CHAPTER 1: Introduction

1. General Introduction

1.1 Introduction to biosensors

A biosensor is comprised of a biologically sensitive material immobilized in intimate contact with a suitable transducing system converts the biochemical signal into a quantifiable and process able electrical signal. The biologically sensitive materials are normally an enzyme, multi-enzyme systems, antibody, membrane component, organelle, bacterial or other cell or whole slices of mammalian or plant tissues that can recognize and bind to the target analyte.[1]

1.2 Component of a biosensor

1.2.1 The transducer element

Biosensors are classified according to their biological element or their transduction element. The method of transduction depends on the type of physicochemical change resulting from the sensing event. The transducer is responsible for developing the biochemical modification of the substrate by the bioreceptor by transforming it in to an electrical signal.[2]

There are two types of transduction methods [2]

1. Chemical transduction including amperometry, potentiometry, conductimetry and optical detection.
2. Physical transduction including micro gravimetric detection and colorimetry.

1.2.2 The sensing element: Enzymes

The selection of the biological recognition element is the crucial decision while developing a novel biosensor design.[3] Enzymes were the simplest and historically first recognition elements to be incorporated into biosensors.[4]

Enzymes are efficient catalysts for biochemical reactions. They speed up reactions by providing an alternative reaction pathway of lower activation energy. Like all catalysts, enzymes take part in the reaction that is how they provide an alternative reaction pathway. They increase the rate of reactions by a factor of between 10^6 to 10^{12} times by allowing the chemical reactions that make life possible to take place at normal temperature. Enzymatic reactions are mainly affected by temperature, pH, concentration of enzyme and as well as that of the substrate.[5]

Enzymes are proteins; their role is determined by their complex structure. The reaction takes place in a small part of the enzyme called the active site, while the rest of the protein acts as "scaffolding". This makes the enzyme specific for one reaction only; as other molecules won't fit into the active site as their shape is wrong. Many enzymes need cofactors (coenzymes) to work properly. Cofactors are metal ions (Fe^{2+}, Mg^{2+}, Cu^{2+}) or organic molecules (haem, biotin, FAD, NAD or coenzyme A).The complete active enzyme with its cofactor is called a holoenzyme, while the protein part without its cofactor is called the apoenzyme.

1.2.2.1 The Michaelis-Menten Mechanism of Enzyme catalysis

Experimental studies of enzyme kinetics are performed by monitoring the initial rate of product formation in a solution where enzyme is present at very low concentration. The principal features of many enzyme-catalysed reactions are as follows:

1. For a given initial concention of substrate , $[S]_{0,}$ the initial rate of product formation is proportional to the total concentration of the enzyme $[E]_0$
2. For a given $[E]_0$ and low values of $[S]_{0,}$ the rate of product formation is proportional to $[S]_0$.
3. For a given $[E]_0$ and high values of $[S]_0$, the rate of product formation becomes independent of $[S]_0$, reaching a maximum value known as maximum velocity, V_{max}

Michalies-Menten mechanism accounts for these characteristics. According to this mechanism, an enzyme-substrate complex is formed in the initial step and either the substrate is released unchanged to form product.

$$E + S \underset{k_{a'}}{\overset{k_a}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{k_b} P$$

Scheme 1.1 Michalies-Menten mechanism

The above mechanism leads to the Michalies-Menten equation for the product formation rate as follows

$$V = \frac{k_b[E]_0}{1 + K_m/[S]_0} \quad (1.1)$$

Where $K_m = (k_a' + k_b)/k_a$ is the Michalies constant, characteristic of a given enzyme working on a given substrate.[6]

Further simplification of equation 1.1 can be made. When all the enzyme has reacted with substrate at high concentration, $[S]_0 \gg K_m$ the reaction will go at maximum rate and independent of $[S]_0$,

$$v = v_{max} = k_b[E]_0 \quad (1.2)$$

Substitution of 1.2 in to equation 1.1 gives

$$v = \frac{v_{max}}{1 + \frac{K_m}{[S]_0}} \quad (1.3)$$

We can rearrange this expression in to a form that is responsive to data analysis by linear regression:

$$\frac{1}{v} = \frac{1}{v_{max}} + \left(\frac{K_m}{v_{max}}\right)\frac{1}{[S]_0} \quad (1.4)$$

A Lineweaver- Burk plot (double reciprocal plot) is a plot of $\frac{1}{v}$ against $\frac{1}{[S]_0}$ and according to equation 1.4, it should yield a straight line with slope of $\frac{K_m}{v_{max}}$, a y-intercept at $\frac{1}{v_{max}}$ and x intercept at $-\frac{1}{K_m}$. Thus this plot provides a useful graphical method for analysis of a Michalies-Menten equation in a enzyme –catalyzed reaction.[6]

1.2.2.2 The advantages of using enzymes in biosensor constructions

1. Enzymes exhibit a very high catalytic activity with a turnover on a per mole basis which makes them exceptional bio electro catalysts for effective signal amplification in biosensors.[3]
2. They have a high selectivity for their substrates.[3]
3. When enzymes were immobilized on transducer surfaces an improvement in enzyme stability was found.[3]

4. The driving force, the redox potential that is needed to achieve enzymatic biocatalysis, is often very close to that of the substrate of the enzyme. Therefore, biosensors can operate at moderate potentials.[3]

1.2.2.3 Disadvantages

1. Enzymes are large molecules. In spite of the high catalytic turnover at the active site of the enzyme, the overall catalytic (volume) density is low. For example, at most about a few picomoles of enzyme molecules per square centimeter are contained in a monolayer of enzymes. Barton and coworkers calculated that the theoretical current density in such a monolayer is about 80 μ A cm^{-2} under the assumption that the "footprint" of the enzyme is about 100 nm^{2} and the turnover frequency is about 500 s^{-1}.[3]
2. The active site of the enzyme is deeply covered within the surrounding protein shell, as a result direct electron transfer is often not possible and there is a requirement of artificial redox mediators.[3]
3. Enzymes have a limited lifetime and therefore, biosensors exhibit only limited long term stability. So far, operational lifetimes of biosensors have been realized to up to 30 to 60 days.[3]
4. Enzymes are very expensive. The cost of extracting; purifying and isolating of enzymes are relatively high. Sometimes the cost of source of the enzyme is high.[3]

1.3 The different techniques for immobilization of biomolecules

The most important factor in stability and sensitivity of biosensor is the stability of biomolecules. Biomolecules (enzymes) may loss their activity rapidly in aqueous solutions, because enzymes can undergo oxidation reactions and their tertiary structure can be destroyed at the air-water interface, thus making enzymes and reagents both complicated. This problem can be resolved by immobilization of biomolecules. Immobilization is a process of fixing in which biological molecules are fixed onto preferred solid supports so that they can retain activity for long periods. The solid support to which a biomolecule is fixed is known as the matrix. Mechanical and chemical properties of a matrix decide the method of immobilization and the operational stability of a biosensor. The solid supports used for biomolecule immobilization are the inorganic materials such as glass, organic materials such as polymers

films of either conducting or non-conducting, self-assembled monolayers of silanes/thiols, Langmuir Blodgett films, nano materials or screen printed electrodes etc.[7]

Immobilization method should be simple and fast, biocompatible, inert, have high retention capacity, controllable porosity and the film should be stable at different temperature and pH.[8] Immobilization of a biological component on a matrix refers to restricting the fouling of a biomolecule and to keep it in a relatively defined region of space which leads to enhanced stability and reusability of biological component. Commonly used methods of immobilization are physical adsorption, physical entrapment, covalent binding and cross-linking.[9-11]While immobilizing a biomolecule to a matrix, it is important to choose a method of attachment that may prevent the loss of enzyme activity by not changing the chemical nature or reactive groups in the binding site of an enzyme.

1.3.1 Physical Adsorption

This is the oldest and easiest method of immobilization of biomolecules. Here, biomolecules are physically adsorbed on to the surface of a matrix.[12,13]This method requires no reagents and a minimum activation steps. This method causes little or no conformational change of the enzyme or destruction of its active center. But main disadvantage is due to weak binding force between the matrix and biomolecules. As a result the adsorbed enzyme may leak from the matrix during use. This method is suitable for short time analysis. In this immobilization technique, weak binding forces such as hydrogen bonds, multiple salt linkages, Vander-wall's forces and formation of electron transition complexes are involved.[14-16]

Sharma et al. described a method of the physical adsorption for immobilization of enzyme glucose oxidase onto electrochemically deposited poly (2-fluoroaniline) thin films on ITO coated glass plates. They studied the redox behavior of poly (2-fluoroaniline) and poly (2-fluoroaniline)/GOX films by cyclic voltammetry technique. Amperometric detection of glucose was carried out by using the GOX enzyme immobilized polymeric film as a working electrode and Ag/AgCl as a reference electrode at a bias voltage of 0.2 V over a physiological range. The GOx immobilized electrode was found to be stable up to 32 days.[17]

1.3.2 Entrapment

Here the biomolecule is mixed with the monomer solution which is then polymerized to a gel to trap the biomolecule. The most commonly used gels are polyacrylamide, starch gel, silastic gel, nylon and conducting polymer such as polypyrroles. The drawback of this technique is the large diffusion barrier to the transport of substrate and product, results in the increase of the response time of the system as compared to other techniques. In this method the biomoecule itself does not bind to the matrix as in the case of covalent or cross linking technique. Sometimes the conditions used in entrapment during the chemical polymerization reaction are relatively severe and result in the loss of activity. Therefore, of the most suitable conditions should be selected for the immobilization.[18]

