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Enzyme-Based Biosensors in Food Industry via Surface Modifications

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7.1 Introduction

Enzyme technology has received great attention with improvements in biological remediation techniques. Enzyme-based biosensors can qualify as a measuring system as they permit the detection of biological events by detecting biochemical changes in biological processes. Advantages of biosensors are identification of even small quantities, cost effectiveness, and requirement of less power and less volume for identifying a wide linear range for usage [1]. Moreover, biosensors have high sensitivity, selectivity, and good response time for different kinds of applications [2].

Biosensor system processing consists of three main parts: analyte detection, signal conversion, and signal reading. Signal conversion can be carried out by optical, thermal, electrical, or electronical components. In conductive polymer-solution part of the system, there will be an electrochemical conversion that occurs at the exact moment of the biochemical reaction and load velocities get higher than the species mass transport speed toward the electrode. This diffusion-controlled electrocatalytic procedure is used for amperometric studies [3]. Biological information is turned into electrical signals in biosensor applications. Biosensors have two main parts: bioreceptors and biorecognition pieces. Bioreceptors can be either biological species such as enzymes or living biological systems that are used as biological mechanisms for recognizing [4].

One of the most important working fields of biosensors is the food industry [5]. Rising demand for high quality and safe food products as well as prevailing consumer trends are important challenges in this industry. In order to meet these requirements, strict auditions for processing and product development are carried out in the food sector [6]. Quality control analyses of manufacturing processes and monitoring the initial and final products are crucial steps in production. These steps have to be performed to prevent enzymatic inactivation, microbial contamination, lack of hygiene, and formation of chemical or biological toxins [7]. There are various different types of analytical methods employed for processing and product development in the food industry. However, most of them are time-consuming, expensive, and nonpractical methods. For this sector, quick, cheap, practical, and reliable methods and equipments

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are critical. Due to these reasons biosensors can be desired as alternative means to primitive techniques. To illustrate, levels of hypoxanthine and xanthine are important biomarkers as a sign of meat spoilage, which can be followed by xanthine-oxidase-based biosensors. Alcohol oxidase is used in the measurement of alcohol content in brewing, winemaking, and distilling industries. Glucose oxidase (GOx) is used for determination of glucose in fruit juices or in the fermentation of liquors. Lactose is the main disaccharide present in milk and dairy products and can be hydrolyzed by the enzyme galactosidase. Histamine, which is an important amine for determining fish freshness, can be analyzed by monoamine oxidase enzyme. Oxygen-based biosensors, as their content serve as good biomarkers for determining food quality. Moreover, enzyme-based biosensors can be used to determine food freshness, detection of glucose content in beverages, analysis of cholesterol in butter, food components of sugars, pathogenic organisms test, and so on. In this chapter, detail information will be shared about enzymes, immobilization of enzymes, main characteristics of enzyme-based biosensors, and their usage in food analysis.

7.2 Biosensors

"Sensors" are devices or systems containing control and processing electronics, software, and interconnection networks. Sensors respond to physical or chemical changes in the medium to which they are exposed by generating a product which is a measure of that quantity [8]. Physical sensors, chemical sensors, and biosensors are the types of sensors that are mainly used. Distance, mass, temperature, pH, and so on, can be measured by physical sensors [9]. Chemical sensors can provide responses in the form of analytical signals as a result of chemical reactions. Chemical sensors transform chemical information into an analytically useful and measurable signal [10] (Figure 7.1).

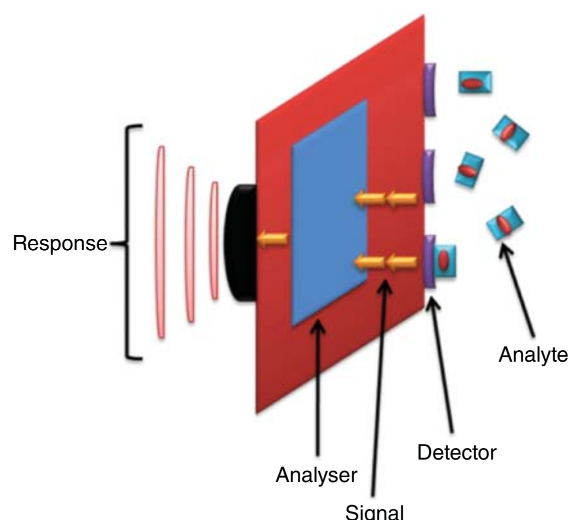


Figure 7.1 Schematic presentation of a sensor. (Kuila 2011 [11]. Reproduced with permission of Elsevier.)

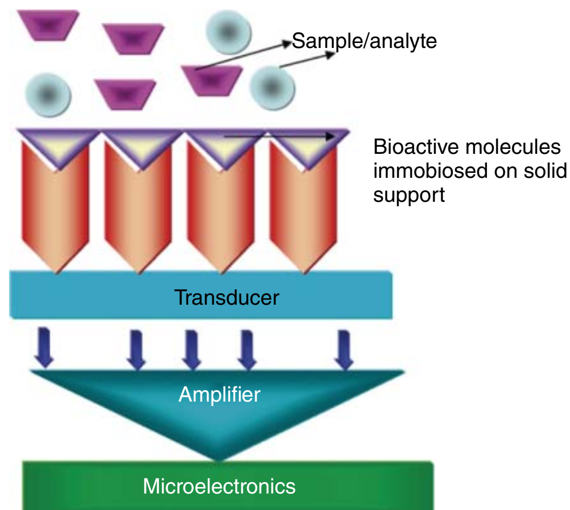


Figure 7.2 Schematic of a biosensor. (Dhawan 2009 [14]. Reproduced with permission of Elsevier.)

Biosensors are a type of chemical sensors that transform a biochemical reaction into an analytical signal. Sensors translate the information from the main domain into an output signal in order to offer the selective sensors for the analyte that can be measured. Biosensors are compact devices and analytical tools that are formed by the following two components:

- a recognition system consisting of a biological material that uses a biochemical mechanism
- a transducer from where the signal of the biosensor is obtained and changed into a meaningful signal [10, 12, 13].

According to the International Union of Pure and Applied Chemistry, a biosensor is exactly defined as a self-contained integrated device, capable of providing detailed quantitative or semiquantitative analytical information using a biological recognition element, which is retained in direct contact with a transduction element (Figure 7.2).

Biosensors can also be defined as analytical devices that combine biological materials such as cells, cell receptors, antibodies, membranes, organelles, enzymes, tissues, and microorganisms either merged within a transducer or closely in contact with a physicochemical transducer [15, 16]. Biosensors are devices that enable rapid analysis of mostly detection and monitoring of biological and chemical agents. Biosensor technology combines biological, chemical, and physical sciences together with engineering for a broad range of applications.

