also, a simple and cost-effective fabrication process of the proposed design can lead to overcome the crucial problems of commercial chromatographic techniques for detection of pesticides.

The recent issue in the development of AChE biosensors is lowering the oxidation potential of thiocholine. It can be directly oxidized to its dimer upon application of +0.7 V. This high potential value may result in a high background current and a possible interference due to oxidizable compounds in the working medium. To overcome this issue, materials with high electron transfer abilities can be used to

oxidize thiocholine at a milder potential. Chauhan et al.^[106] constructed an amperometric biosensor based on AChE immobilized onto nanocomposite of ZnS nanoparticles (ZnSNPs) and poly(indole-5-carboxylic acid) (Fig. 9a). The nanocomposite promoted the electron transfer reaction at a lower potential (+0.2 V) providing a conductive path for that matter. Malathion and chlorpyrifos were used as the model inhibitors. Validation of the sensor design was tested by conventional gas chromatography–mass spectrometry (GC–MS). In another study, we suggested the combined use of carboxylate-modified MWCNT with an amino-functionalized conducting polymer (poly(SNS-NH₂)) for the determination of organophosphorous pesticides: paraoxon, parathion, and chlorfenvinphos^[107] (Fig. 9b). This strategy improved an electron transfer rate even at a lower potential (+0.1 V). The practical use of the present sensor for tap water samples was confirmed by comparing the experimental results obtained by HPLC/DAD. Cesarino et al.^[108] studied the immobilization of AChE on a core–shell structure-based PANI and MWCNT films. The effect of MWCNT on the hybrid film was examined by comparing thiocholine oxidation peaks for the biosensors fabricated with and without MWCNT (Fig. 10). MWCNT-containing electrode causes the peak potential to shift ~100 mV to more negative values as well as increases the current peak of about 32%. In a similar study, AChE was deposited on PPy and PANI copolymer doped with MWCNT.^[109] The copolymer film displayed a porous morphology which provided an ideal structure for enzyme entrapment. MWCNTs catalyzed the oxidation of thiocholine with a promoted electron transfer reaction at a lower potential. The developed biosensor was used for the detection of an irreversible pesticide, malathion.

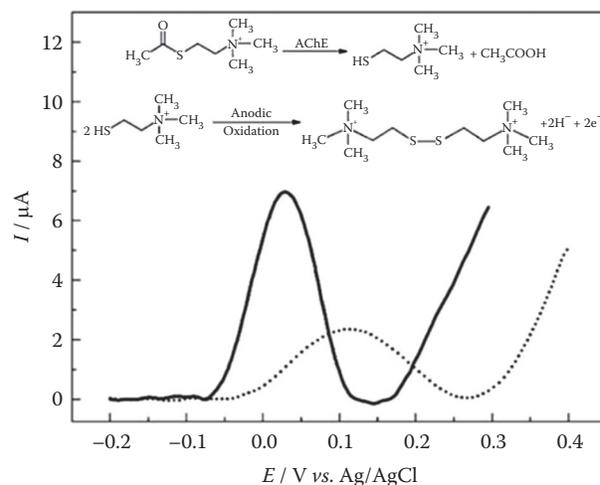


Fig. 10 Square wave voltammogram for GC/MWCNT/PANI/AChE electrode (solid line) and GC/PANI/AChE electrode (dotted line). *Inset:* Electrochemical mechanism of dithio-bischoline dimer formation

Source: Adapted with permission from Wiley-VCH Verlag GmbH&Co. KGaA, © 2011.^[108]

The objective of many studies is to fabricate an amperometric pesticide biosensor to be used to quantify pesticides in any test solution. In other words, applicability of the sensing systems in various test samples demonstrates the selectivity of the proposed biosensors. Yang et al.^[110] investigated the reliability of an amperometric biosensor based on a nanohybrid of gold nanoparticles, PPy, and reduced G oxide sheets in detecting paraoxon-ethyl in tap water. Kaur et al.^[111] tested a PANI-zeolite nanocomposite material-based AChE biosensor for the determination of monocrotophos in different real samples (apple, cabbage, tap water, and river water) with good recovery. In another work, an AChE-immobilized biosensor on a composite of PANI-MWCNTs was developed for electrochemical detection of carbamates in fruits and vegetables.^[112]

Cholesterol Biosensors

The estimation of cholesterol in human blood is one of the central issues in clinical diagnosis since cholesterol is an essential lipid with several biological functions in organisms.^[113] An imbalance of cholesterol level in blood is considered to be one of the most severe threats to human health and is related with several serious illnesses. High levels of cholesterol is associated with cardiovascular diseases, such as atherosclerosis, hypertension, diabetes mellitus, and brain vascular diseases, whereas a low level of cholesterol is associated with anemia and wasting syndrome, etc.^[114] Hence, several researchers have focused on the development of analytical devices for the detection, quantification, and monitoring of blood cholesterol level. Among the various analytical methods developed so far, amperometric enzyme-based biosensors have been mostly preferred to detect cholesterol. In recent years, electrochemical biosensors have shown to be very efficient tools for the analysis of biologically important molecules. They are fast, inexpensive, and portable, and have high selectivity and sensitivity. Moreover, this method offers a sensitive, selective, and quick detection of the cholesterol in blood samples.

Cholesterol oxidase (ChOx) is a flavin adenine dinucleotide (FAD)-containing enzyme that catalyzes the oxidation of cholesterol by molecular oxygen generating cholest-4-en-3-one and H_2O_2 .^[115] ChOx is a key enzyme in the cholesterol metabolism due to its high selectivity to cholesterol. A number of biosensing matrices have been developed previously, including CPs, nanomaterials, or their combinations. Among the diversity of sensing materials, CP containing surfaces are our main focus in this review. In our group, we designed and synthesized different functional group containing CPs for construction of a cholesterol biosensor. Soylemez et al. synthesized a monomer, (Z)-4-(4-(9H-carbazol-9-yl)benzylidene)-2-(4-nitrophenyl)oxazol-5(4H)-one (CBNP), and electrochemically polymerized on an electrode where ChOx was deposited onto

the polymeric support matrix using glutaraldehyde as the crosslinking agent.^[116] They also reported that due to the free nitro group of the polymer, hydrogen bonding with the enzyme molecules was achieved. In addition to this, possible π - π stacking between the aromatic residues of enzyme and polymer enables strong attachment for the enzyme on the polymer surface. Since enzyme molecule is properly attached on the polymeric surface, the proposed biosensor showed a wide linear range for the substrate compared to those of the previously reported studies^[117] (Fig. 11a). The similar fabrication process was followed by Soylemez et al. using a fluorine containing polymer.^[118] In this study, the use of a functional fluorine moiety in the structure provides a sensitive and reliable biosensor where no membrane or covalent bonding is required. Furthermore, through the successful adsorption of the biomolecules on the polymer surface, the three-dimensional feature of the protein molecules was protected due to the efficient H-bonding and the static interactions in the polymer/enzyme interface. The results reveal the changes in the morphology of the surface after immobilization (Fig. 11b).