Sharma et al. used the entrapment technique for developing galactose biosensor by entrapping galactose oxidase into polyvinyl formal membrane. They have estimated galactose in milk and milk product by using dissolved oxygen analyzer and found the electrode to be linear in the range of 0.5–3.0 g dl^{-1}galactose.[19]

1.3.3 Cross linking

In this method, the biomolecules are chemically bonded to the solid support or to a supporting material such as gel. Cross-linking of biomolecules with a matrix occurs by the use of some compounds having two or more functional groups which are capable of binding two different materials under different conditions. Intermolecular cross-linking of biomolecules has been achieved by using bifunctional or multifunctional reagents e.g. 4-azido-1-fluoro-2-nitrobenzene, glutaraldehyde, hexamethylene di-isocyanate, 1, 5 difluoro 2, 4-dinitro benzene and bis diazobenzidine-2, 2' disulphonic acid.[20-22] Since the biomolecule is covalently linked to the support matrix, very little desorption occurs. The most common reagent used for cross-linking is glutaraldehyde. Attempts are being made to find new cross linkers for binding different types of biomolecules under mild conditions so that cross-linking reactions take the advantages of both strong binding to prevent the leakage and the loss of activity during binding.

Singh et al. had used the cross linking technique via glutaraldehyde for fabrication of a cholesterol biosensor by coimmobilization of enzymes cholesterol oxidase, cholesterol esterase and peroxidase onto electrochemically prepared polyaniline films.[23]

1.3.4 Covalent binding

One of the most intensively used immobilization technique is the formation of covalent bonds between and biomolecule and the support matrix.[24] This method of immobilization is generally applicable in most of the cases using different functional groups of the matrices capable of covalent coupling or being activated to give such groups. Generally, nucleophilic functional groups present in amino acid side chains of proteins and groups such as amino, carboxylic, sulfohydryl imidazole, thiol, hydroxyl, phenolic, threonine, indole etc. are used for covalent coupling. In general the covalent binding involves the conditions for the immobilization which are much more complicated and harsh than in the case of physical adsorption. Therefore, covalent binding may alter the conformational structure and active center resulting in the major loss of activity or changes of the substrate. However, the binding force between an enzyme and the carrier is so strong that no leakage of the enzymes occurs, even in the presence of substrate or solution of high ionic strength.[7]

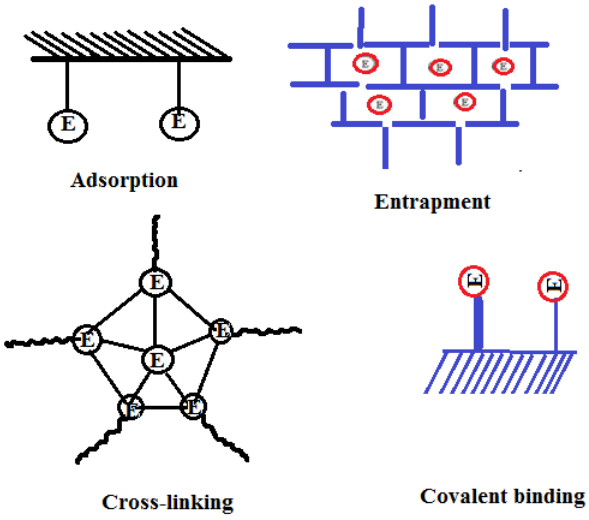


Fig.1.1 Four different immobilization techniques[17]

1.4 Electrochemical biosensors

The electrochemical biosensors are considered as a substitute to the expensive, time-consuming and sophisticated chromatographic techniques currently applied for pesticides detection and quantification. Electrochemical sensors continue to pay an important role in many environmental, clinical, industrial and security applications because of the high

sensitivity of the determinations, the simplicity of the operational procedure, the availability and the reasonable cost of the equipment.

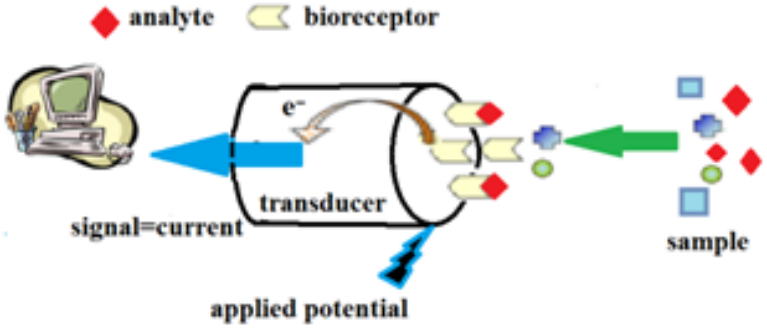


Fig.1.2 Schematic representation of an electrochemical biosensor

Electrochemical biosensors are a subclass of chemical sensors. They combine the sensitivity as indicated by low detection limits of electrochemical transducers (electrode) with the high specificity of biological recognition processes. The biological recognition elements (enzyme, proteins, antibodies, cells etc) selectively react with the analyte to produce an electrical signal feature of electrochemical biosensors is the presence of a suitable enzyme in the bio recognition layer providing electroactive substances for detection by the physico-chemical transducer providing the measurable signal.[25]

1.4.1 Working principle of a electrochemical biosensor

The working principle of electrochemical sensors is that when an electroactive analyte is subjected to fixed or varying potential of some predefined patterns causes oxidation or reduction of analyte on the working electrode surface, which leads to the generation of an electrochemically measurable signal by the variation on electron fluxes. Finally this signal is measured by the electrochemical detector.[26]

1.4.2 Advancement from 1st to 3rd Generation Electrochemical Biosensors

1.4.2.1 1st generation biosensor

In first generation biosensors the normal product of a reaction diffuses to the transducer (electrode) and causes the electrical response.[30] In 1962 Clark and Lyons developed the first generation glucose biosensor. Their sensor used glucose oxidase (GOx) entrapped over an oxygen electrode by a semipermeable membrane to select for β-D-glucose in the presence of

oxygen gas. The oxygen consumption, when it reacted with protons and electrons to produce water was detected by the electrode as a change in potential. The increasing concentration of the product H_2O_2 or the decrease in O_2 concentration as natural co substrate can be electrochemically detected in order to monitor glucose concentration.[27]

The major drawbacks of the first generation biosensor approach are the following:

1. If the concentration of oxygen is monitored, it is challenging to maintain a reasonable reproducibility due to varying O_2 concentrations within the sample.[3]
2. Working electrode potentials used for either the oxidation of H_2O_2 or the reduction of O_2 is not optimal because these potentials are prone to the impact of interferences present in biological samples.[3]

1.4.2.2 2nd generation biosensor

1. In order to get biosensors that can operate at moderate redox potentials, the use of artificial redox mediators was introduced. These biosensors are called the "second generation" biosensors.[3] Cass et al. were the first who showed the application of ferrocene, the artificial redox mediator for an amperometric glucose biosensor.[28] The employed redox enzyme for the analyte of interest is able to donate or accept electrons to or from an electrochemically active redox mediator. It is important that the redox potential of this mediator is in tune with the cofactor(s) of the enzyme. Preferably, the redox mediator is highly specific for the selected ET (electron transfer) pathway between the biological recognition element and the electrode surface. A large variety of compounds such as ferrocene derivatives, organic dyes, ferricyanide, ruthenium complexes and osmium complexes have been used as free diffusing mediators.[3]

1.4.2.3 3rd generation biosensor

3rd generation biosensors are constructed by wiring an enzyme to the electrode by co immobilizing the enzyme and mediator directly onto the electrode surface or into an adjoining matrix such as a conductive polymer film. The immobilized mediators allow efficient electron transfer, resulting in a higher current density. Close proximity of the enzyme and the mediator to the transducer surface minimizes the electron transfer distance thereby resulting in faster response times. As they are immobilized, mediators cannot escape the biosensor film and leach into the surroundings thereby allowing sensor use for in vitro measurements. The modified electrode can be operated at the desired voltage, eliminating

background interference. This design also provides itself to repeated and prolonged measurements because there are no reagents to replace.[27]

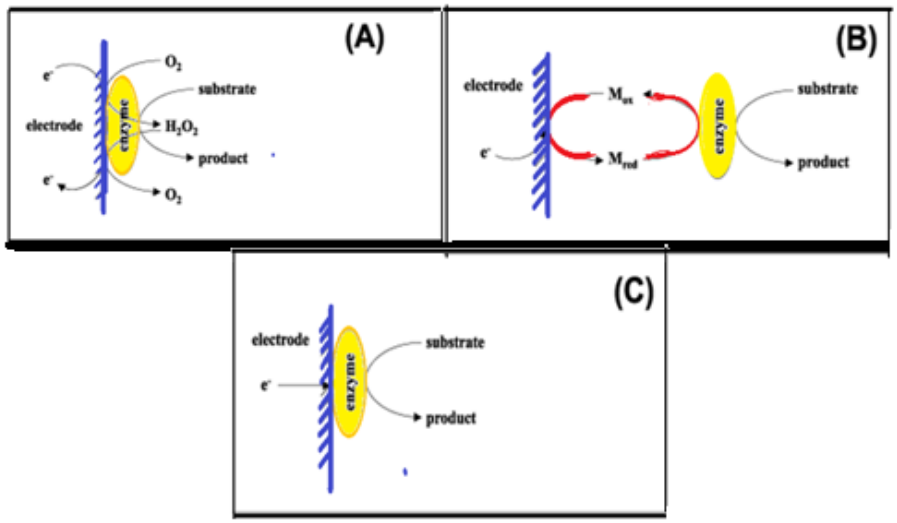


Fig.1.3 Generation wise biosensor design. (A) first generation biosensor, (B) second generation biosensor and (C) third generation biosensor.[29]