7.2.1 Historical Perspectives of Biosensors

The first "biosensor" was discovered by Clark in 1956 who is well-known for his invention of the Clark electrode that was later improved by Clark and Lyons in 1962 as an enzyme electrode. Updike and Hicks expanded the studies on the enzyme electrode, as they developed the first enzyme sensor in 1967 via trapping GOx in a layer of polyacrylamide gel attached to the plastic membrane of an

oxygen electrode. Clark's ideas on the glucose analyzer were commercialized in 1975, which was the first of biosensor-based analyzer device to be built by companies around the world, based on the amperometric detection of hydrogen peroxide. In 1974, thermal biosensors were introduced, together with thermal enzyme probes and enzyme thermistors. Subsequently, Divi's study achieved alcohol detection via microbial electrodes with the help of bacteria. In 1975, carbon dioxide- or oxygen-based sensors were created by Lubbersand Opitz. In 1982, the *in vivo* application of glucose biosensors was presented by Shichiri *et al*. In the early 1970s, the piezoelectric or potentiometric transducer was designed. Liedberg *et al*. described the use of surface plasmon resonance to monitor affinity reactions in real time; starting from the early to the mid-1980s mainly made of modified electrodes. The first commercial lab-on-a-chip (LOC) system was announced in 1999 by Agilent, and various LOC products on glass chips exist in the literature. It is believed that the future of biosensors will depend on LOC systems due to their miniaturized biochemical analysis systems and their ability to gain miniaturized skills to biochemical analysis.

7.2.2 Parts of Biosensors: Bioreceptor and Transducer

A biosensor is composed of two parts, a bioreceptor and a transducer. Enzymes, antibodies, cell receptor, nucleic acid, tissue, and so on, can be used as a bioreceptor that selectively recognizes the analyte which is the target molecule for the receptor. The interaction between the analyte and bioreceptor produces a physical change close to the transducer surface and transducer and converts the biorecognition event into a signal by measuring the changes in the reaction. These two components, namely, the bioreceptor and transducer, are combined into one single sensor that measures the analyte under concern. The biosensor depends directly on a selective biorecognition where a conversion of biochemical information to electrical information occurs. The working principle of a biosensor depends on the diffusing of an analyte from a bulk solution to the bioreceptor immobilized sensor and on the selective and efficient reactivity with the bioreceptor. Subsequently, the biochemical reaction leads to a change depending on the transducer such as its optical, electronic properties, and so on. The alteration in the biosensor surface is converted into a signal, depending on the transducer model. Various transducers such as electrochemical, calorimetric, optical, piezoelectric are available in literature.

The transducer of a biosensor has the ability to convert a specific biological reaction (binding or catalytic) into a response that can be processed into a useable signal [17]. The working principle of the transducer is to transfer the signal from the output domain of the biorecognition system and convert a response to a signal [10]. According to the transducer type, biosensors can be classified as electrochemical (amperometric, impedimetric, potentiometric, conductimetric) optical, acoustic, thermal, and piezoelectric. Biosensing responses can be followed and converted into signals by a transducer through several ways such as oxygen consumption, hydrogen peroxide formation, changes in NADH concentration, fluorescence, absorption, pH change, conductivity, temperature, or mass [10, 17].

Electrochemical biosensors, first seen by Clark and Lyons, are the most used transducers employing amperometric glucose biosensing using the Clark oxygen electrode. Up to now, due to their high sensitivity, selectivity, inherent simplicity, ease of mass production, low cost, and availability of instrumentation, electrochemical biosensors have been used and improved now and are better in quality. In general, electrochemical transducers are used for designing a biosensor, which can have conducting, semiconducting, and ionic conducting material to coat the electrode surface combining with the biological receptor. When the electrochemical species is consumed or produced during the biological reaction, the electrochemical signal can be recorded using an electrochemical detector. The working principle of electrochemical biosensors is based on the electrochemistry of the substrate or product. Effective, simple to handle, low cost, and fast detection properties in electrochemical biosensor is related to the analytical power of the electrochemical techniques used as well as the process employed for a specified biological recognition element [13]. Amperometric, impedimetric, potentiometric, and conductimetric biosensors are the electrochemical techniques mainly used in biosensing of foods (Figure 7.3).

Amperometric electrochemical biosensors have attracted great attention in biosensor design since amperometric detection is a useful technique for substrate analysis because of its selectivity, sensitivity, rapid response, ease of construction, and reproducible performance. In amperometric biosensors, the change in the current on the working electrode upon applied constant potential is measured, which is the result of oxidation or reduction of the species in the sensing matrix due to a biological reaction. Chronoamperometry is the preferred technique in electrochemical detection systems. The main advantage of chronoamperometry is that it allows monitoring either oxygen consumption or hydrogen peroxide production. In amperometric measurements, there exists a current change that results from the reaction, which can be monitored as a function of time, which correlates with the amount of analytes in the reaction solution. The electron transfer between the analyte and the electrode surface occurs in instances where oxidation or reduction reactions are involved. Therefore, the transducer surface directly effects the functioning of amperometric biosensors. Generally, modified electrodes with nanoparticles, conducting polymer, and so on, are used in amperometric transducer design. Moreover, since constant potential is applied, maintaining the applied potential is very important to perform the process, which is correlated with the quality of the reference electrode. As a result of a biocatalytic reaction, the release of an electroactive product or consumption of reactant can easily be followed by amperometry in an inert working electrode. Due to their simple construction systems, high sensitivity, and rapid response amperometric biosensors are preferred [19]. Conductometric biosensors are based on the principle of the change of conductivity of the medium as a consequence of the biological element, designed by a two-electrode device. They are used to detect the changes in the conductance either due to consumption or to generation of charges in the species involved in the enzymatic reactions. Due to their nonspecificity and poor signal to noise ratio, conductometric biosensors have not been much used [15, 20]. The potentiometric biosensors are based on ion-selective electrodes and ion-selective field effect transistors, designed to

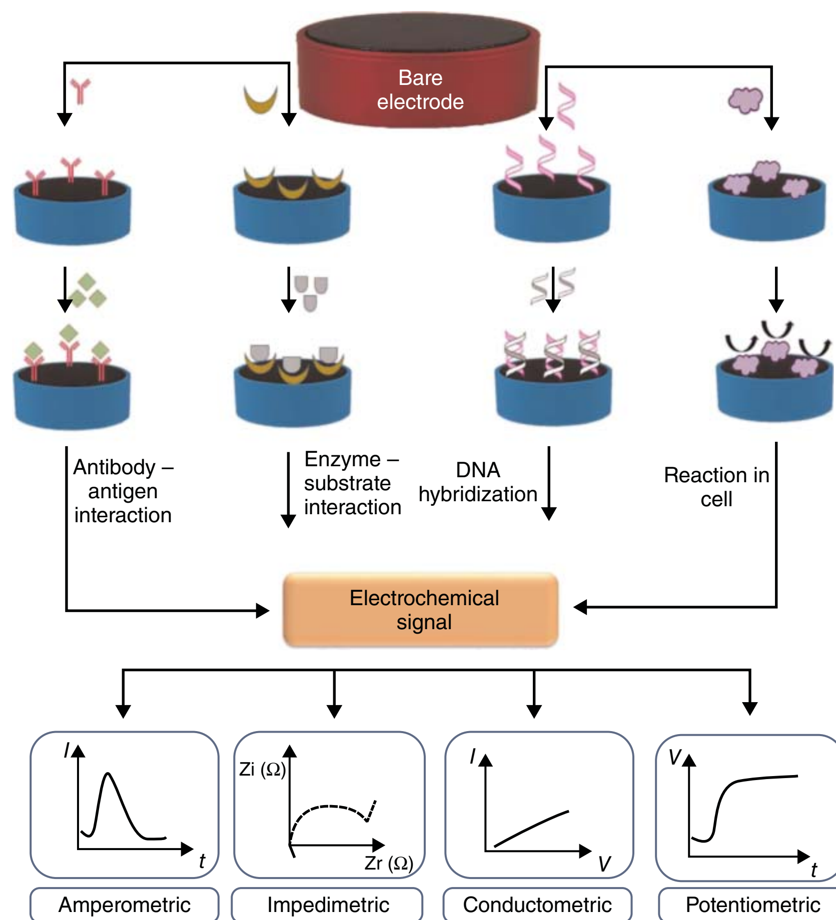


Figure 7.3 Scheme of an electrochemical biosensor. (Bahadır 2015 [18]. Reproduced with permission of Elsevier.)