The polymer backbone can be adjusted according to desired purposes. Copolymerization is a new approach enabling the covalent attachment of the enzyme molecules that are widely used to improve the biosensor performance. For instance, 2-heptyl-4,7-di(thiophen-2-yl)-1H-benzo[d]imidazole (BImTh) and 2-(((9H-fluoren-9-yl)methoxy)carbonylamino)acetic acid (Fmoc-Gly-OH)BImTh were chosen as comonomers. BImTh has a hydrophobic alkyl chain as the pendant group, whereas Fmoc-Gly-OH has a free carboxyl group which is open to amide bonding. Hence, the functional groups on the polymer in a same scaffold lead to a long-lived and highly selective biosensor.^[119] In order to work on various analytes comfortably, the biosensor was tested in the presence of several interferences such as urea, ascorbic acid, and glucose. No interference effect was reported for the proposed biosensor (Fig. 12a).

One approach to obtain stable biosensor is to use clay to fix enzyme molecules to a polymer-coated electrode surface by adsorption. Sepiolite ($Mg_2H_2(SiO_3)_3xH_2O$) is a naturally occurring clay-like mineral with a microfibrillar morphology which provides high surface area and extended porous volume. Therefore, sepiolite as a support material is a good choice for immobilizing enzymes via adsorption. In literature, amperometric behavior of ChOx was studied where sepiolite was introduced into the polymeric surface.^[120] The combined use of poly(10,13-bis(2,3-dihydrothieno[3,4-b][1,4]dioxin-5-yl)dibenzo[a,c]phenazine) (PPHED) and sepiolite enhanced the stability of the tertiary structure of proteins. Although PPHEDs do not have functional pendant groups to link the enzyme molecules with the support, efficient binding was achieved using sepiolite through adsorption. Moreover, after the enzyme immobilization, the morphology of the surface changed drastically (Fig. 12b).

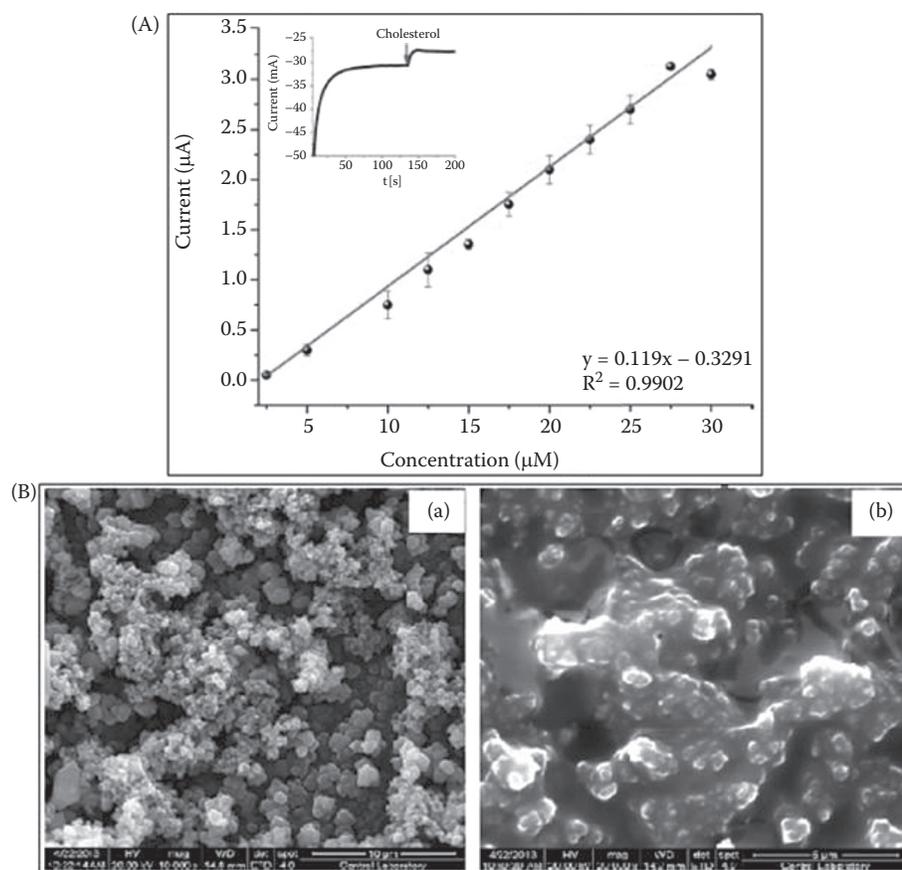


Fig. 11 Schematic representation illustrating (A) calibration curve for cholesterol (in 50 mM phosphate buffer, pH 7.0, 25°C, -0.7 V). Error bars show the standard deviation of three measurements (a typical amperometric response to 25 µM cholesterol given as inset). (B) Surface characteristics of (a) pristine CP (b) ChOx-immobilized CP-coated biosensor SEM images
 Source: (a) Adapted with permission from Elsevier Science Inc., © 2015.^[116] (b) Adapted with permission from Elsevier Science Inc., © 2014.^[118]

Rather than using a pristine polymer, creating a modified one for a target application and tuning the material properties for a certain purpose is a worthy step in scientific progress. Cyclodextrins (CDs) are cyclic oligosaccharides that are composed of both hydrophobic and hydrophilic moieties in their molecular structure. This enables them to form new supramolecular complexes with a variety of molecules. An alternative approach for the easy preparation of biosensing surface based on a poly(2-(2-octyldodecyl)-4,7-di(selenophen-2-yl)-2H-benzo-[d][1,2,3]triazole)) (PSBTz)-bearing β-CD was described.^[121] The best interaction and satisfactory immobilization of the enzyme molecules were achieved with an optimum amount of β-CD that provides an ideal conformation to create a host-guest system. The proposed biosensor showed a perfect linearity in a range of 0.15–22.5 µM cholesterol, as given by $y = 0.4093x - 0.0797$ and $R^2 = 0.9908$. The morphology of the electrode surface was also examined by SEM. As seen in Fig. 13, the surface morphology was notably changed after each modification. The pristine (PSBTz)-coated surface has a typical cauliflower-like structure. After the electropolymerization of monomer, a wrinkled surface

structure was obtained. Such surface properties allow easy penetration of the enzyme molecules into the polymeric layer, where enzyme molecules are freely oriented. The successful coating of electrode surface allows reliable and accurate detection of cholesterol in real samples.