1.5 Conducting polymers

Since the chemical and physical properties of polymers may be adapted by the chemist for particular needs, they gained importance in the construction of sensor devices. A new class of polymers known as intrinsically conducting polymer or electro active conjugated polymers (extended p-conjugation along the polymer backbone) has newly materialized. Because of their electrical, electronic, magnetic and optical properties, conducting polymers (CPs) are of great scientific and technological importance.[30] CPs can be synthesized by both chemical and electrochemical oxidation; the second approach is preferable as it results in polymeric films deposited on electrode surface, which can be removed to give free standing films. Besides, in electrochemical synthesis, reactions can be carried out at room temperature. Due to these reasons, electrochemically polymerized CPs has received considerable attention over the last two decades.[32]

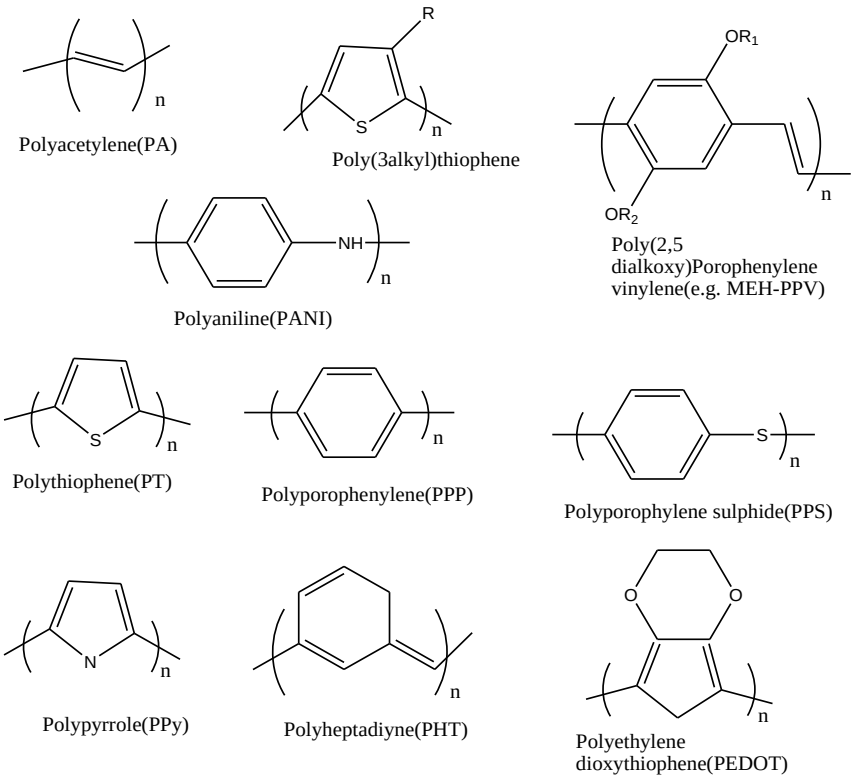
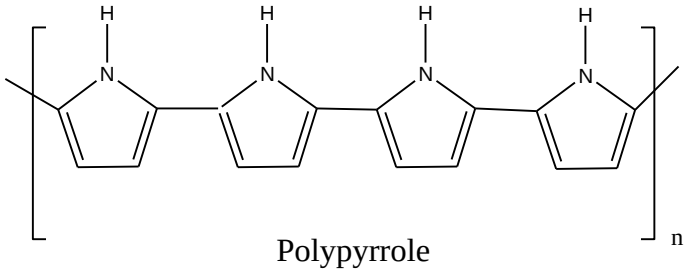


Fig.1.4 Structure of some conducting polymers.

The entrapment of enzymes in CPs films provides a controlled method of localizing biologically active molecules in defined area on the electrodes. CPs has the ability to efficiently transfer electric charge produced by the biochemical reactions to electronic circuit. Besides, CPs can be deposited over defined areas of electrode. This unique property along with possibility to entrap enzymes during electrochemical polymerization has been widely used for fabrication of amperometric biosensors.[32]

1.6 Polyprrole as versatile immobilization matrix in biosensor architecture



Polypyrrole

Fig.1.5 Structure of polypyrrole

CPs belonging to polyenes and polyaromatics such as polyacetylene(PA), polyaniline (PANI), polypyrrole (PPy), polythiophene(PT), poly (p-phenylene)(PPP), poly (phenylene vinylene) classes has been extensivelystudied.[32] Among the three most important conducting polymers polypyrrole, polythiophene and polyaniline, polypyrrole is most widely employed for the development of electrochemical sensors. It is due to the fact that polypyrrole can be obtained from aqueous solution as well as non aqueous solution and can be prepared at lower anodic potentials. Secondly polypyrrole can be electrochemically generated and deposited on the conducting surfaces. This characteristic is successfully utilized for development of different types of electrochemical sensors and biosensors.[31]

Polypyrrole is versatile. It is biocompatible, has capacity to transduce energy arising from interaction of analyte and analyte recognizing-site into electrical signals that are easily monitored, it has the capability to protect electrodes from interfering materials, easy ways for electrochemical deposition on the surface of any type of electrodes etc. As polypyrrole is biologically compatible, it has been widely studied for the immobilization of enzymes, antibodies and nucleic acids.[32] Immobilization of biologically active material is of pivotal importance in the creation of biosensors, since it allows application of the same biologically active material for a number of analysis series. The requirements for successful biomaterial immobilization are: (i) biological recognition properties and catalytic properties of biomaterial should remain after immobilization; (ii) the biomaterial should be well fixed on/within the substrate, otherwise the biosensor will lose its activity (iii) improve or at least minimally decrease selectivity of constructed biosensor (iv) improve electron transfer if amperometric measurements are applied as signal transduction system. To solve the majority and sometimes all these tasks, conducting polymers can be considered as a very effective substrate for biomolecule immobilization. Among other conducting polymers, polyaniline is often used as immobilizing substrate for biomolecules and sometimes as efficient electro catalysts. However, the necessity to detect bioanalytes at neutral pH range leads to electro inactivity of the deposited films, discouraging the use of polyaniline as biosensing materials. As opposed to polyaniline, polypyrrole can be easily deposited from neutral pH aqueous solutions containing pyrrole monomer, making this polymer very attractive and at present it is one of the most extensively studied materials useful for immobilization of different biomolecules and even living cells.[33] Biomolecule immobilization on polypyrrole film by various methods are already discussed in paragraph 1.3

From the viewpoint of electrochemical biosensing, polypyrrole has a number of very attractive characteristics: (i) it can be synthesized electrochemically and modified by enzymes in several different ways. This gives different analytical characteristics for constructed biosensors; (ii) it protects electrodes from fouling and interfering materials such as electroactive anions (iii) Since it is biocompatible, causes minimal and reversible disturbance to the working environment (iv) in some particular cases it can be used as redox mediator to transfer electrons from the redox enzymes towards electrodes.[35]

1.6.1 Synthesis of polypyrrole

PPy is generally synthesized by both chemical and electrochemical methods. In chemically induced polymerization, large quantities of material are required and it involves mixing a strong oxidizing agent (typically $FeCl_3$) with monomer solution.[34,35] Secondly, the PPy is mainly produced in the bulk solution and just some amount of synthesized polypyrrole is covering the surface of introduced materials. It implies that chemically induced polymerization is not very efficient with respect to deposition of PPy over some surfaces.[33]

The pioneering work on PPy electrodeposition was done by Diaz et al in 1979. They synthesized continuous andfreestanding films of conducting PPy using platinum electrodes. These films showed much higher electricalconductivity (100 Scm^{-1}) and excellent air stability.[36]

Since then, huge work has been done on PPy film characterization.

Electrochemical synthesis is preferred for research purposes due to the simplicity of the technique, control over material thickness, geometry and location, and the facility for doping during synthesis, the wide choice of available dopant ions and the generation of good quality films.[37,38] It leads to the development of adherent surface conformal deposits i.e. thin solid films, from the bulk solution phase of monomer units.