measure the potentials at the working electrode with respect to the reference electrode under no current flow. They monitor electric potential related to ion concentration in the system created by selective binding of the electrode and generally possess long lifetimes and acceptable mechanical stability. The electrode measures the change in electrode potential and determines several ions in the reaction matrix due to the enzymatic reaction, while the ions accumulate and bind to a suitable ion-selective membrane. Simple instrumentation, low cost, and suitability for continuous monitoring are the advantages of potentiometric biosensors, while, on the other hand, leaching of the membrane components into the sample is a disadvantage and a major problem with potentiometric biosensors [21].

Optical biosensors are also another type of transducers that are mainly used in biosensor design. The working principle is based on the detection of changes in the refractive index. Optical biosensors contain heterogeneous group of sensors

in which the interaction of light with an immobilized biorecognition material is sensed. The binding of the analyte to its specific bioreceptor produces a change in the refractive index and therefore, a response occurs at the sensing surface. Detection principle of optical biosensors is based on the changes in absorbance, fluorescence, luminescence, reflectance, light scattering, and refractive index. Thermal and acoustic transducers are adequately used in analytical applications; they have the advantage of miniaturization and the possibility of construction of arrays of sensors for simultaneous determination of several compounds. One of the disadvantages of these transducers is that they lack selectivity [15]. The change in energy is followed in thermal transducers-based biosensors but this energy can be lost and the produced heat is partly wasted by irradiation, conduction, or convection [15]. Calorimetric biosensors probe the amount of change in heat during a biochemical reaction. In general, thermistors are used to find out the modification of temperature in the solution. The recorded peak height is proportional to the enthalpy change with respect to a substrate concentration [22, 23]. A piezoelectric biosensor working principle mainly depends on measuring the decrease in frequency of vibration of an oscillating crystal by the adsorption of a foreign material on its surface. A piezoelectric biosensor uses the piezoelectric effect to probe pressure, acceleration, strain, or force by converting them to an electrical charge. The measurement of the mass changes on a piezoelectric crystal, which is sensitized by covering it with material binding or by reacting with the analyte, is the main concern. Piezoelectricity is described as a material that generates mechanical movement in response to electric potential.

In biosensor-based analyses, there exist some parameters that should/may be reported to prove that the suggested biosensor is analytically valuable. Some criteria must be satisfied in accordance with standard IUPAC protocols. Sensitivity; which is the response of the sensor to analyte concentration over a unit change, is not an obligatory to report, although reporting it is beneficial for the users. The range of biosensor where there is a linear relationship between the concentration of the substrate and the response of biosensor should be reported. This parameter is very crucial, since the measured concentration should be within the characterized linear range. Limit of detection and limit of quantification values should also be calculated, under optimized conditions of the biosensor. These parameters also show the performance of the biosensor. Selectivity of the biosensor is also important as it shows the ability of the sensor to respond only to the target analyte. IUPAC defines the response time as the time passing between the substrate added before and after or at which the activity of the enzyme starts with adding its substrate and the reaction/response has finished. Rapid response time is desired in biosensors and, therefore, response time should also be reported to support the designed biosensor. Reproducibility of the biosensor shows how accurate it is. This parameter should also be given and can be given as relative standard deviation values. Life time of the biosensor can also be reported, which is the time period of the biosensor, used without significant deterioration in performance characteristics. Shelf-life stability studies can also be shared with the readers which defines the change in its baseline or sensitivity over a fixed period of time. In addition to these parameters, a biosensor should be cheap, small and

portable, biocompatible, nontoxic, nonantigenic, and stable under ambient conditions.

The major key points to note in the improvement of biosensor design involve a proper bioreceptor or a recognition molecule, suitable immobilization method, transducer to convert binding reaction into a measurable signal, range, linearity, enhancement, and sensitivity. These are crucial factors to design a biosensor and finally packaging of the biosensor into a complete device.

7.3 Enzymes

Different amino acids play important roles in metabolism; they can be found in living organisms and are the building blocks of proteins. Proteins are macromolecules containing long chains of amino acids, consisting of thousands of amino acids. Proteins are found in all forms of living organisms and perform a wide variety of tasks; for example, fibrous, elongated proteins that are insoluble in water and provide structural support, and globular, spherical proteins that are water soluble and have specific functions in the immune system and metabolism.

Many chemical reactions occur spontaneously but some of them need to be catalyzed for determined reaction speed. Catalyzers are molecules that decrease the required activation energy for chemical transformation of substrates to different materials. Thermodynamical amount of energy is defined as transversion of free energy. Catalyzers are not consumed or changed during reactions. Limitless used catalyzers can restrict the reaction that turns the substrate to product due to their stability [24]. Enzymes are the protein packages that are biological catalysts that speed biochemical reactions without being permanently changed (Figure 7.4).

A large number of biochemical reactions occurring in living organisms are catalyzed by enzymes that are protein structured biocatalysts [26]. Biomolecules

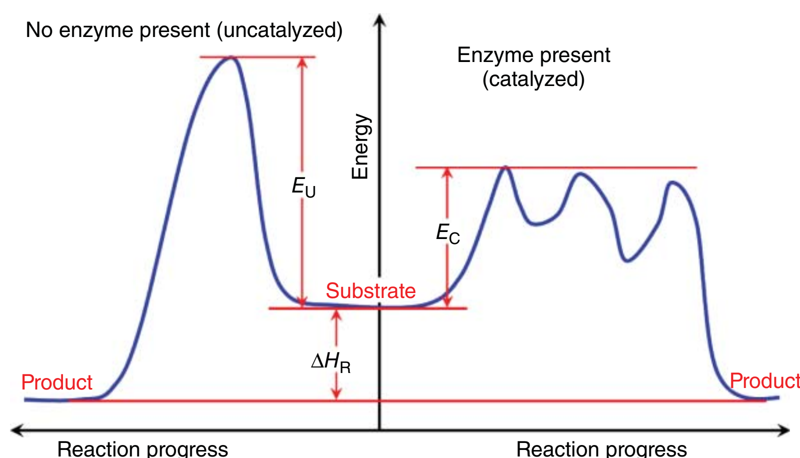


Figure 7.4 Activation energies of enzymatically catalyzed and uncatalyzed reactions. (Liu 2013 [25]. Reproduced with Permission of Elsevier.)

most used as bioreceptors are enzymes. Enzymes have high affinities toward their substrates. They select from a variety of substrates to which they are related chemically and induce the reactions. Enzymes do not change the equilibrium of a reversible reaction; they only facilitate access into balance. Characteristic properties of enzymes are correlated with the differences in their molecular structures. They are constructed by hundreds of amino acids that are bonded to each other covalently by the carbon atoms of carboxyl groups and next amino acids α-amino groups N atoms. Amino acids show either hydrophilic or hydrophobic property according to their radical groups. Enzymes are more often colorless and soluble in salt or water.