Kakhki et al.^[122] developed a PEDOT/PMB-modified glassy carbon electrode (PEDOT/PMB/GCE) as a platform for a cholesterol biosensor. It was reported that the poor stability of PMB-modified glassy carbon electrodes was improved using a hydrophobic PEDOT electropolymerized film. The proposed biosensing system showed a superior sensitivity value ($79.0 \text{ mA cm}^{-2} \text{ mM}^{-1}$). Moreover, the developed ChOx/PEDOT/PMB/GCE biosensor was tested for cholesterol detection in whole cow milk and egg yolk.

To construct cholesterol biosensor, PANI is one of the most preferred CPs due to its excellent stability in different solutions and good electronic properties. Additionally, it has a number of amino groups ($-\text{NH}_2$) which are open to covalent bonds with biomolecules. Shin et al.^[123] designed a PANI-coated polyester film to obtain an amperometric cholesterol biosensor. In this study, PANI and polystyrene

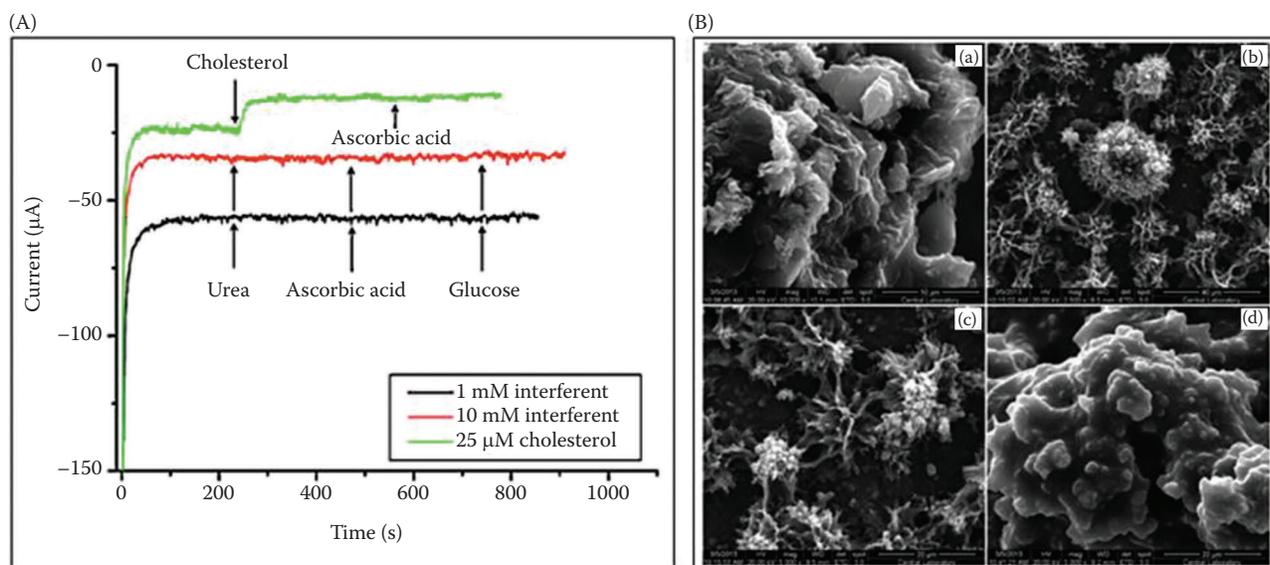


Fig. 12 (A) Amperometric responses of P(BImTh:Fmoc-Gly-OH)/ChOx biosensor to cholesterol and interference studies with ascorbic acid, urea, and glucose solutions (1 and 10mM) in 50mM phosphate buffer, pH 7.0, 25°C, -0.7V. (B) Surface characteristics of (a) sepiolite, (b) conducting polymer (PPHED) (with 2,500 \times magnification), (c) PPHED (with 5,000 \times magnification), and (d) ChOx immobilized on a conducting polymer-coated sepiolite

Source: (a) Adapted with permission from Elsevier Science Inc., © 2013.^[119] (b) Adapted with permission from Elsevier Science Inc., © 2013.^[120]

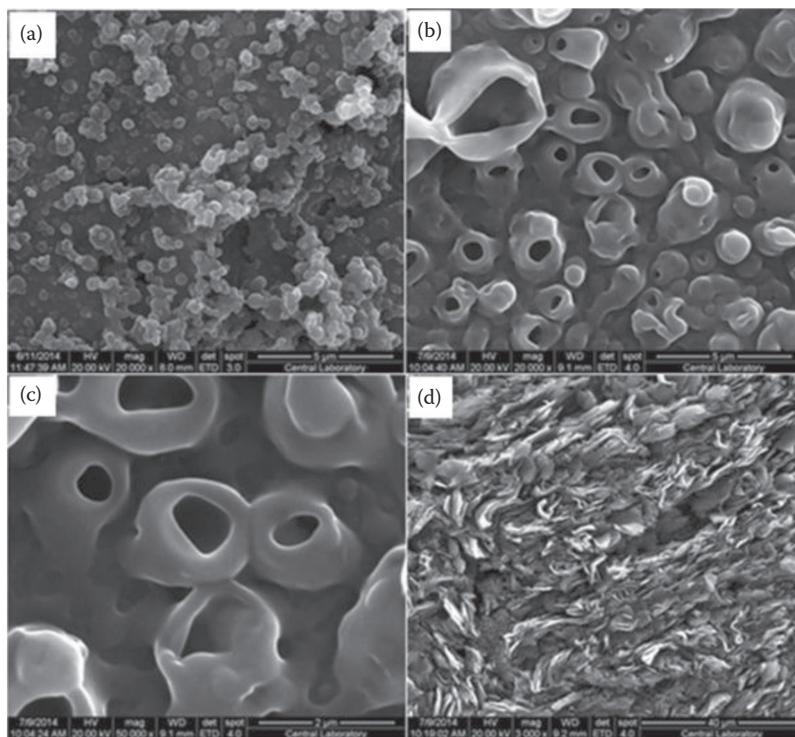


Fig. 13 SEM images showing the surface characteristics of (a) PSBTz, (b) PSBTz/ β -CD, (c) PSBTz/ β -CD, and (d) ChOx-immobilized PSBTz/ β -CD via under optimized conditions