The working electrode used for electro synthesis of polypyrrole is usually made of corrosion resistant materials which are stable at high anodic potential, namely Pt, Au, Pd, Rh, Ir, conducting In_2O_3-SnO_2 glasses and carbonaceous materials (graphite, vitreous carbon). Syntheses of polypyrrole on Al, Ta, Fe, Cu, Ti, Ni, Cr, Nb stainless steel were also reported.[34]

The electrodeposition on the positively polarized working electrode proceeds through a condensation reaction between the monomer units of the five-membered heterocycle pyrrole. Concurrently negatively charged counter ions must be present in solution to maintain charge

balance within the polymer since positive charges are developed along the PPy backbone. This latter process is referred to as doping and the choice of counter ion, including biomolecules, affects formed polymer properties.[39]

The growth of the PPy depends on its electrical characteristics, if it was non-conducting, its growth would be self-limiting, producing very thin films as in the case of polyphenol and its derivatives reported by Eddy et al. 1995.[40] In contrast, PPy growth is virtually unlimited due to inherent conductivity and therefore charge connectivity to the subjacent anode. There are a large number of experimental formulations for the preparation of PPy each of which significantly modify the phenomenological properties of the polymer. Normally, electrochemical polymerization is carried out at potentials above 600-700 mV versus Ag/AgCl reference electrode. The morphology of the prepared film depends on the nature of the supporting electrolyte, the crystallographic structure of the underlying anode, the kinetics of the process related to the electrode material, the potential used for deposition, the nature of the dopant and the concentration of the original monomer solution. pH and temperature also have an effect on the resultant film. For formed films, conductivity arises from electronic transfer along the conjugated p-molecular orbital backbone coupled with the motion of charge carriers in the material. On oxidation, an electron is removed from the p-system of the backbone producing a cation and a local distortion due to a change in geometry every four pyrrole units. This radical cation coupled with the local deformation constitutes a polaron. Upon further oxidation, at higher charging levels, pairs of polarons combine to form bipolarons, those are energetically more favourable. Bipolarons have the ability to migrate along the conjugated polymer chain and provide the main charge transport mechanism within the conducting polymer.[41, 41] Final conductivity reflects the charge transfer between the dopant and the polymer segment, charge carrier mobility within the conjugated segments of a single polymer chain and charge transfer (or 'hopping') between individual chains.[42] Essentially, it was postulated that it is the least efficient one of any of these mechanisms under any given condition of say temperature and pH that determines the final conductivity of the material at a macroscopic level.[43]

1.7 Cholinesterase enzymes

Cholinesterases enzymes (ChE) are formed in vertebrates and insects; they hydrolyze the neurotransmitter acetylcholine and butyrylcholine in the nervous system.[44] In the body, ChE is responsible for the transmission of nerve impulses to the cholinergic synapses and is

connected with human memory and Alzheimer's disease. Two types of cholinesterases are known which are used for biosensor fabrication: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). The principal biological role of AChE is the termination of the nervous impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. BuChE has similar molecular structure to that of AChE but is characterized by different substrate specificity. AChE preferentially hydrolyses acetyl esters such as acetylcholine, whereas BuChE hydrolyzes butyrylcholine.[45] AChE enzyme has a very high catalytic activity. Each molecule of AChE degrades approximately 25,000 molecules of ACh (acetyl choline) per second into choline and acetic acid.[46,47] Commercially available ChE enzymes are extracted from the Drosophila melanogaster and the Electric Eel and are most widely used for biosensor fabrication.[48]

1.7.1 Acetylcholinesterase: working principle

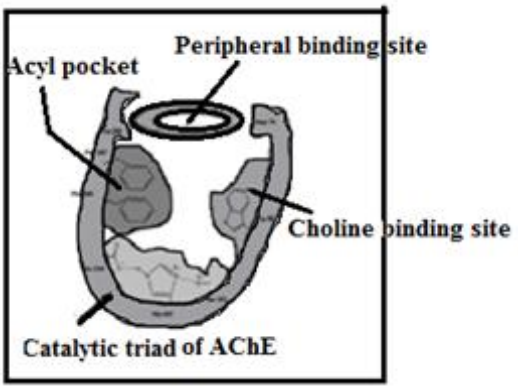
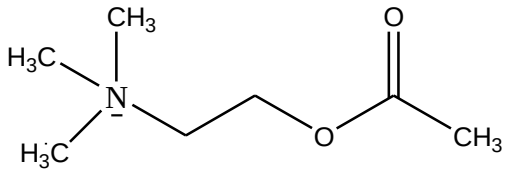


Fig.1.6 Schematic representation of AChE binding sites.[49]

AChE enzyme molecule has an ellipsoidal shape. The active site of AChE comprises of two subsites anionic subsite and esteratic subsite corresponding to the choline binding pocket and the catalytic machinery.[50] The anionic subsite, uncharged and lipophilic, binds the positive quartenary amine of choline moiety of ACh, as well as both quartenary ligands (edrophonium, N-methylacridinium) acting as competitive inhibitors[51,52]and quartenary oximes which effectively reactivate organophosphate inhibited AChE.[53] The cationic substrates are not bound by a negatively charged amino acid in the anionic site, but by interaction of 14 aromatic residues that line the gorge leading to the active site.[54-56] All 14 amino acids in the aromatic gorge are highly conserved across different species.[57] Among the aromatic amino acids, tryptophan 84 is critical and its substitution with alanine causes a 3000-fold decrease in the enzyme activity.[58]The esteratic subsite where ACh is hydrolyzed to acetate and choline contains similar to the catalytical subsites of other serine hydrolases, the

catalytic triad of three amino acids: serine 200, histidine 440 and glutamate 327. In addition to two subsites of active centre, AChE contains one or more peripheral anionic sites distinct from the choline-binding pocket of the active site (Fig.1.6). It serves for binding ACh and other quartenary ligands acting as uncompetitive inhibitors that bind at a site clearly distinct from that occupied by the mono quaternary competitive inhibitors.[59] This site is involved in the substrate inhibition characteristics of AChE.[60]

1.7.2 Acetyl choline



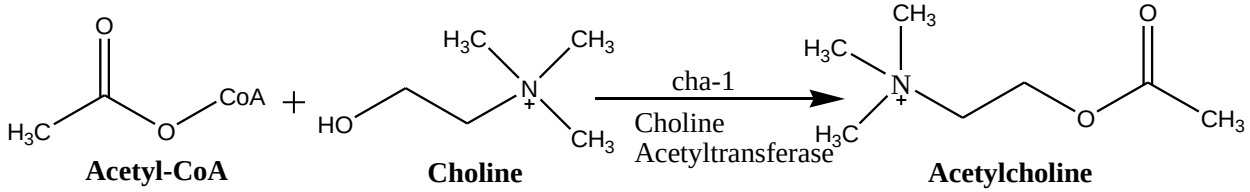
Acetylcholine

Fig.1.7 Structure of acetylcholine[61]

Acetylcholine (ACh), the first neurotransmitter discovered, was initially described as "vagus stuff" by Otto Loewi, because of its ability to mimic the electrical stimulation of the vagus nerve. It is currently known to be neurotransmitter at all autonomic ganglia, at many autonomically innervated organs, at the neuromuscular junction, and at many synapses in the Central Nervous System (CNS).[64]

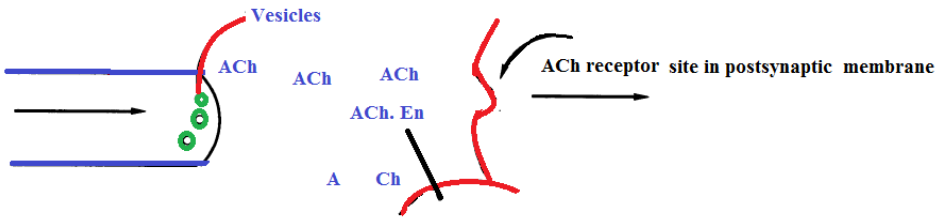
1.7.2.1 Synthesis of ACh

ACh is synthesized from choline and acetyl coenzyme A through the action of the enzyme choline acetyltransferase and becomes packaged into membrane bound vesicles (Scheme1.2). After the arrival of a nerve signal at the termination of an axon, the vesicles fuse with the cell membrane, causing the release of acetylcholine into the synaptic cleft. For the nerve signal to continue, acetylcholine must diffuse to another nearby neuron or muscle cell, where it will bind and activate a receptor protein.[64]



Acetyl-CoA **Choline** **Acetylcholine**

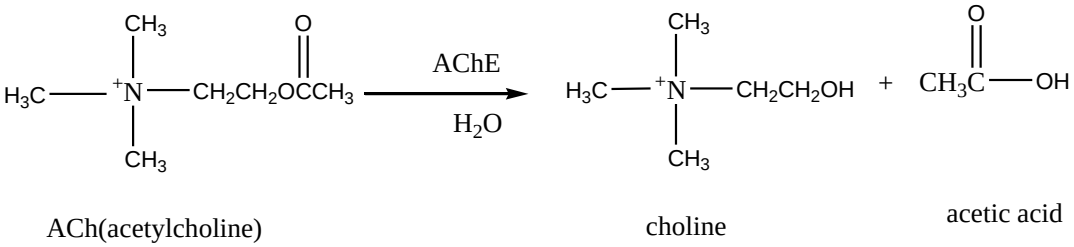
Scheme1.2 Synthesis of ACh[61]



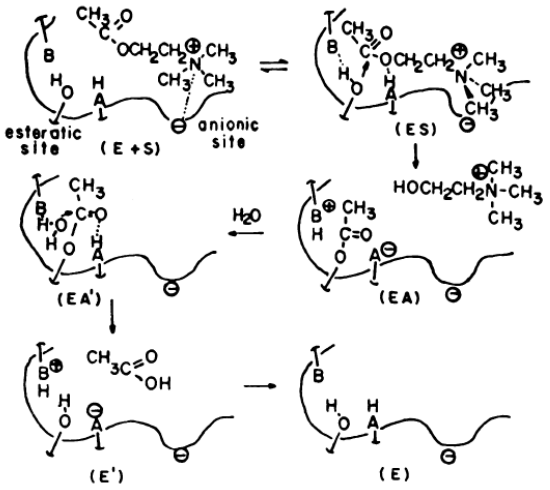
Scheme1.3 The role of AChE in cholinergic transmission[62]