The part that directly participates in the catalytic activity is the specific binding part of enzymes called the active site which was postulated by Emil Fischer in 1860 [27]. The enzyme – substrate complex enters a catalytic reaction directed by amino acid residues in the active site. The active site is a three-dimensional niche in the protein, which constitutes a small portion of the overall protein structure. The specificity of the enzyme depends on the arrangement of atoms in the active site and the substrate – enzyme binding process involves a relatively small amount of energy.

Apoenzyme is called the protein and glycoprotein part of an enzyme. The catalytic properties of enzymes are dependent on cofactors or coenzymes, which are non-present in a peptide structure. If these structures are firmly connected to the polypeptide it is called a prosthetic group. Apoenzymes and a prosthetic group create active holoenzymes. Specificity of an enzymatic reaction is usually the result of steric harmony [28].

Emil Fischer offered "lock and key" model to fit the enzyme and substrate complex with each other.

In 1962, the "induced fit model" was offered by Daniel Koshland, suggesting that the enzyme is induced to undergo a structural rearrangement upon substrate binding due to the change in shape of the active site that facilitates the reaction [29]. As the enzymatic reaction is completed, the products are released, and the active site returns to its original state (Figure 7.5).

7.3.1 Enzyme Commission Numbers

The nomenclature of enzymes is generally based on recommendations of the Enzyme Commission in 1961, by the International Union of Biochemistry and Molecular Biology. According to the International Union of Biochemistry and Molecular Biology, every type of characterized enzymes is required to contain Enzyme Commission Numbers. The systematic name of the enzyme describes the substrate or substrates and defines the type of reaction catalyzed [29]. The Enzyme Commission Number, is associated with a recommended name, alternative names, catalytic activity, cofactors, pointers to the SWISS-PROT [31] protein sequence entries that correspond to the enzyme, pointers to the PROSITE [32] entries describing the protein families of which the enzyme is a member, pointers to human diseases associated with a deficiency of the enzyme [33].

Classification of enzymes by the International Union of Biochemistry and Molecular Biology is based on the type of chemical reaction catalyzed. The

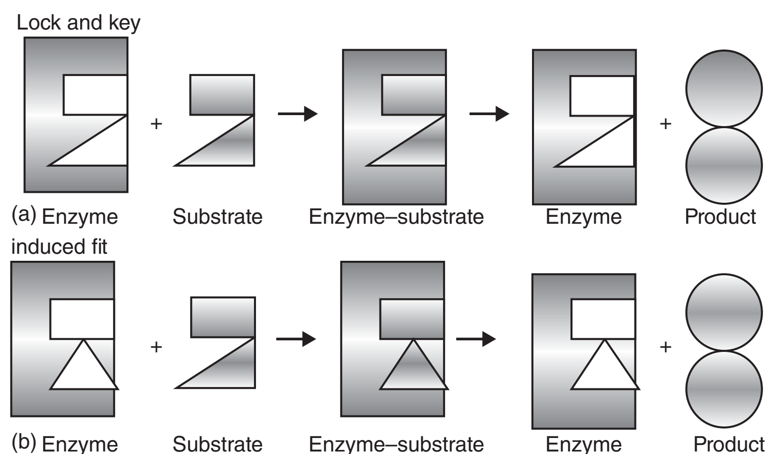
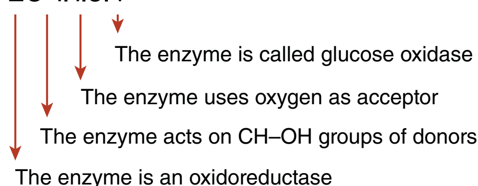


Figure 7.5 Enzyme–substrate complex with (a) lock-and-key and (b) induced fit model. (Whiteley 2006 [30]. Reproduced with permission of Elsevier.)

Enzyme Commission of the International Union of Biochemistry and Molecular Biology assigns a four digit number for each enzyme: the first number indicates the family, the second indicates the subclass of the family and also indicates its actions of chemical group type, the third indicates a subgroup within a subclass and is related to the particular chemical groups involved in the reaction, and the forth indicates the correlative number of identification within a subgroup. By using Enzyme Commission numbers, quick searching of enzymes and enzymes functions and properties can be achieved. Moreover, Enzyme Commission numbers provide original classification of enzymes [34].



Enzymes are generally named with reference to the reaction they catalyze. The name of an enzyme is often formed by adding -ase to the name of its substrate. Suffix -ase is added to the name of the enzyme's primary substrate. Enzyme Commission has recommended nomenclature of enzymes based on the reaction being catalyzed. Enzymes are classified into six different groups. The six main types of enzyme-catalyzed reactions are as follows:

- EC1: Oxidoreductases catalyze oxidation–reduction reactions
- EC2: Transferases catalyze group transfer reactions
- EC3: Hydrolases catalyze hydrolytic reactions
- EC4: Lyases catalyze cleavage and elimination reactions
- EC5: Isomerases catalyze isomerization reactions
- EC6: Ligases catalyze synthetic reactions.

7.3.1.1 EC1 Oxidoreductases

Oxidoreductases catalyze oxidation–reduction reactions, transfer of electrons, hydrogen or oxygen atoms is the main cause of enzymes catalyzing oxidation/reduction reactions. Oxidoreductases are also called “oxidases,” “dehydrogenases,” or “reductases.” Oxidoreductase enzymes that are also called electron donors catalyze the transfer of electrons, and are included in the central *meta*. Oxidoreductases have 22 subclasses; they all have different technological importance such as, dehydrogenases that oxidize a substrate by transferring hydrogen atoms to a coenzyme (NAD^+, $NADP^+$, FAD^+, FMN), which acts as an acceptor [29, 35].

The class Oxidoreductase includes the following:

- Dehydrogenases and oxidases (1.1–1.10)
- Peroxidases (1.11), which use H_2O_2 as the oxidant,
- Hydroxylases (1.99.1), which introduce hydroxyl groups
- Oxygenases (1.99.2), which introduce molecular O_2 in place of a double bond in the substrate.

Oxidoreductases are enzymes that catalyze oxidoreduction reactions. Simply, oxidoreductases catalyze reactions like; $A^- + B \rightarrow A + B^-$, where A is the oxidant and B is the reductant. These enzymes can be located in glycolysis, TCA cycle, oxidative phosphorylation, and amino acid metabolisms. They (dehydrogenases) use electron acceptors other than oxygen, such as the cofactor NAD^+ or $NAD(P)^+$. These cofactors have an important advantage when combined with electrochemical sensors that ensure the outwardly supplied, immobilized, and regenerated cofactor. It is reported that there are more than 250 commercially and highly specific dehydrogenases available [36].