Source: Adapted with permission from American Chemical Society, © 2014.^[121]

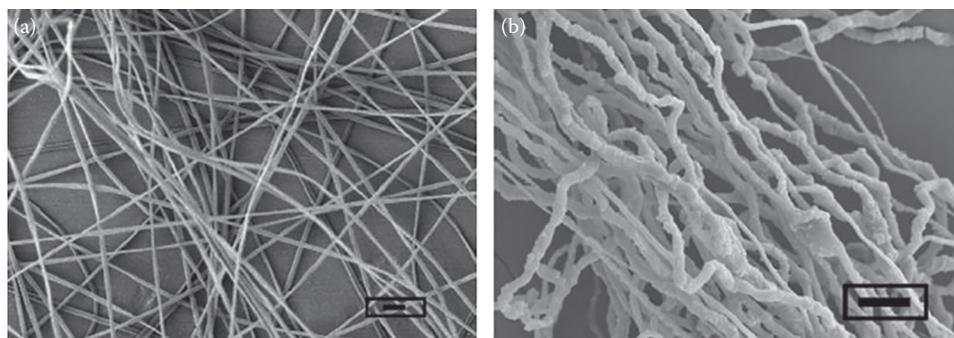


Fig. 14 (a) SEM images of the blend of PANI and polystyrene (bar represents 5 μm) and (b) ChOx immobilized nanofibers (bar represents 5 μm)

Source: Adapted with permission from Elsevier Science Inc., © 2012.^[124]

were used to improve sensor properties. When PANI was used alone, proper film was not generated on the electrode surface. Therefore, the combination of the PANI and polystyrene served as a proper immobilization matrix by making use of an electrostatic layer-by-layer adsorption technique. In another study, Shin and Kameoka^[124] designed a cholesterol biosensor using electrospun PANI nanofibers. It was reported that the electrospun nanofibers are promising nanomaterials for enzyme immobilization matrices since the nanofibrous platforms provides high surface area and long-time stability. After they constructed PANI nanofibers, biomolecule was immobilized on the PANI nanofibers using an electrostatic layer-by-layer adsorption method. The SEM images of the PANI nanofibers before and after enzyme immobilization are given in Fig. 14a and b.

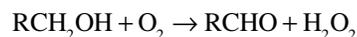
Molecular architecture plays a crucial role to obtain accurate, sensitive, and low-cost biosensors. Considering their charming properties, paper-based biosensors are promising candidates for biomolecule immobilization in biosensing systems since they offer low cost, biocompatibility, and disposability. To fabricate G, polyvinyl pyrrolidone (PVP), and PANI nanocomposites on a paper-based biosensor (G/PVP/PANI), Ruecha et al.^[125] designed a cholesterol sensor via an electrospraying technique. Under optimum conditions, linear range and LOD values were determined as 50 μM–10 mM and 1 μM for cholesterol, respectively (Fig. 15a and b). The amperometric measurements were achieved in 2 weeks to test the reproducibility of the sensor. It was reported that there was only 10.9% decrease in its initial response indicating that the constructed paper-based sensor had good stability for bio-applications. Moreover, this sensing system was successfully applied to detect the cholesterol level in human serum.

The wrapping of silver nanoparticles (AgNPs) with PANI represents a new class of material to construct highly sensitive and stable cholesterol biosensor. This way a high conductivity was maintained compare to that of pure PANI. For this reason, PANI-p-toluene sulfonic acid-silver (PANI-pTSA-Ag) nanocomposite film on indium tin oxide coated glass plate was utilized to fabricate a cholesterol

sensor.^[126] Cholesterol esterase (ChEt) and cholesterol oxidase were covalently attached to modified surface. The authors observed that the proposed sensing system offers high sensitivity of 36.3 μA/mg/dL with a fast response time of 10 s. In addition, the proposed sensor shows low K_M^{app} value estimated as 0.107 mM, indicating high affinity between ChEt and ChOx to cholesterol.

Alcohol Biosensors

To date, a number of matrices and a wide range of polymers have been employed for the development of different ethanol biosensors. In this part, we will focus on the most recent studies related to the CP-based biosensors prepared with *Pichia pastoris* (28 units per mg protein) enzyme molecule since AOX from *P. pastoris* (EC 1.1.3.13) is one of the most used enzymes due to its substrate specificity and availability and stability over a useful range of reaction conditions, which makes this enzyme a promising catalyst for biosensor applications. Alcohol oxidase (AOx; Alcohol: O₂ oxidoreductase, EC 1.1.3.13) is an oligomeric enzyme consisting of eight identical subunits arranged in a quasi-cubic arrangement, each containing a strongly bound FAD molecule.^[127] AOx oxidizes low-molecular-weight alcohols to the corresponding aldehyde in the presence of molecular oxygen (O₂) as the electron acceptor, according to the following reaction:



CP-based ethanol biosensing systems have attracted considerable attention than the others due to the unique properties of CPs. Conjugated polymeric materials led scientists to explore their distinctive and charming properties in various applications. Recently, there is a need for rapid and reliable detection of ethanol in many areas due to its commercial importance. At this point, choice of appropriate matrix and stability of the immobilization are the most significant factors to create this fascinating analysis. An appropriate immobilization matrix should be

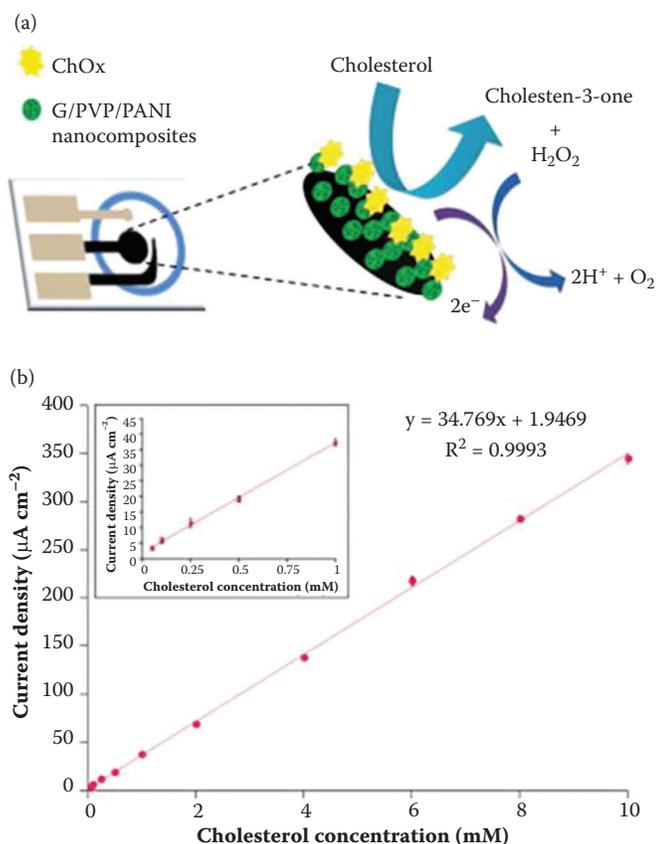


Fig. 15 (a) The schematic representation of the reaction between cholesterol and ChOx on G/PVP/PANI modified paper-based biosensor and (b) The calibration graph for cholesterol and calibration graph between 50 μM and 1 mM (inset) in 0.1 M PBS pH 7.0