The action of AChE in cholinergic transmission at a synaptic junction is represented in the basic scheme1.2.When a nerve impulse moves down a parasympathetic neuron and reaches a nerve ending, the ACh stored in vesicles in the ending is released into the synaptic or neuromuscular junction. The released ACh interacts with the ACh receptor site on the post synaptic membrane, causing stimulation of the nerve fiber or muscle within 2 to 3 millisecond. AChE serves as a regulating agent of nervous transmission by reducing the concentration of ACh in the junction through AChE catalyzed hydrolysis of ACh into choline (Ch) and acetic acid (A) (scheme1.3). These products do not stimulate the post synaptic membrane. In the above scheme1.3, En indicates the enzyme AChE, ACh.En is the enzyme-substrate complex formed prior to hydrolysis of ACh into choline and acetic acid. When enzyme AChE is inactivated by an organophosphorus or carbamate ester, the enzyme is no longer able to hydrolyze ACh; as a result concentration of ACh in the junction remains high and continuous stimulation of the muscle or nerve fiber occurs, resulting in exhaustion and tetany.[62]

1.8 Mechanism of hydrolysis of acetylcholine (ACh) by Acetylcholinesterase



Scheme1.4 Catalytic hydrolysis of ACh in presence of AChE[62]



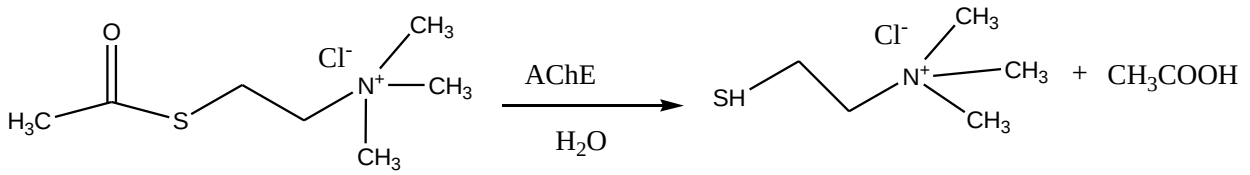
Scheme1.5 Mechanism of hydrolysis of ACh by acetylcholinesterase (Reproduced from Environmental Health Perspective) [62]

ACh is attracted to the active site of the enzyme through an electrostatic interaction between the positive charge on the acetylcholine (ACh) nitrogen atom and negative charge in the anionic site of the enzyme (structure E + S) resulting in the enzyme-substrate complex (ES). Acetylation of a serine hydroxyl (OH) in the esteratic site is catalyzed by the basic imidazole moiety B (histidine) and acidic moiety AH (tyrosine hydroxyl), leading to the acetylated enzyme EA. Deacetylation then takes place very fast, resulting in the free enzyme (E) within milliseconds. As presented, ACh hydrolysis by AChE has the elements of an acid-base catalyzed reaction, including both the acetylation and deacetylation reaction. The negative charge at the anionic site is attributed to the carboxylate anion of aspartic or glutamic acid. The reaction step given in scheme 1.5 provides a basic presentation of the AChE active site and a reasonable mechanism for the hydrolysis of ACh. In reality, the

enzyme is a highly complex protein having in addition to the esteratic and anionic sites, a number of peripheral sites and hydrophobic areas.[62]

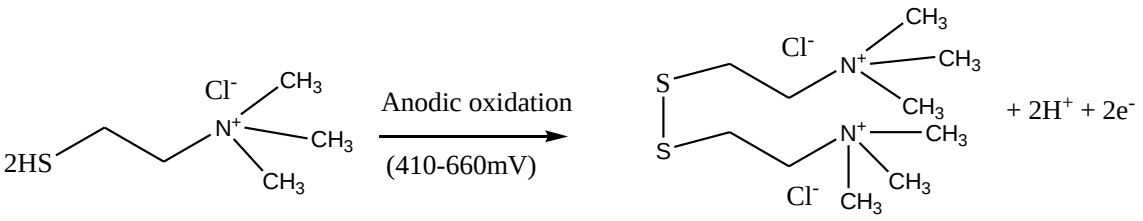
1.9 Acetylthiocholine as an alternate substrate for *in vitro* monitoring of AChE activity

Acetylcholine cannot be used as a substrate in amperometric electrochemical biosensing because choline, (the product formed in its hydrolysis) is not an electro active species, indicates that it cannot be detected by electrochemical techniques involving current measurements, hence used only in potentiometric applications. However acetylthiocholine (ATCh), an artificial analogue of acetylcholine, can be used as a substrate.[63] In particular ATCh is hydrolysed to thiocholine (TCh) which is an electroactive species, by AChE in the enzymatic reaction nearly identical to that of acetylcholine. Characterisation of the electrochemical behaviour of TCh is essential for development of sensors for pesticides. All existing literature on pesticide detection by AChE amperometric biosensors propose that TCh is anodically oxidised, resulting in the respective disulfide form [Scheme1.7].[64] Thiocholine (TCh) oxidation is broadly used to evaluate the activity of acetylcholinesterase (AChE) and, going further, to develop biosensors for pesticides detection based on AChE inhibition .The oxidation of thiocholine occurs at a relative high potential (~700mV) on solids electrodes that cause interferences from other electroactive species and also a high background current . The reduction of the working potential can be achieved by using chemically modified electrodes that provide higher selectivity and sensitivity. Mediators of the electron transfer such as tetracyanoquinodimethane, cobalt (II) phthalocyanine (CoPC), Prussian blue, CdS (Cadmium sulphide) quantum dots or cobalt hexacyanoferrate are used to lower the oxidation potential of thiocholine.[65]



Acetylthiocholine choride Thiocholine chloride Acetic acid

Scheme1.6 Hydrolysis reaction of Acetylthiocholine in presence of AChE



Dithio-bis-choline

Scheme1.7 Thiocholine anodic oxidation

1.10 Pesticides

Pesticides are substances which are extensively used for preventing, destroying, repelling or mitigating pests, intended as insects, rodents, weeds, and a host of other unwanted organisms.[66]

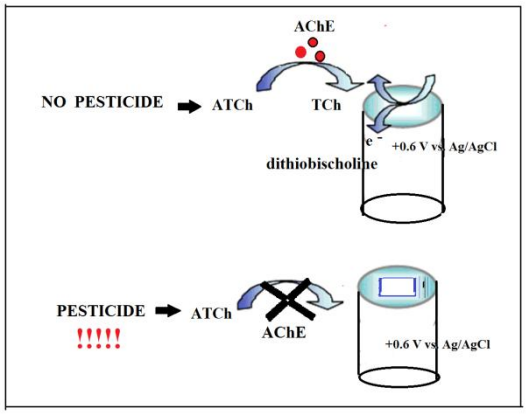


Fig.1.8 Pesticide inhibition

1.10.1 Pesticides Classification

There are several different classes of pesticides with different uses, mechanisms of action in target species, and toxic effects in non target organisms. Pesticides are classified according to their target species. There are four major classes and they are insecticides (insects), herbicides (weeds), fungicides (fungi, molds) and rodenticides (rodents). In addition to a number of "minor" classes such as, for example, acaricides (mites) or molluscides (snails, other mollusks). Within each class, several subclasses exist depending upon the different chemical and toxicological characteristics.[67]

1.11 Organophosphates

Organophosphorus compounds or organophosphates (OPs) form a large group of chemicals insecticides which are used over the past 60 years to protect crops, livestock, and human health and as warfare agents.[68] The general formula of organophosphate is as follows

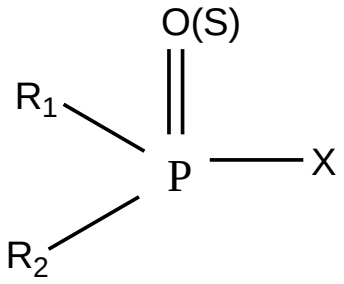


Fig.1.9 The general formula of OPs. 'X' represents the leaving group. $R_1$and R_2, the side, normally alkoxy group.[69]

OPs are esters of phosphoric acid and its derivates. The general chemical structure of an organophosphate includes a central phosphorus atom (P) and the characteristic phosphoric (P=O) or thiophosphoric (P=S) bond. The leaving group represents by symbol X which is replaced (by nucleophilic substitution) by the oxygen of serine in the AChE active site. The rate of AChE inhibition depends on the leaving group, higher tendency of leaving results in higher affinity of the inhibitor to the enzyme.[71]

OPs and their metabolites are the most extensively used pesticides worldwide across different populations[70-72] The unpleasant short-term effects of exposure to these chemicals have been studied mostly in the nervous system, which is their primary target[73], but there is a growing concern about their possible toxic effects in non-target tissues and (long-term) chronic effects that have not been studied in detail. The majority of people are continually exposed to low OP concentrations, and long-term epidemiologic studies reveal linkage to higher risk of cancer development.[74,75] The World Health Organization estimates approximately that every year 3 million people experience acute poisoning by OPs. Humans are exposed to OPs via ingested food and drink and by breathing polluted air.[76] The exposure of workers in closed areas and in agriculture or people living near farms is also very important.[77]