Oxidoreductases have 22 subclasses:

- EC 1.1 contains oxidoreductases the CH—OH group of donors (alcohol oxidoreductases)
- EC 1.2 contains oxidoreductases the aldehyde or oxo group of donors
- EC 1.3 contains oxidoreductases the CH—CH group of donors (CH—CH oxidoreductases)
- EC 1.4 contains oxidoreductases the $CH—NH_2$ group of donors (amino acid oxidoreductases, monoamine oxidase)
- EC 1.5 contains oxidoreductases CH—NH group of donors
- EC 1.6 contains oxidoreductases NADH or NADPH
- EC 1.7 contains oxidoreductases and other nitrogenous compounds as donors
- EC 1.8 contains oxidoreductases a sulfur group of donors
- EC 1.9 contains oxidoreductases a heme group of donors
- EC 1.10 contains oxidoreductases diphenols and related substances as donors
- EC 1.11 contains oxidoreductases peroxide as an acceptor (peroxidases)
- EC 1.12 contains oxidoreductases hydrogen as donors
- EC 1.13 contains oxidoreductases single donors with incorporation of molecular oxygen (oxygenases)
- EC 1.14 contains oxidoreductases paired donors with incorporation of molecular oxygen

- EC 1.15 contains oxidoreductases superoxide radicals as acceptors
- EC 1.16 contains oxidoreductases that oxidize metal ions
- EC 1.17 contains oxidoreductases CH or CH_2 groups
- EC 1.18 contains oxidoreductases iron–sulfur proteins as donors
- EC 1.19 contains oxidoreductases reduced flavodoxin as a donor
- EC 1.20 contains oxidoreductases phosphorus or arsenic in donors
- EC 1.21 contains oxidoreductases X—H and Y—H to form an X—Y bond
- EC 1.97 contains other oxidoreductases

7.3.1.2 EC2 Transferases

Transferases catalyze functional group transfers; these are of the general form [29]. Transferase enzymes catalyze the transfer of a functional group from a donor to an appropriate receiver. According to the transferred groups' chemical properties they are classified into nine subclasses. In cell metabolisms, transferases, such as methyltransferases, acyltransferases, transaminases, phosphotransferases, and glycosyltransferases, have vital assignment. Transferases need purely intracellular coenzymes. They have limited applications but some of them are commercial enzymes of relevance in research [24].

7.3.1.3 EC3 Hydrolases

Hydrolases catalyze hydrolysis; these are of the general form [29]. Reactions of hydrolysis that is enzymes catalyzing, is the breaking of a chemical bond by water reaction. There are 12 subclasses of hydrolases according to the type of susceptible bond [37]. Hydrolases are necessary for destructive metabolism by procuring assimilable nutrients to the niche. The most incontestable hydrolase enzymes with their technological function are esterases, proteases, and glucosidases. Hydrolases can catalyze the overturn reactions of chemical bond formation with water elimination under suitable circumstances [38].

7.3.1.4 EC4 Lyases

Lyases catalyze lysis reactions, in other words nonhydrolytic removal of functional groups from substrates, often creating a double bond in the product; or the reverse reaction, that is, addition of function groups across a double bond [29]. Lyases are enzymes that catalyze the breaking of various chemical bonds nonhydrolytic, nonoxidative, and mostly forming a new double bond or a new ring structure. Lyases have seven subclasses according to the type of precise bond: C—C, C—O, C—N, C—S, C—X (halides), P—O, and other bonds. For forming double bond or a new ring, lyase enzymes act as a single substrate and eliminate a molecule. Lyase enzymes have dissimilar metabolic performance by their cell-destructive metabolism and biosynthesis by acting in reverse. Aldolases, generally acting in reverse reactions of C—C bond formation, carboxylases and decarboxylases, hydratases, and dehydratases are the most riveting lyases. Some lyases are intracellular and some do not need coenzymes. As an example pectate lyase ((1 → 4)-α-D galacturonan lyase; EC 4.2.2.2) from a separate supplier is extracellular and requires Ca^{++} as cofactor but does not need a coenzyme [39, 40].

Having different properties like, for instance, requirement of substrate is only one for one direction reactions but two for reverse direction, from other enzyme species Lyases have much more technological applications. As an example, a recognizable enzyme of lyases, nitrile hydratase (nitrile hydrolyase; EC 4.2.1.84) has a use with acrylonitrile for acrylamides industrial production [41, 42]. There are more concerned essays for lyases; production of L-aspartate with aspartase (aspartate ammonia-lyase; EC 4.3.1.1) [43–45] the production of fumarate with fumarase (fumarate hydratase; EC 4.2.1.2) [46, 47] and the production of urocanic acid with histidase (L-histidine ammonia lyase; EC 4.3.1.3) [48]. Moreover, Lyases have a role for optically active organic compounds asymmetric synthesis [49, 50].

Lyases have seven subclasses:

- EC 4.1: broken carbon–carbon bonds lyases, such as decarboxylases (EC 4.1.1), aldehyde lyases (EC 4.1.2), oxo acid lyases (EC 4.1.3), and others (EC 4.1.99)
- EC 4.2: broken carbon–oxygen bonds lyases, such as dehydratases
- EC 4.3: broken carbon–nitrogen bonds lyases
- EC 4.4: broken carbon–sulfur bonds lyases
- EC 4.5: broken carbon–halide bonds lyases
- EC 4.6: broken phosphorus–oxygen bonds, such as adenylate cyclase and guanylate cyclase lyases
- EC 4.99: other lyases, such as ferrochelatase.

7.3.1.5 EC5 Isomerases

Isomerases catalyze molecular isomerization, including racemizations and cis–trans isomerizations [29]. Isomerase enzymes convert a molecule from one isomer to another isomer. They can simplify intramolecular rearrangements in the presence of broken and formed bonds. Moreover, they can catalyze the conformational transfers. Transition of a substrate into an isomer enzyme catalyzing reactions is a substance which has the same kind and number of atoms. Isomerases have six subclasses depending on the produced kind of isomers such as racemases and epimerases, cis–trans-isomerases, intramolecular oxidoreductases, intramolecular transferases (mutases), intramolecular lyases, and other isomerases. Most of them are intracellular but some of them need cofactors but do not need organic coenzymes. Isomerases do not have much technological applications but glucose isomerase (xylose isomerase; D-xylose aldose–ketose-isomerase EC 5.3.1.5) is an impressive example of those applications [51]. This enzyme is used for the production of high fructose syrups (HFS), usually from corn starch [52].

7.3.1.6 EC6 Ligases

Ligases catalyze ligation, a synthesis reaction involving condensation of two molecules with the hydrolysis of a pyrophosphate bond [29]. Ligases are enzymes that can catalyze the covalent bonding of two large molecules by making a new chemical bond. Generally, this covalent bonding occurs by hydrolysis or a small chemical group added on one of the bigger molecules or catalyzes the bonding

of two molecules with an enzyme. These enzymes have a crucial role in cell anabolism by making synthesis in the cell. Ligases have six subclasses according to the C—O, C—S, C—N, C—C, phosphoric esters, and C-metal bonds. Ligases have high molecular weight and they need intracellular coenzymes. Ligases have no wide application in technology. But they are priceless trading enzymes for research practices. For instance, T4 DNA ligase (poly deoxy ribonucleotide synthase; EC 6.5.1.1) is routinely used in genetic engineering protocols [53].