Source: Adapted with permission from Elsevier Science Inc., © 2014.^[125]

biocompatible, nontoxic to biomolecule, durable, and long life via conserving the bioactivity of the biological molecules. At this point, functional surfaces generated by CPs assess functionality on the electrode surfaces with the pendant groups on their backbones. Kekec et al.^[128] designed and synthesized a novel monomer, 9-methyl-9H-carbazole-3-carbohydrazine (MCCH), and electrochemically polymerized it on a graphite electrode to achieve an effective immobilization matrix for biomolecule deposition. The presence of amino groups in the structure of poly(MCCH) allows covalent immobilization of AOx onto the electrode surface. *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride/*N*-hydroxysuccinimide (NHS) chemistry was used for linking carboxylic acid groups of the enzyme molecules to amino groups of the polymer. The biosensor showed a considerably wide linear range, high sensitivity, and very low limit of detection. Moreover, the poly(MCCH)/AOx biosensor was used for the determination of ethanol content in some commercial alcoholic beverages and the results were comparable with the labels provided by the manufacturers (Fig. 16a and b).

Proper enzyme immobilization platform was achieved with the polymerization of CPs of 3,4-ethylenedioxythiophene and (PEDOT) and 4,7-dithien-2-yl-2,1,3-benzothiazole (TBTD).^[129] In this study, entrapment method was

used to immobilize AOx to achieve amperometric alcohol biosensor. Platinum electrode was coated with polymer and AOx was entrapped by polymerizing EDOT with constant potential onto CP coated surface. The novel designed biosensor was tested for kinetic parameters, optimum pH, and optimum charge. Moreover, kinetic parameters were obtained for PEDOT/TBTD/AOx and also for only PEDOT matrix. The best results were obtained for the PEDOT/TBTD/AOx biosensing system (Fig. 17a and b).

The amplification of polypeptides into polymeric structures is of great importance in the field of biotechnology since they are fascinating biomaterials mimicking natural proteins. Polypeptides are excellent candidates for bio-applications due to their remarkable mechanical and biological durability and biocompatibility. Ferrocene derivatives are well-known electron transfer mediators because of their superior characteristics.^[51] Especially, incorporating ferrocene units within the polymeric chain has revealed great importance since such a combination brings high electronic communication features. Kesik et al.^[130] designed a surface containing conducting polymer bearing polypeptide segments and ferrocene moieties (Fig. 18). To construct such a surface, the electroactive polypeptide macromonomer and independently synthesized ferrocene imidazole derivative of dithiophene were

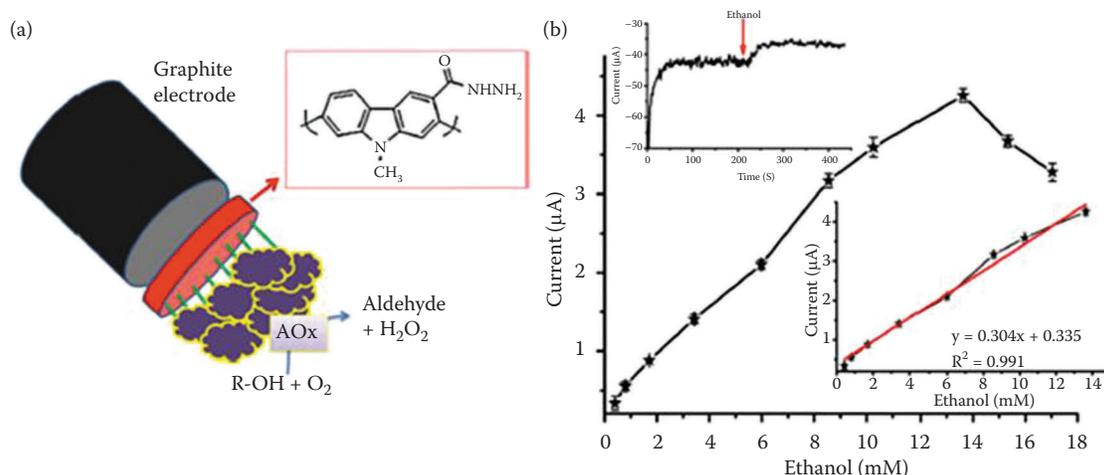


Fig. 16 (a) The schematic construction of the proposed biosensor and (b) Calibration curve for ethanol (in 50 mM phosphate buffer, pH 7.0, 25°C, -0.7 V). Error bars show standard deviation of four measurements (a typical amperometric signal to 8.55 mM ethanol in phosphate buffer, 50 mM, pH 7.0 given as inset)

Source: Adapted with permission from Elsevier Science Inc., © 2014.^[128]

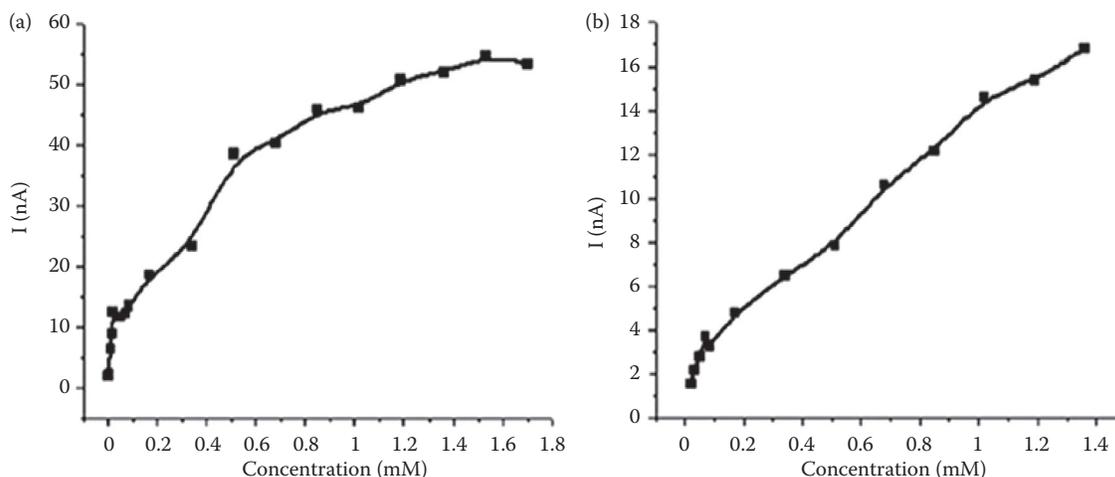


Fig. 17 Calibration curves for (a) TBTD/PEDOT and (b) PEDOT matrices

Source: Adapted with permission from Taylor & Francis Group, LLC, © 2013.^[129]