1.12 Carbamate

Carbamates are *N* -substituted esters of carbamic acid and have different degrees of acute oral toxicity, ranging from moderate to low toxicity (carbaryl) to extremely high toxicity (e.g. aldicarb).The mechanism of toxicity of carbamates is similar to that of OPs, as they also inhibit AChE. However, in case of carbamates, the inhibition is transient and rapidly reversible because there is rapid reactivation of the carbamylated enzyme, and carbamylated AChE does not undergo the aging reaction. Nevertheless, the sign and symptoms of carbamate poisoning are the same observed following intoxication with OPs, and include urination, diarrhea, salivation, muscle fasciculation, and CNS effects. However, differently from OPs, acute intoxication by carbamates is normally resolved. Carbamates are direct AChE inhibitors and do not require metabolic bioactivation. Oximes have been shown to aggravate the toxicity of carbaryl, but may have beneficial effects in case of other carbamates, such as aldicarb.[78]

Their general formula of carbamate is

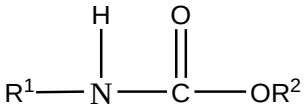


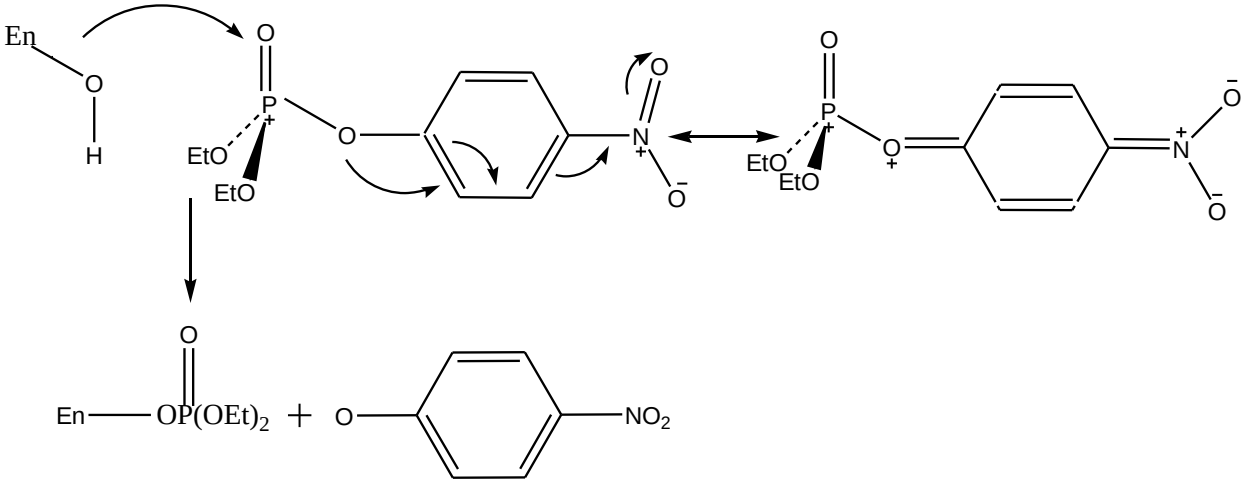
Fig.1.10 The general formula of OCs

Where R^2 is an aromatic or aliphatic moiety. Three main classes of carbamate pesticides are known

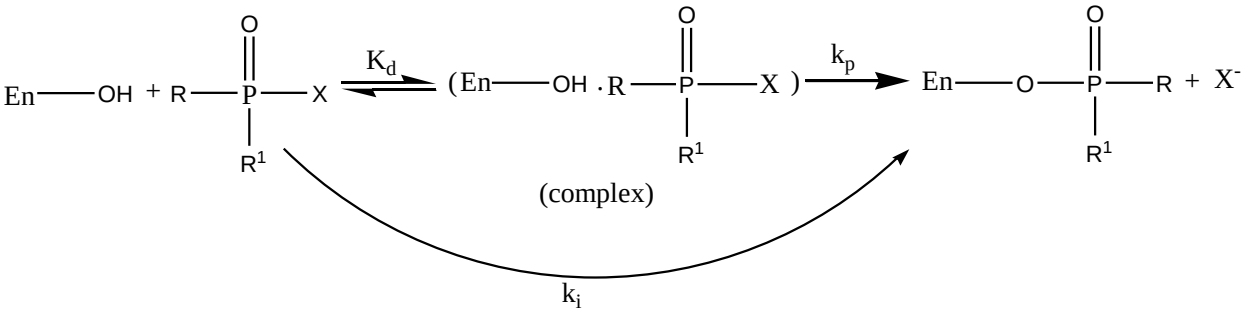
(a) Carbamate insecticides where R^1 is a methyl group
(b) Carbamate herbicides where R^1 is an aromatic moiety
(c) Carbamate fungicides where R^1 is a benzimidazole moiety. [79]

1.13 Mechanism of inhibition of organophosphates and carbamates

OPs and OCs are known to be cholinesterase inhibitors; they block the function of acetylcholinesterase, causing the accumulation of excessive acetylcholine in the synaptic cleft. This causes neurotoxin effects such as neuromuscular paralysis (i.e. continuous muscle contraction) throughout the entire body.[77]



Scheme1.8 The effect of an electron-withdrawing substituent on the reactivity of paraoxon [62]



Scheme1.9 Organophosphate pesticide inhibition mechanism [62]

The inhibition of AChE by an organophosphorus ester takes place through a chemical reaction (Scheme1.7). In this reaction the serine hydroxyl moiety in the enzyme active site is phosphorylated in a manner analogous to the acetylation of AChE (Scheme1.4 EA). The acetylated enzyme rapidly breaks down to give acetic acid and the regenerated enzyme on the other hand, the phosphorylated enzyme is highly stable, and in some cases, depending on the groups attached to the phosphorus atom (R and R'), it is irreversibly inhibited. The serine hydroxyl group is blocked by a phosphoryl moiety, no longer able to participate in the hydrolysis of ACh. The inhibition reaction takes place in a two-step process, as indicated by

(Scheme1.8) In this equation En-OH represents AChE in which the serine hydroxyl moiety (-OH) is highlighted, R and R' are a variety of different groups (alkoxy, alkyl, amino, thioalkyl etc.), X is the leaving group, K_d is the dissociation constant between the enzyme-inhibitor complex and reactants, k_p is the phosphorylation constant, and k_i is the bimolecular rate constant for inhibition. Since K_d provides a measure of the dissociation of the enzyme-inhibitor complex, it is regarded as an estimate for binding and is dependent on the structural and steric properties of the molecule. On the other hand, the phosphorylation constant k_p is regarded as an estimate of the reactivity of the organophosphorus ester. The bimolecular rate constant k_i is dependent on the values of K_d and k_p and is generally regarded as the most useful parameter for the estimation of the inhibitory strength of an organophosphorus (and carbamate) anticholinesterase. In the Scheme1.8, the moiety X is displaced from the phosphorus atom by the serine hydroxyl of the enzyme and is, therefore referred to as the leaving group.[62]

1.14 Cholinesterase reactivator

The treatment of an inhibited enzyme with particular reactivators make it possible to accelerate the spontaneous recovery of the enzyme activity to a degree, depending on the nature of the enzyme-inhibitor complex and the period of time from the initial contact with the inhibitor to the reactivation procedure. Reactivation prolongs the lifetime of a biosensor but complicates the whole cycle of the inhibitor determination. Reactivation is specific for a particular inhibitor and an enzyme. In several cases, pharmaceutical medications, i.e. antidotes are used. 2-Pyridinealdoxime methiodide (2-PAM) or 1, 1%-trimethylenebis-4-(hydroxyiminomethyl) pyridinium bromide (TMB-4) and NaF [80] are a well-known example of reactivators used for phosphorylated cholinesterase.[81]

1.15 pH value of working solution and concentration of supporting electrolyte

The pH value of the working solution is considered as the most important factor determining the performance of the biosensor and its sensitivity toward inhibitors. Like all natural proteins, enzymes have tertiary structure which is sensitive towards pH. At extreme pH, denaturation of enzymes can occur. The pH maximum of the enzyme activity is reviewed as the most appropriate, both for the substrate and inhibitor determination. In fact, the pH-dependence of the observed inhibiting effect often corresponds to that of the response of a

biosensor. Thus, enzyme sensors employing various esterases are most sensitive to organophosphates at the pH 8–9 when the response of a biosensor is maximal.[81]

The activity of the immobilized acetylcholinesterase as a function of pH had been studied between pH 2 and 9 by Stoytcheva.[82] In her study, reported a decrease in the activity of approximately 70% at pH 2 compared to of that at pH 7.