7.3.2 Enzyme Immobilization

Enzymes are biological catalyzers that speed up biochemical reactions quickly and efficiently. Due to their large application area, usages of enzyme biosensors have great attention in areas such as fine chemistry, food chemistry, therapeutics applications, protein engineering, and so on. Even though enzyme biosensors have great potential, due to economical and stabilization on storage aspects their usages are limited. Usage of enzymes has some drawbacks due to some of their characteristics that are not appropriate for industrial application such as being natively unstable, easily inhibited and working well only on natural substrates and under physiological conditions [54]. In this point, procedures to improve their stability and enable them to be reused, gains importance. The term *immobilization* refers to "restricted movement" or "making unable to move." The term *immobilized enzymes* refers to "enzymes localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." Subsequently, immobilization means physical localization of biocatalysts or connection of the biocatalysts with an insoluble matrix or immobilized proteins and cells to an insoluble support. Mixing together the enzyme and the support material under optimized conditions and following a period of incubation, is the main procedure for immobilization (Figure 7.6).

Enzyme immobilization offers benefits in comparison with soluble enzymes or alternative technologies [56, 57]. Enzyme immobilization technology has become reasonable in the design of enzyme biosensors due to their many advantages such as:

- Increase in enzyme stability
- Easily separating or removing from solution without contamination by the contents of the reaction mixture
- Repetitive usage with the same amount of enzyme in solution and immobilized enzyme
- Greater control and follow of the catalytic process
- Decrease in effluent problems
- Noninfected product by the enzyme
- Developing continuous process.

The enzyme immobilization process consists of three main components such as the enzyme, the matrix, and the mode of attachment or entrapment. An ideal matrix must be inert, physically strong, stable, regenerative, and be able to increase enzyme activity and decrease product inhibition, nonspecific adsorption, and microbial contamination [58]. There can be different carriers

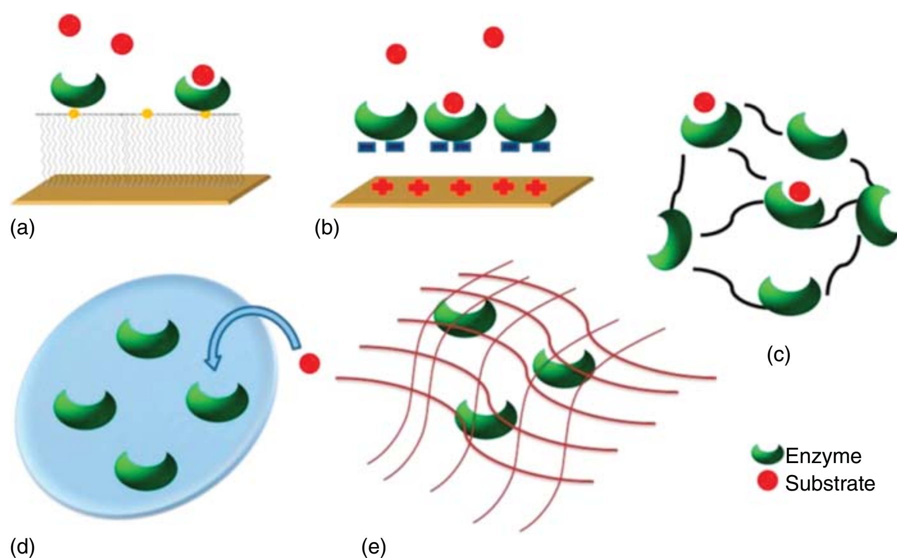


Figure 7.6 Common methods of immobilization used in biosensing: (a) covalent binding; (b) adsorption; (c) cross-linking; (d) encapsulation; and (e) entrapment. (Rodríguez-Delgado 2015 [55]. http://www.sciencedirect.com/science/article/pii/S0165993615002290 created under creative common license http://creativecommons.org/licenses/by/4.0/.)

used for enzyme immobilization in terms of organic and inorganic [59, 60]. Organic carriers are polysaccharides such as cellulose, agar, agarose, chitin, alginate dextrans; proteins such as collagen and albumin; carbon, polystyrene, and other polymers such as polyacrylate polymethacrylates, polyacrylamide, polyamides, vinyl, and allyl-polymers. Inorganic carriers are natural minerals such as bentonite, silica, sand; processed materials such as glass (nonporous and controlled pore), metals, controlled pore, and metal oxides such as ZrO_2, TiO_2, Al_2O_3. Chemical characteristics of enzymes, different properties of substrates and products, and the range of potential processes employed should be considered while selecting the immobilization methods. For enzymes, immobilization techniques can be classified based on the entrapment of enzyme or bounding of enzyme. Entrapment can be matrix entrapped or membrane entrapped. Bounded methods are classified as adsorption and covalent binding (Figure 7.7).

For food analysis applications, enzymes have to be immobilized, with very simple and cost-effective procedures [61]. In immobilization methods, how well the enzyme bonds to the sensor surface is crucial, since it directly affects the success of the enzyme biosensor. Moreover, not only the immobilization method, but also immobilization steps are important since there can be a loss of enzyme activity in the immobilization process and, therefore, it should be carefully monitored. Reuse of the enzyme is very critical because enzymes are expensive catalysts [15]. The selection of an appropriate immobilization method depends on the nature of the enzyme, type of transducer used, physicochemical properties of the analyte, and operating conditions for the enzyme biosensor

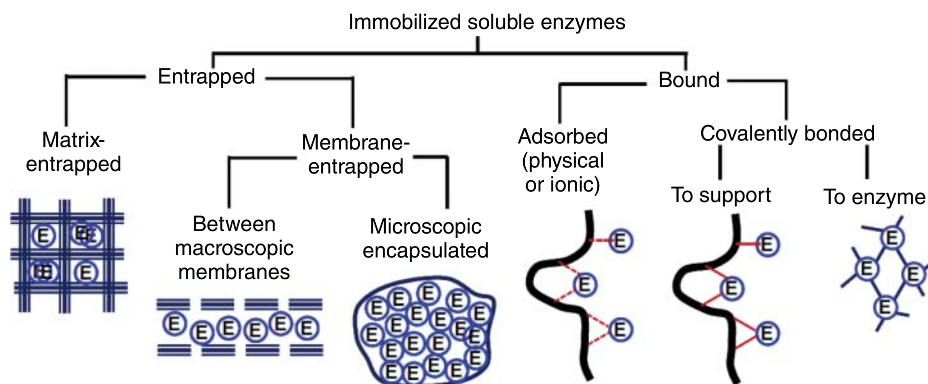


Figure 7.7 Immobilization techniques. (Liu 2013 [25]. Reproduced with permission of Elsevier.)

[15]. A successful matrix should immobilize the enzyme at a transducer surface efficiently, while maintaining the functionality of the enzyme and providing accessibility toward the target analyte with an intimate contact with the transducer surface. The development of a good biocompatible matrix for enzyme immobilization is very crucial to improving the analytical performance of a biosensor [62]. Although the most common methods for immobilization of biocomponent are adsorption and covalent bonding, the suitability of a method for a particular task is at present still being empirically elucidated.