electrochemically polymerized on the graphite electrode surface. It was reported that novel biosensor was evaluated as an ethanol sensing system providing fast response time (9s), wide linear range (0.17–4.25 mM), low detection limit (0.28 mM), and high sensitivity value ($12.52 \mu\text{A mM}^{-1} \text{cm}^{-2}$). K_M^{app} and I_{max} values of this sensor were calculated as 2.67 mM and 2.98 μA , respectively. Moreover, obtained biosensor was tested by determining the alcohol content in several alcoholic beverages, and results revealed that it is a reliable sensing strategy for alcohol determination in real samples.

Nanomaterials have been the key to recent successful developments in the area of biosensors. CNTs are excellent candidates for the development of biosensors due to their properties such as non-toxicity, good biocompatibility, and fast electron transfer.^[65] Especially,

carboxylic acid functionalized CNTs have been mostly preferred since acid functionalized nanotubes produce hydrophilic groups that can provide covalent linkage between the biomolecule and electrode surface. A novel monomer was synthesized and applied for the formation of a composite layer with carboxyl functionalized nanotubes by Soylemez et al.^[131] The combination of a novel conducting polymer of 2-(4-nitrophenyl)-4,7-di(thiophen-2-yl)-1H-benzo[d]imidazole (BIPN) with functionalized MWCNTs (f-MWCNT) is not explored by other researchers for the detection of ethanol. This combination offers better enzyme immobilization platform and maximum enzyme loading through the interaction between the enzyme molecule and functional groups of the polymers. After the formation of f-MWCNT/poly(BIPN), a possible improvement in the electrical

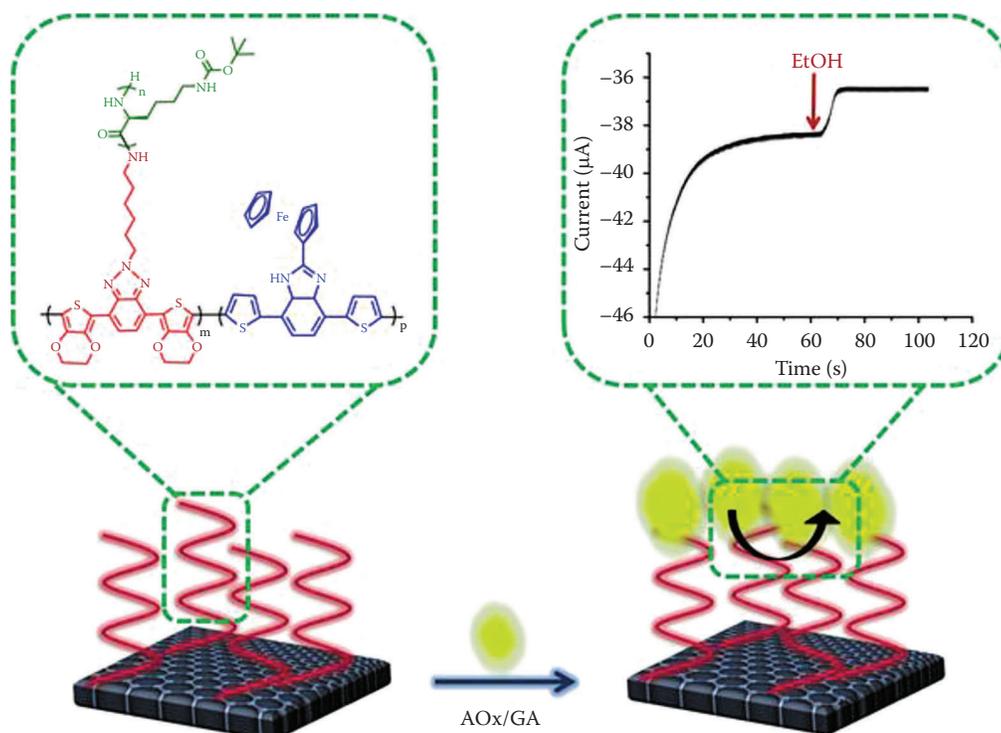


Fig. 18 Preparation of the amperometric ethanol biosensor for proposed biosensor
 Source: Adapted with permission from Royal Society of Chemistry, © 2014.^[130]

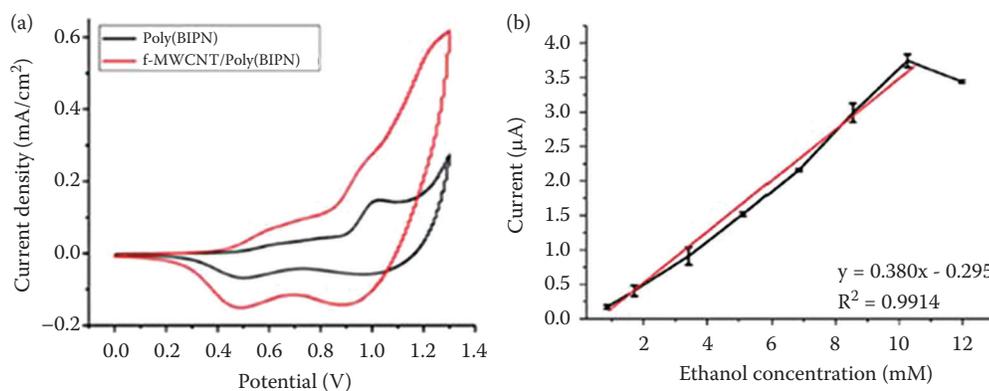


Fig. 19 (a) Cyclic voltammograms of poly(BIPN) and optimum f-MWCNT/poly(BIPN) electrode in 0.1 M LiClO₄/NaClO₄, DCM-ACN (5 : 95, V/V) solution at 100 mV s⁻¹ and (b) Calibration curve for ethanol (in 50 mM phosphate buffer, pH 7.0, 25°C, -0.7 V). Error bars show the standard deviation (SD) of three measurements
 Source: Adapted with permission from Royal Society of Chemistry, © 2013.^[131]

properties of polymers was observed (Fig. 19a). The proposed ethanol sensor shows good kinetic parameters and wide linear range (Fig. 19b). To investigate the performance and reliability of the constructed biosensor, it was tested for various beverages.