Urease is proposed for the determination of heavy metal ions at pH 8–10, whereas glucose oxidase or catalase is sensitive to heavy metal ions at pH 5–8. Enzyme immobilization can shift the pH optimum to a higher value relative to the measurements with a native enzyme owing to the accumulation of H ions in the membrane body. The increase of electrolyte concentration contributes to the reproducibility of biosensor response together with the decay of the response value and the lifetime of the immobilized enzyme. This can be followed by the decrease of the sensitivity of the enzyme toward reversible and irreversible inhibitors.[81]

1.16 Pesticides Measurements in organic media

The effect of organic solvents on the response and sensitivity of biosensors for the determination of pesticides has been studied by many workers. The characterization of pesticides in non aqueous media is very attractive because they present new opportunities for the application of such biosensors, as inline control of industrial processes. The utilizing enzymes in organic media allow the increase in the solubility of non-polar substrates and inhibitors and to shift the sensitivity of the enzymes toward various substrates and effectors.[83]

The measurement in organic solvents places more strict requirements upon the immobilization of the enzyme. The appropriate carrier must neither dissolve nor swell in organic media and the immobilized enzyme must retain its activity in the working conditions.[81]

The effects of organic solvents on enzyme activity in general found to be quite variable and depend on the configuration in which the enzyme is employed [84] Both retention and loss of activity have been reported.[85-87] Degradation of enzyme activity in organic solvents caused through removal of the water layer around the enzyme molecules by solvents. Thus the general observation is that hydrophilic solvents can deactivate the enzyme more rapidly than hydrophobic solvents.[88]

Organophosphorous and Organocarbamate pesticides are characterized by a low solubility in water and a higher solubility in organic solvents. It is for this fact that the extraction and concentration of pesticides from fruits, vegetables, etc. are carried out in organic solvents. The application of enzymatic biosensors for pesticide detection in real samples necessitates development of methods for enhanced sustainability of enzymes in the organic media.[89-91]

Organic phase enzyme electrodes (OPES) were developed to circumvent this problem. OPES relied on the partitioning of an organic analyte from a non aqueous phase across a permeable selective membrane which retained enzyme in an aquoues phase. [92-93] However, the technique is not able to draw much attention due to difficulties associated with the fabrication process.

1.17 Overview of the methodologies used for pesticide quantification

The methodologies for pesticide analysis in food and other environmental sample are very well documented and many examples are available in the literature. Earlier methodologies used for pesticide detection were chromatographic methods like Gas Chromatography (GC), Liquid chromatography (LC),[94 ,95] High Performance Liquid Chromatography (HPLC) along with Mass Spectrometry (MS) etc. Among all the techniques, gas chromatography is the most widely used method for detection and quantification of most of the pesticides. However, liquid chromatography is also used for measuring some pesticide residues such as carbamate, triazines. [94]

These methods were sensitive and reliable. However, they involves a complex analysis procedure and time consuming, require highly trained technicians and unable to perform on site detection.[94]

To circumvent these problems, newer techniques are being developed. The new techniques use more sensitive devices like chromatographic techniques with various detection methods, electro analytical techniques, chemical and electrochemical biosensors, spectroscopic techniques and flow injection analysis (FIA) etc. [94]

Enzyme-linked immunosorbent assay (ELISA) has proven to be rapid, sensitive and a cost effective analytical tool for routine monitoring of pesticides.[96] Use of class specific ELISA for OP pesticide has been reported by Xu et al. 2011. The authors had utilized monoclonal Ab based indirect ELISA technique for detection of organophosphate pesticides. This method

had LOD in ppb level. However the method has broad specificity, therefore, can be used only for screening of organophosphates from water samples.[97]

Surface plasmon resonance technique has secured an important place among the newly developed sensing techniques due to its high sensitivity and reliable procedure. Surface plasmon resonance (SPR) based sensors have been widely applied for quantitative detection of pesticides, nucleic acid, enzymes etc. Rajan et al. 2007 developed a Surface plasmon resonance based fiber-optic sensor for the detection of organophosphate chloropyrifos.[98] However, SPR immunosensors may suffer from disturbances caused by changes in the refractive index or temperature.

In the domain of chemical sensors, quartz crystal microbalance (QCM) or surface acoustic wave sensors functionalized with organic polymers and molecular imprinted polymers have been developed for pesticide detection.[99]

A combination of enzymatic reactions with the electrochemical method allowed developing different enzyme based electrochemical biosensors for determination of pesticides due to the good selectivity, sensitivity, rapid response, and miniature size. A variety of enzymes, such as organophosphorous hydrolase, alkaline phsosphatase, ascorbate oxidase, tyrosinase and acid phosphatase have been employed for preparation of pesticide biosensors. Among these, amperometric AChE biosensors based on the inhibition to AChE have shown satisfactory results for pesticides analysis in which the enzymatic activity was employed as an indicator of quantitative measurement of pesticides. [100]

1.18 Review of few recent work on AChE biosensor fabrication

The most important step in the development of an enzyme based electrochemical sensor/biosensor is the dense attachment of the enzyme onto the surface of the working electrode. This process is controlled by the various interactions between enzyme and the electrode material that strongly affects the performance of a biosensor. There are a variety of techniques for immobilization of enzyme for construction of AChE biosensor.

1.18.1 Nanomaterial-based AChE biosensors

In recent years, nanomaterials are gaining more importance in the field of biosensor construction.[101] Nanomaterials (Carbon nano tube, Gold nano particles) based AChE biosensors have many advantages both in terms of stability and in terms of promoting the

catalytic reduction of redox species. Nano materials have high surface area and high sensitivity, excellent conductivity, making them suitable carrier to immobilize enzymes in fabrication of electrochemical biosensor.[102] The limitation of nanomaterial based sensor is that, the nanomaterial synthesis involves cumbersome steps and the materials are costly.

1.18.2 Use of Carbon nano tube for AChE biosensor fabrication

The use of CNTs for AChE immobilization has been reported. Their unique electronic properties suggest that CNs have the ability to promote the electron transfer reactions of biomolecules in electrochemistry. Their mechanical properties, high-aspect ratio, electrical conductivity and chemical stability make CNs perfect for a wide range of applications. Another approach is to use metallic nanoparticles deposited onto the surface of the electrodes. Gold nanoparticles (GNPs), in particular, are preferred because of their biocompatibility which makes them an ideal support for the immobilization of a number of biologically active substances such as enzymes and antibodies and DNA. Another reason to choose GNPs is their conductivity properties which make them suitable for enhancing the electron transfer between the enzyme active centers and electrodes. [103-108]

Joshi et al. 2005, fabricated an AChE based biosensor by deposition of acid-purified carbon nanotubes onto the working electrode surface of a screen-printed electrode, followed by drying and simple deposition of AChE in solution. The sensor was characterized by a high surface area and exhibited enhanced electrocatalytic activity toward thiocholine which facilitated operation at low applied potential without the use of electronic mediators.[103]

Ivanov et al. 2010 developed an amperometric biosensor based on a site-specific immobilization of acetylcholinesterase via affinity bonds on a nanostructured polymer membrane with integrated multiwall carbon nanotubes. The combination of the highly conductive and electrocatalytic behavior of MWCNs with the controlled site-specific enzyme immobilization helped in development of a stable and sensitive sensor towards ATCh and paraoxon.[104]

Development of an amperometric biosensors based on the self-assembly of acetylcholinesterase (AChE) on a CNT electrode using the LBL approach for OPs and nerve agents was reported by Liu and Lin, 2006. The electrocatalytic activity of CNT leads to a greatly improved electrochemical detection of the enzymatically generated thiocholine product, including a low oxidation overpotential (+150 mV), higher sensitivity, and stability.

The biosensor was used to measure as low as 0.4 pM paraoxon with a 6-min inhibition time under optimal conditions.[105]

Marinov et al. 2010 developed an amperometric acetylthiocholine sensor based on acetylcholinesterase immobilized on nanostructured polymer membrane containing gold nanoparticles. In their work, they used poly (acrylonitrile-methylmethacrylate-sodium vinylsulfonate) membranes which are chemically modified and loaded with gold nanoparticles. Acetylcholinesterase was immobilized on the prepared membranes in accordance with two distinctive procedures, the first of which involved immobilization of the enzyme by convection, and the other by diffusion. The prepared enzyme carriers were used for the construction of amperometric biosensors for detection of acetylthiocholine and organophosphate paraoxon. Their sensor showed good storage stability up to fifty days.[106]

Du et al. 2010 developed a sensitive acetylcholinesterase biosensor based on assembly of beta cyclodextrins on to multiwall carbon nanotubes for organophosphate dimethoate detection. The developed biosensor exhibited good reproducibility and stability.[107]

Vamvakaki et al. 2008 utilized biomimetically synthesized silica and conductive activated CNFs for the development of a novel electrochemical biosensor system. The enzyme, acetylcholine esterase from Drosophila melanogaster (Dm. AChE), was directly immobilized onto the CNFs. Subsequently, bioinspired poly (L-lysine) templated silica was grown under mild conditions around the enzyme. CNFs provided high surface area for the immobilization of enzyme as well as the mediator and transducer for signal monitoring. The initial sensitivity of the silica/nanofiber/AChE biosensor was calculated to be 7.8 mA mM^{-1} the response of this biosensor was linear to acetylthiocholine concentrations from 0.04 to 0.3 mM. The biosensor presented a remaining activity of 70% after 3.5 months of continuous polarization. Importantly, this silica/nanofiber architecture could improve enzymes stability against thermal denaturation. [108]

1.18.3 Sol–gel-based AChE biosensors

Sol–gel matrices have been known for their rigidness, chemical inertness, thermal and photochemical stability, negligible swelling in aqueous solution, tunable porosity, and optical transparency. They have been widely used in the fabrication of biosensors because most of the biological materials tend to retain their activity owing to the attractive low-temperature

process of immobilization for various enzymes and antibodies. To design an effective biosensor, a good optical quality and stable sol-gel-based glassy matrix is required. [109-111]

An example is the construction of an acetylcholinesterase biosensor by Du et al. 2008, in which the sol–gel-derived silicate network assembling gold nanoparticles provided a biocompatible microenvironment that stabilized the enzyme bioactivity and prevented its leaking out from the interface. Typical pesticides such as monocrotophos, methyl parathion and carbaryl were determined with high sensitivity, accuracy, low cost and simplified procedures.[109]

Anitha, 2004 fabricated an amperometric silica sol-gel immobilized biosensor doped with acetylcholinesterase for organophosphate pesticide detection.[110]

Kuswandi et al. 2008 developed an optical fiber biosensor for detection of chlorpyrifos by using a single sol-gel film containing acetylcholinesterase and bromothymol blue.[111]

1.18.4 Use of graphene and hybrid graphene in AChE biosensor fabrication

Graphene, a new two dimensional carbon nano material, has attracted much attention during recent years because of its excellent electrocatalytic ability.[112] . Reduced graphene oxide (rGO) could maintain the bioactivity of biomolecules and exhibit catalytic activity to small molecule.[113]

Thus it shows outstanding application in construction of nanoelectronic and sensor devices in recent years.