7.3.2.1 Physical Adsorption

Physical adsorption is the simplest and oldest method made up of mixing together enzyme and support under suitable conditions which are pH, ionic strength; incubation period for immobilization of enzyme. In the physical adsorption procedure, reversible surface interactions between enzyme and support material occurs. Weak forces of electrostatic such as van der Waals forces, ionic and hydrogen bonds are involved in the physical adsorption steps. Surface chemistry between enzyme and support by these forces enable the necessary binding. In this method, there is no need for activation and chemical modification and, therefore this is a simple immobilization technique.

Since the adsorption of enzyme to a surface is a reversible process, changes in pH, ionic strength, substrate concentration, and temperature may remove the enzyme from the surface [63]. On the other hand, there exists a major disadvantage of physical adsorption such as leakages of the enzyme from the support with desorption and contamination of the solution as a result of flowrate, bubble agitation, particle–particle abrasion, and so on. Consequently, usage of physical adsorption immobilization method has its limitations. Despite having some limitations and disadvantages, immobilization by physical adsorption is still a simple procedure since it does not need physiological coupling conditions or chemicals which is an additional advantage [63]. Currently, physical entrapment is no longer used due to the short life of enzymatic activities achieved. Therefore, chemical

immobilization techniques are more frequently used in enzyme sensors such as cross-linking, entrapment, and so on.

7.3.2.2 Covalent Binding

Formation of covalent bonds is the main principle of this method. Functional groups such as —NH_2, —COOH that are available on the surface of the enzymes mainly originate from the side chain of the amino acids attached covalently to chemically activated supports [15]. The immobilization by covalent binding is conducted by activating the carrier, coupling of the biomolecule, and removal of the adsorbed biomolecules. A disadvantage of covalent coupling is the frequently occurring loss of enzyme activity [63]. In their work, Xu *et al*. covalently immobilized the enzyme using glutaraldehyde as a spacer arm on the surface of polyvinyl alcohol (PVA)/chitosan (CS)/multiwalled carbon nanotubes (MWNTs) and composite nanofibrous membrane as shown in Figure 7.8.

7.3.2.3 Entrapment

Immobilization by entrapment differs from adsorption and covalent binding in that enzymes are free in solution but are restricted by the lattice structure of the entrapment system. Entrapment is the easiest immobilization method where there is no alteration in the structure of the enzyme.

Three general methods for entrapment are as follows:

- Entrapment behind a membrane: a solution of enzyme, a suspension of cells, or a slice of tissue is simply confined by an analyte permeable membrane as a thin film covering the detector.

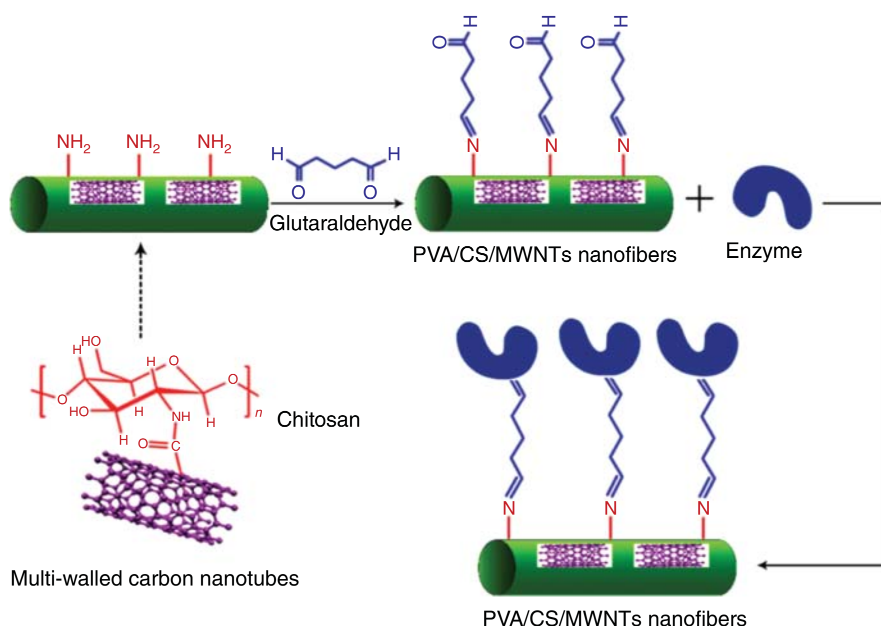


Figure 7.8 Schematic illustration of laccase immobilization on PVA/CS/MWNTs nanofibrous membranes via activation. (Xu 2015 [64]. Reproduced with permission of Elsevier.)

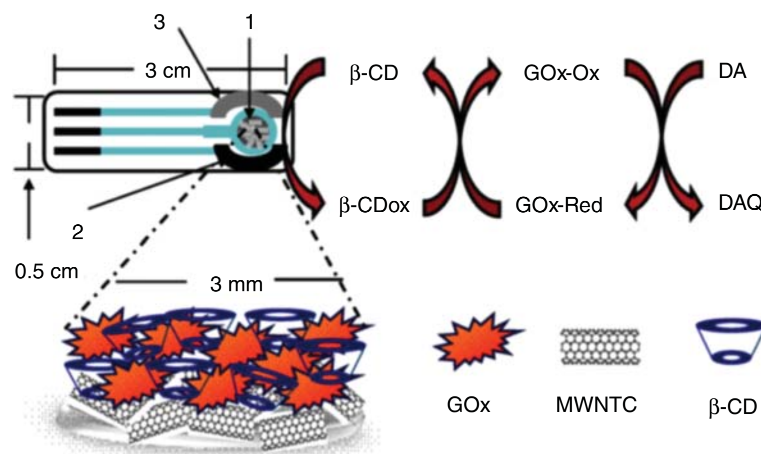


Figure 7.9 Entrapment of glucose oxidase. (Alarcón-Ángeles 2010 [65]. Reproduced with permission of Elsevier.)

- Entrapment of biological receptors within a polymeric matrix: polyacrylonitrile, agar gel, polyurethane or polyvinyl-alcohol membranes, sol gels, or redox hydrogels with redox centers.
- Entrapment of biological receptors within self-assembled monolayers or bilayer lipid membranes [10].

The entrapment method for enzyme immobilization was first carried out by Bernfeld and Wan in 1963 by entrapping them into lattices of synthetic polymers. Till date, entrapment method is the commonly used method, which is quite simple and easy. The main difference from other techniques is that the enzyme does not bind directly to the matrix. The main disadvantage of an enzyme entrapment method is that, the response time is longer compared to other methods due to difficulties in the accessibility of entrapped biomolecules. The immobilized support should allow the diffusion of substrates and products to overcome this problem.

In a recent work, Alarcón-Ángeles *et al*., developed a novel enzyme entrapment approach based on an electropolymerization process using multi-walled carbon nanotubes (MWCNTs), ß-cyclodextrin (ß-CD), and GOx as shown in Figure 7.9.

7.3.2.4 Encapsulation

Encapsulation is the other immobilization method that can be used to immobilize enzymes within a semipermeable membrane. Similar to entrapment, in this method the enzyme is restricted in a limited space. Depending on their molecular weight, small substrates and products can be moved in and out of the semipermeable membrane. The main disadvantage of this method is that if the diffusion of the product or substrate is high, or the density of the encapsulated enzyme is high, the membrane can be damaged. As an advantage, this encapsulation method can lead to immobilization of biological materials in any desired combination.