Other Enzymes

In literature, different types of polymeric films were carried out for amperometric biosensor design and the proposed biosensors were tested for various analyses. The

focus of this part will be the use of polymeric films in amperometric biosensors using horseradish peroxidase (HRP), laccase (Lac), and urease. We reviewed several recent studies and emphasized the most preferred ones in current technology and innovative biosensor designs. The objective of the researchers is to fabricate a CP-based amperometric biosensor to be used to quantify analytes in any test solution.

Hua et al.^[132] prepared a PANI/carboxy-functionalized multiwalled CNT (PANI/MWCNT-COOH) nanocomposite to obtain a hydrogen peroxide (H₂O₂) sensor. HRP was

immobilized onto the PANI/MWCNTCOOH nanocomposite-modified Au electrode (PANI/MWCNT-COOH/Au) (Fig. 20a). The authors reported that the obtained surface brings a broad linear range (86 μM –10 mM) and good sensitivity (194.9 $\mu\text{A mM}^{-1}\text{cm}^{-2}$). The surface morphologies of the biomolecule-deposited surface with and without PANI were investigated by FE-SEM. HRP/PANI/MWCNT-COOH/Au surface showed porous “island”-like structures compared to HRP/MWCNT-COOH/Au surface. They suggested that this porous structure offered cavities for biomolecule immobilization and a highly accessible surface area for substrate (Fig. 20b).

In another study, Jabłońska et al. designed a surface to obtain stable biosensor platform.^[133] In this study, different kind of platforms (PANI films, PANI nanotubes, thermally treated PANI films, and thermally treated PANI nanotubes) were prepared and the performances of the biosensors were evaluated. The obtained structures and electrochemical properties of the PANI nanotubes and PANI bulk films were elucidated using resonance Raman spectroscopy, cyclic voltammetry, and scanning electron microscopy. Further work in this type of biosensor involved biosensors employing films prepared from copolymer of aniline and a disulfide derivative of aniline (dithiodianiline) as a mediator for the analysis of heavy metal ions. It was concluded that such a modification brings lower detection limit for the detection of Cd^{+2} , Pb^{+2} , and Hg^{+2} metal ions, and good stability, repeatability, and reproducibility compared to the Pt/PANI/HRP biosensor.

Many polyphenols are essential antioxidants and excess amounts of them may inhibit the growth of cells. On the other hand, their low quantities may lead to tumors.^[134] Therefore, their quantification is an essential task for human health and sensing of polyphenols attains significance importance. As we stated in other parts, PPy is one of the most studied polymer in biosensor construction. A new biosensor was developed by the electrocodeposition of MWCNTs, PPy, and Lac on platinum (Pt) to obtain a neurotransmitter biosensor.^[135] The Pt electrode was used as the working electrode and modified with a PPy/MWCNT nanocomposite and then, successfully utilized for the immobilization of the Lac enzyme without using any mediator. This sensor was applied for the detection of dopamine in urine samples using differential pulse voltammetry and the obtained results were found to be comparable with those determined with HPLC.

In addition to the abovementioned procedure for Lac biosensor, PPy can be modified with different types of nanoparticles to improve biosensor performance. For example, AgNPs,^[136] nickel nanoparticles,^[137] iron oxide nanoparticles ($\text{Fe}_3\text{O}_4\text{NPs}$),^[138] and manganese dioxide nanoparticles^[139] were decorated with carboxylated multiwalled CNTs /PANI composite onto gold (Au) electrode separately. The main objective of these studies is to improve the analytical performance of amperometric biosensor using nanomaterials for detection of total phenolic content. In a similar study, Sethuraman et al.^[140] prepared a new nanocomposite of copper nanoparticles (CuNPs/MWCNTs/PANI) using CS for the fabrication of a phenol

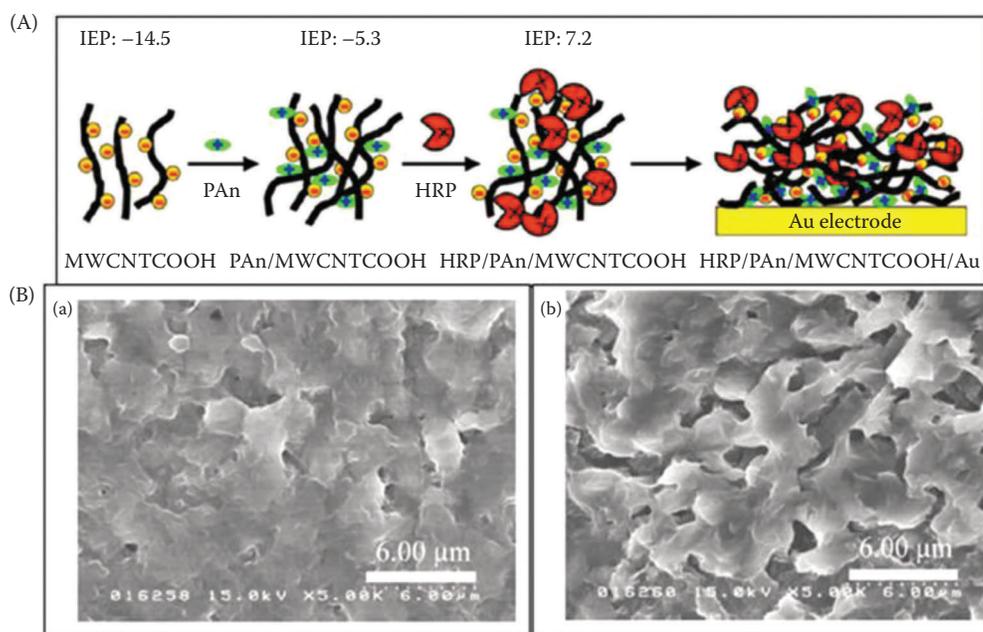


Fig. 20 (A) Presentation of the HRP/PANI/MWCNT-COOH/Au electrode and (B) FE-SEM images of (a) HRP/MWCNT-COOH/Au and (b) HRP/PANI/MWCNT-COOH/Au surfaces

Source: Adapted with permission from Elsevier Science Inc., © 2011.^[132]