Wu et al. 2013 demonstrated the application of GRO/nafion in AChE loading for the electrochemical sensing of pesticide.[114]

Liu et al. 2011 reported the immobilization of AChE on reduced graphene oxide (rGO)/AuNPs modified electrode.[115] AChE retained activity after being immobilized on the electrode by boronic acide-diol interaction. Integration of rGO and AuNPs enhanced the signal of electro-oxidation of thiocholine.[112]

Zhang et al. 2012 proposed a stable AChE biosensor fabrication technique based on simple and efficient self-assembling AChE to graphene nanosheets for detection of different pesticide.[116]

1.18.5 Polymer supported AChE biosensor

Polymer matrices as a support have enhanced speed, sensitivity, and versatility in diagnostics of desired analytes using biosensors. Conducting polymers (CP) serves as the immobilizing

matrices for biomolecules and provides a suitable environment for their immobilization. Conducting polymers are the conjugated polymers that can be synthesized by chemical methods as well as electrochemical methods; provide easy modulation of various properties (film thickness, conductivity, fictionalization, use of various supporting electrolytes, ability to serve as an electrochemical transducer itself). Additional merits include entrapping of enzyme molecules during electropolymerization in one step and also uniform covering of the surface of substrate electrodes of any shape or size by polymer film.[99] Among the conducting polymers polypyrrole and polyaniline have potential application in AChE biosensor fabrication due to their relative stability, ease of preparation, and good conductivity.

The first use of polypyrrole in biosensor fabrication was reported by Umana, M in 1986.[117] They reported a novel approach to electrode immobilization of enzyme glucose oxidase, by electropolymerisation of pyrrole in the presence of the enzyme. Their sensor was applied for the indirect determination of electrochemically active products of glucose oxidase catalysed reaction. Since then a huge work have been done on polypyrrole based biosensor construction.

Gong. et al. in 2009 developed a simple strategy for designing a highly sensitive electrochemical biosensor for organophosphate pesticides (OPs) based on acetylcholinesterase (AChE) immobilized onto Au nanoparticles-polypyrrole nanowires composite film modifid glassy carbon electrode (labeled as AChE-Au-PPy/GCE).In their work, the generated Au nanoparticles (AuNPs) were homogenously distributed onto the interlaced PPy nanowires (PPy NWs) matrix, constructing a three-dimensional porous network. This network-like nanocomposite not only provided a biocompatible microenvironment to keep the bioactivity of AChE, but also exhibited a strong synergetic effect on improving the sensing properties of OPs. The combination of AuNPs and PPyNWs greatly catalyzed the oxidation of the enzymatically generated thiocholine product, thus increasing the detection sensitivity. The detection limit was 2 ppb for methyl parathion. The developed biosensor exhibited good reproducibility and acceptable stability.[118]

Du.D et al. in 2010 develpoed a simple method to immobilize acetylcholinesterase (AChE) on polypyrrole (PPy) and polyaniline (PANI) copolymer doped with multi-walled carbon nanotubes (MWCNTs).The synthesized PAn-PPy-MWCNTs copolymer presented a porous and homogeneous morphology which provided an ideal size to entrap enzyme molecules. Due to the biocompatible microenvironment provided by the copolymer network, the obtained composite was devised for AChE attachment, resulting in a stable AChE biosensor

for screening of organophosphates (OPs) exposure. MWCNTs promoted electron-transfer reactions at a lower potential and catalyzed the electro-oxidation of thiocholine, thus increasing detection sensitivity. Based on the inhibition of OPs on the AChE activity, using malathion as a model compound, with a detection limit was found to be 1.0ppb. The developed biosensor showed good reproducibility and acceptable stability.[119]

Somerset. VS et al. in 2007 developed an acetylcholinesterase-polyaniline biosensor for the investigation of organophosphate pesticides in selected organic solvents. In their work gold electrode was modified first with a mercaptobenzothiazole self-assembled monolayer, followed by electropolymerization of polyaniline in which acetylcholinesterase as enzyme was immobilized. The developed sensor was then used for organophosphorous pesticide diazinon and fenthion detection. Detection limits was found to be 0.147 ppb for diazinon and 0.172 ppb for fenthion.[120]

1.19 Scopes, aim and objectives of the present investigation

It is known from the available literature that considerable efforts have been devoted in last two decades towards the development of better efficient AChE biosensors for OP and OC pesticide quantification. However the field is still an open area of research, to achieve further improvement of the AChE based biosensorsin terms of their stability (both operational and storage), reproducibility, reusability, specificity, and selectivity. The ease and cost of fabrication also important factors those need attention from the perspective of applicability toroutine analysis. Moreover,most of the AChE based pesticide biosensors developed till date are capable of operating in either phosphate buffer or in a solution of 5% acetonitrile in phosphate buffer, or in 10% ethanol in select cases. As the amount of residues present in produce or food items are generally very low, such high dilution may lower the amount of residues in the real samples even below the detection limit of the sensor. Attempt to address this problem through development of organic phase enzyme electrodes (OPES) was also not successful up to the mark as far as AChE biosensor is concerned.

So our primary goal in the present investigation is to develop an easy, cost effective method for AChE biosensor fabrication with focus on achieving high stability, reproducibility and reusability. Also we aim to evolve some efficient mechanism for analyzing pesticide residue in organic extract using the developed biosensor.

Under the above back ground the objectives of the present investigations are as follows

1. To select a suitable matrix for AChE immobilization.
2. To perform preliminary electrochemical study of the feasibility of enzyme immobilization in the selected matrix.
3. To optimize the conditions for stable immobilization, conditions for maximum signal output and reproducibility.
4. To fabricate the sensor probe under the optimized conditions and application to test sample (pesticide) analysis.
5. To check the low potential workability of the prepared sensor so as to have some added advantages, and hence to optimize the parameters for low potential application, if possible.
6. To work out suitable method or optimized conditions for pesticide analysis in real samples (organic extract) using the prepared biosensor.
7. To check the validation of the new method through comparison of the results with the conventional gas chromatographic method.
8. To work out correlation procedures for mapping the results of the new method onto the true value.

1.20 Plan of work

1. Since conducting polymers PPy is easily available and advantageous material for enzyme immobilization, has been used by different workers as enzyme hoisting matrix, and also not easily degraded by organic solvents, so we will attempt to fabricate the sensor on PPy support.
2. Few workers have already used PPy for construction of AChE biosensor. In all those work AChE was immobilized on the surface of the PPy film. Surface immobilization results in poor sensor life time due to washing out of the enzyme. Electro immobilization of the enzyme inside the film expected to provide a longer operational lifetime of the sensor. So we will use electroentrapment method for fabrication. Electrochemical chronoamperometric method will be used for film deposition as well as for enzyme immobilization.
3. Cross linker gluteraldehyde will be used for preventing leaching out of the enzyme and gelatin will be used for providing biocompatible micro environment to the enzyme inside PPy matrix.
4. The immobilization will be tested through electrochemical response measurement as well as through study of the film morphology by using SEM images.

Both chronoamperometry and cyclic voltammetry will be applied for electrochemical characterization.

5. Optimum parameter such as pH, enzyme amount, concentration of supporting electrolyte, saturated substrate concentration etc. will be studied for stability of the sensor as well as for reproducibility and maximum signal output.
6. Inhibition characteristic of organophosphosphate and organocarbamate towards the immobilized enzyme and hence towards the sensor response will be studied.
7. The sensor will be applied to sample analysis in both phosphate buffer and 5% acetonitrile in phosphate buffer.
8. Validation will be checked through analysis of fortified samples extracted in acetonitrile and diluting to 5%.
9. For developing a process/method for real sample analysis at minimum possible dilution, it will be attempted to make feasible biosensingin QuECHERS extract, since QuECHERS is the most recent and most efficient work up technique for pesticide residues. It will be attempted to link between the two through the use of ethylacetate as the final phase QuECHERS solvent followed by lipase catalyzed transformation of ethylacetate to enzyme friendly mixture.
10. The transformation will be studied through spectroscopic methods.
11. Validation of the new method will be checked through gas chromatography.
12. Calibration curves under both methods (the new method and the conventional 5% acetonitrile method) will be obtained and will be utilized for deriving correlation formula.

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