To illustrate this method, Macario *et al*. reported an encapsulation strategy for lipase enzyme in a highly ordered mesoporous matrix by a sol–gel method. The

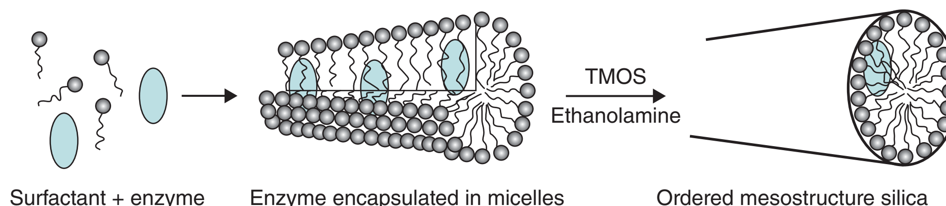


Figure 7.10 Enzyme encapsulation in micelles. (Macario 2009 [66]. Reproduced with permission of Elsevier [66].)

micellar phase of the surfactant, which is self-assembled with silica is used to encapsulate the enzyme. The authors came up with the result that an immobilization procedure saves the mobility of the enzyme and there is a sixfold increase in enzyme activity with increased stability (Figure 7.10).

7.3.2.5 Cross-Linking

One of the commonly used methods for enzyme immobilization is the cross-linking method. In this method, enzyme form cross-linked or covalent bonds with the support material and this method is a support-free procedure and involves joining the receptor molecules with each other to form a large three-dimensional complex structure. Cross-linking or co-cross-linking can be achieved by chemical or physical methods. The chemical method normally involves the formation of covalent bonds between the enzymes by means of bi- or multifunctional reagents such as bovine serum albumin, glutaraldehyde, or carbodiimide. The cross-linking method offers advantages of being a simple method and having a strong chemical binding of the enzymes. Moreover, the choice of the degree of cross-linking influences the physical properties and the particle size. The main disadvantage is that intermolecular cross-linking is difficult to control; therefore, there is a possibility of activity loss due to chemical alternations of the active site of the enzymes [63] (Figure 7.11).

7.4 Application of Enzyme-Based Biosensors in Food Industry

Poly(ethyleneoxide)/polypyrrole (PEO-*co*-PPy) and 3-methylthienyl methacrylate-*co*-*p*-vinyl benzyloxy poly(ethyleneoxide)/polypyrrole (CP-*co*-PPy) are covered with thiophene and yeast cells (*Saccharomyces cerevisiae*) and invertase enzyme were immobilized in conducting copolymers by electrochemical polymerization of pyrrole that was studied in another research [68].

Alcohol biosensor, which has a photoelectrochemical property, was constructed by using alcohol oxidase enzyme and 4-(2,5-di(thiophen-2-yl)-1*H*-pyrrol-1-yl)benzenamine (SNS-NH2) and carbon nanotubes (CNTs) modified gold slides, for detection of alcohol amount in real samples. According to research, the modified alcohol biosensor should be an alternative method for photobiocatalytic nanoscale systems based on alcohol oxidation reactions in various biotechnological, biological, and environmental analyses [69].

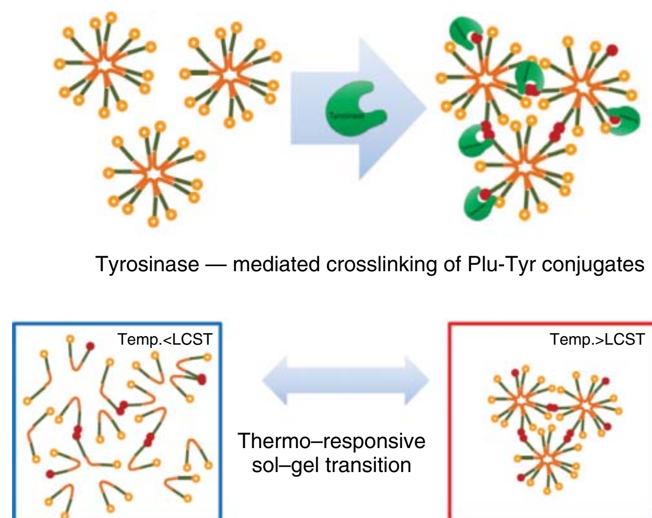


Figure 7.11 Schematic illustration of Plu-Tyr hydrogel formation by enzyme mediated cross-linking and its thermo-responsive sol–gel transition. (Lee 2011 [67]. Reproduced with permission of Elsevier.)

Another substantial leading study for biosensor application was designed by using cholesterol oxidase for cholesterol determinations. P(SNS-NH2)/ChOx/[Ru(bpy)3]2+ and ChOx/[Ru(bpy)3]2+, were covalently bonded cholesterol oxidase to modified electrode and gold slide, modified by thioaniline [70].

For cholesterol determination there has been a significant study. In this regard, thiophene capped PEO-*co*-PPy and CP-*co*-PPy were synthesized with electrochemical techniques. Cholesterol oxidase was immobilized on electrodes by electrochemical polymerization [71].

Bis(4-(3-thienyl ethylene)-oxycarbonyl)diazobenzene (TDAZO), photosensitive and thermosensitive monomer, was synthesized recently. In this study, thiophene and pyrrole were copolymerized on TDAZO, then immobilization of invertase, by pyrroles electropolymerization, was carried out for biosensor production [72].

Another important amperometric GOx biosensor was constructed by immobilization of biomolecule by thiol-containing calixarenes. For determination of the glucose amount Calix-SH was formed on a gold surface. Fabricated calix-SH/GOx biosensor was successfully used in real samples [73].

Calixaren was used in another study with modification with montmorillonite (Mt) for biosensor applications. Amino-functionalized calixarenes (Calix-NH2) were modified with Mt (Calix-NH2/Mt) and the pyronose oxidase was immobilized on the electrode for real sample tests [74].

A selenium contented polymer biosensor was improved for glucose detection. Poly(2-(2-octyldodecyl)-4,7-di(selenoph-2-yl)-2*H*-benzo[*d*][1,2,3]-triazole), poly(SBTz) was electropolymerized on graphite electrode. Immobilization was carried out with a mixture of calixaren and gold nanoparticle [75].

Another different study shows a N-functionalized dithienopyrroles (DTP-NH2) modified GOx immobilized biosensor. In addition, experiments were carried out with real samples and a reference to those experiments where commercial glucose kits shows were used shows the reliability of the biosensor [76].

A new amperometric xanthine (X) biosensor was established by immobilization of xanthine oxidase (XOx). Electopolimerized polymer film was used as a conducting polymer; DTP type polymer indicated that xanthine biosensor has high sensitivity, stability, and selectivity in real samples [77].

7.5 Conclusion

In enzyme-based biosensors, the bioreceptor takes the help of an immobilized enzyme as biocatalyst to transform the analyte to a product that can be detected by the transducer. A large number of enzymes are commercially available, but some need to be extracted in specific measurements. Some of the enzymes are naturally present in food materials, whereas others are used to enhance food processing actions or food quality. It is seen that enzyme sensors have potential applications in the food industry. The specific applications are monitoring ingredients, food additives, contaminations, and toxins.

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