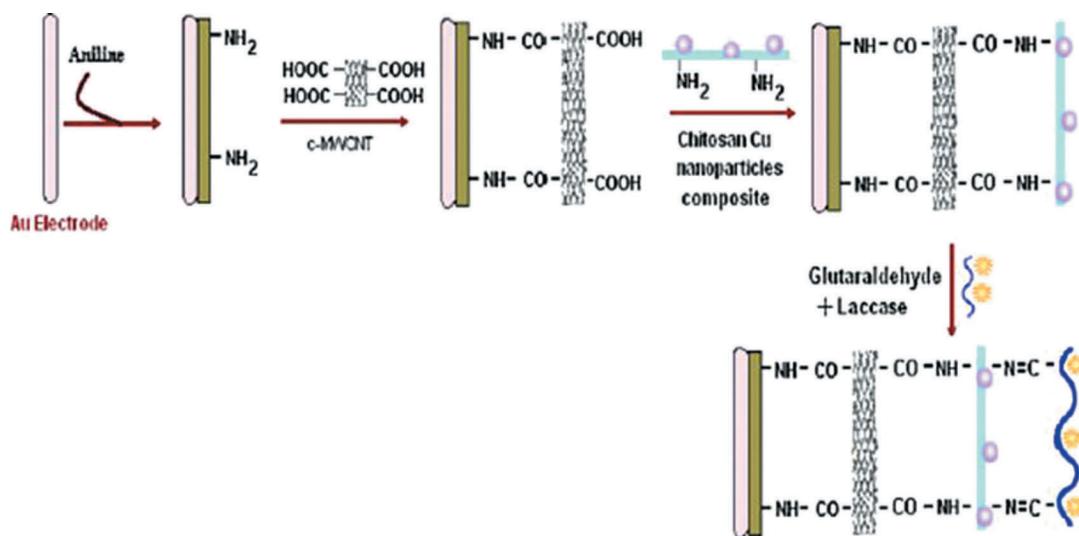


Fig. 21 Biosensor preparation of CuNPs/CHIT/cMWCNT/PANI on gold electrode and chemical reaction of immobilization of enzyme on gold electrode

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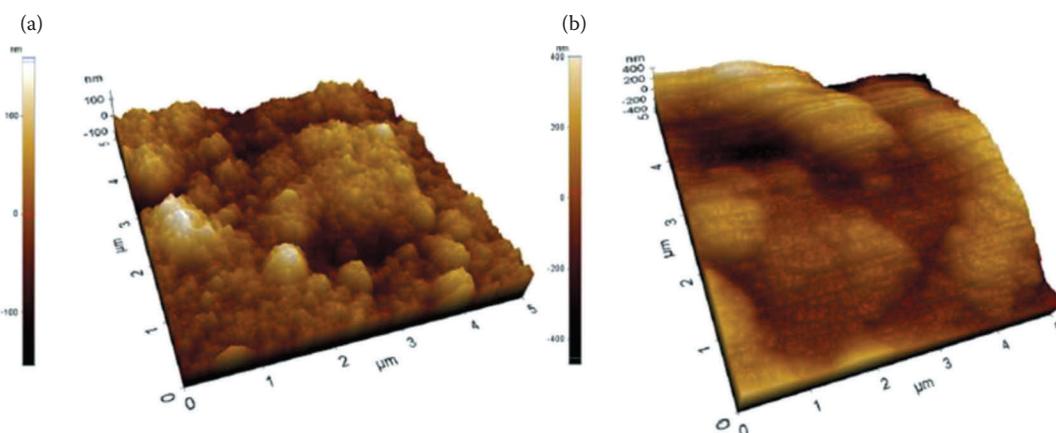


Fig. 22 AFM images for (a) PEP and (b) PEP-urease-modified surfaces

Source: Adapted with permission from Elsevier Science Inc., © 2011.^[143]

biosensor (Fig. 21). It was reported that using this nano-composite layer, which is open to covalent bond formation, prevents the enzyme leakage on the transducer surface.

Urea is an important component in human body since its excess amount in blood serum is known as uricemia causing hypertension, metabolic syndrome, and cardiovascular disease.^[141] Hence, its analysis is significant. One crucial step is immobilization of urease on the proper matrix for developing an electrochemical urea biosensor. In this way, various electrodes were prepared using polymers, sol-gels, CPs and nanomaterials to obtain enzyme-based biosensors. Meibodi and Haghjoo^[142] prepared a PANI-MWCNT composite as a matrix for entrapment of enzyme. Pencil graphite disk electrode as the working electrode was used for further modifications. It was reported that the covalent side wall functionalization of MWCNT provides a feasible direction to incorporate CNTs in polymer matrix. K_M^{app} , I_{max} ,

and sensitivity $\left(\frac{I_{max}}{K_M^{app}}\right)$ values were determined as 2.02 mM, $2.5 \times 10^{-5} \text{ Acm}^{-2}$, and $12 \times 10^{-5} \text{ AmM}^{-1}\text{cm}^{-2}$, respectively. In another study, an amperometric urea biosensor based on poly(*N*-glycidylpyrrole-*co*-pyrrole) (PEP) conducting film was reported.^[143] For urea detection in blood serum samples, urease was immobilized onto the polymer modified electrode surface via the covalent binding technique. The results of constructed biosensor are comparable with the hospital data. Moreover, the surface morphologies of the PEP and PEP-urease-modified electrodes were apparently different from each other, confirming that biomolecules were well-immobilized onto the PEP surface (Fig. 22). Considering the results, the proposed biosensor shows an efficient platform to produce a reliable biosensor and can be properly applied for detection of urea in serum samples.

CONCLUSION AND OUTLOOK

Considering the recent progress summarized in this review, CP-based amperometric biosensors have attractive merits in material science and chemistry and have a variety of applications for detection of various analytes in any test solution. For development of an excellent sensory system, an appropriate support material should be provided. This entry is devoted to the use of conjugated polymers for the fabrication of electrochemical biosensors that allow the achievement of enhanced biosensor performance with respect to other designs.

Accordingly, CP films accomplish desired properties, producing sensitive and reproducible microenvironment for biological reactions to mimic the naturally occurring environments of biological molecules. Moreover, incorporation of CPs with other structures such as nanostructures, mediators, or macrocyclic scaffolds exhibits superior properties for the fabrication of a biosensor. This entry provides an overall survey of up-to-date literature results concerning the very promising use of CPs for biosensor construction in terms of use of different enzymes. Hence, in the near future, an efficient CP design with a promising sensor architecture may become a complementary method to conventional chromatographic sensing techniques for detection and quantification of target analytes in our daily life.

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