Chapter 4

Amperometric biosensors for enantioanalysis

4.1. Introduction

A biosensor is a device that incorporates a biological sensing element connected to a transducer. Possible combinations of sensing element and transducer are summarized in Table 4.1 [1]. The first biosensor was reported in the early 1960's and comprised enzyme immobilized to an oxygen electrode. Continued development of this kind of biosensor led to the commercialization of various devices for different applications e.g., the measurement of glucose in blood.

Table 4.1 Biological elements and transducers used in biosensor's design

Biological elements	Transducers
Organisms Tissues Cells Organelles Membranes Enzymes Enzymes components Receptors Antibodies Nucleic acids Organic molecules	Potentiometric Amperometric Conductometric Impedimetric Optical Calorimetric Acoustic Mechanical Molecular electronic

In previous works different amperometric biosensors were constructed for the determination of chemical species regardless their stereochemical configurations (L- or D-enantiomer).

The evolution concerning the design of enantioselective amperometric biosensors made their utilization a reliable alternative for chromatographic techniques in high throughput enantioselective screening analysis [2-5].

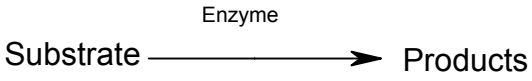
The principle of enantiorecognition used by biosensors is catalyst selectivity. Enzymes are used to catalyze only the reaction of one of the enantiomers. The enzyme can be coupled with different types of electrochemical transducers such as field effect transistor, potentiometric and amperometric. The most used enzymes pairs are L-amino acid oxidase (L-AAOD) and D-amino acid oxidase (D-AAOD) for the assay of L- and D-enantiomers of amino acids.

4.2. Design of amperometric biosensors

The reliability of the response characteristics and the analytical information obtained by using amperometric biosensors is correlated with the design of the biosensor. Different types of membranes have been utilized with amperometric sensors. Distribution of the enzyme in the matrix plays an important role in sensor design and response characteristics. The quality of the matrix of the membrane and the type of immobilization of the enzyme recorded several improvements in sensor design [6-12]. New generations of biosensors are continuously being developed (e.g., amperometric biosensor) based on the direct enzymatic regeneration at the electrode surface [13].

The most reliable matrices used for enantioselective amperometric biosensors are carbon paste and diamond paste. The type of sensor and utilized enzyme must be selected in

concordance with enantiomer configuration (L- or D-enantiomer). The biochemical reaction is very selective and sensitive: due to the presence of immobilized enzyme, the substrate (enantiomer) is transferred under the action of the enzyme into product that will be detected by the transducer:



Biosensors design is related to two main parts (Figure 4.1):

1. the immobilization techniques for the sensing medium (enzymes, cells, or the biological active substances).
2. the type of transducer that will be used.

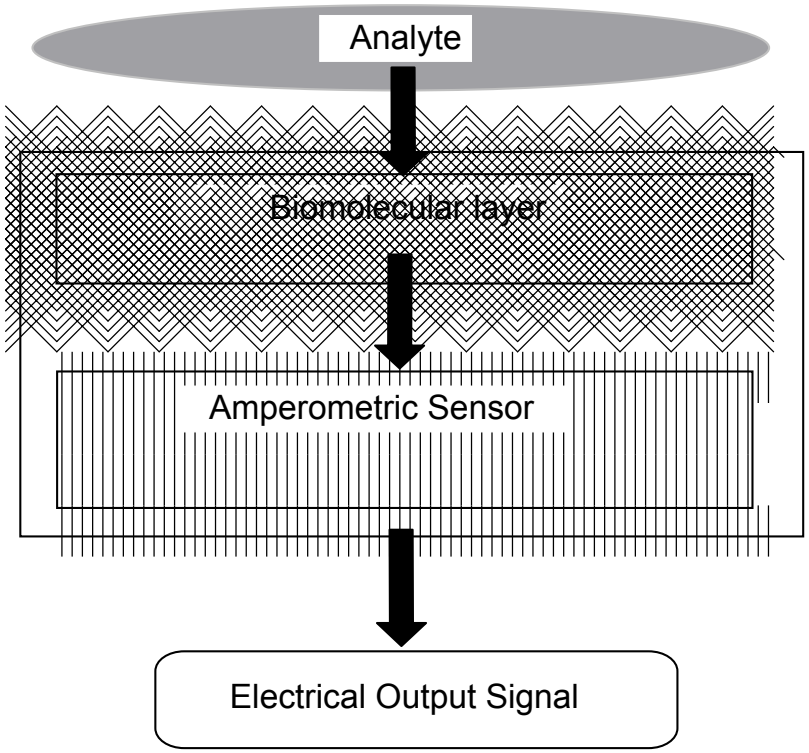


Figure 4.1 Amperometric biosensor design.

The biomolecular layer consists of a biological sensing substance (e.g., enzyme, antibodies, receptors, etc.) impregnated in a matrix (PVC, imprinting polymers, carbon paste, diamond paste). The fast development evolution of transducer influenced the improvement of biosensor design in terms of sensitivity and reliability. The type of transducer must be selected in biosensor design according to the type and quantity of the products formed in the enzymatic reaction, e.g.,

(i) when H^+ is forming, a pH electrode is used as transducer,

(ii) when H_2O_2 is forming, an amperometric transducer for determination of H_2O_2 is recommended.

(iii) when carbon dioxide or ammonia are formed, the gas sensor is utilized as transducer.

The sensitivity of the enzymatic reaction must be correlated to the sensitivity of the transducer or biosensor's design, the best electrochemical transducer is amperometric electrode [4].

The nature of the matrix, as well film technology will influence the behavior of the enzyme because the biotransformation equilibria of the substrate are taking place at the membrane-solution interface. Carbon [4] and diamond [14] pastes proved to be the best matrices for enzymes immobilization. To immobilize an enzyme, three facts must be taken into account: the enzyme activity and its stability with temperature, type of the matrix where the enzyme will be immobilized and finally the possible types of immobilization. These pastes are prepared by mixing graphite powder or diamond

powder with nujol or paraffin oil. There are several principles to add the sensing part in the biosensor: membrane entrapment, physical adsorption, matrix entrapment and covalent bonding (Figure 4.2). A covalent bond of the enzyme to the matrix will decrease its activity, therefore it is necessary to check the enzyme activity after immobilization. This type of immobilization will also decrease the sensitivity of the electrode and its lifetime.

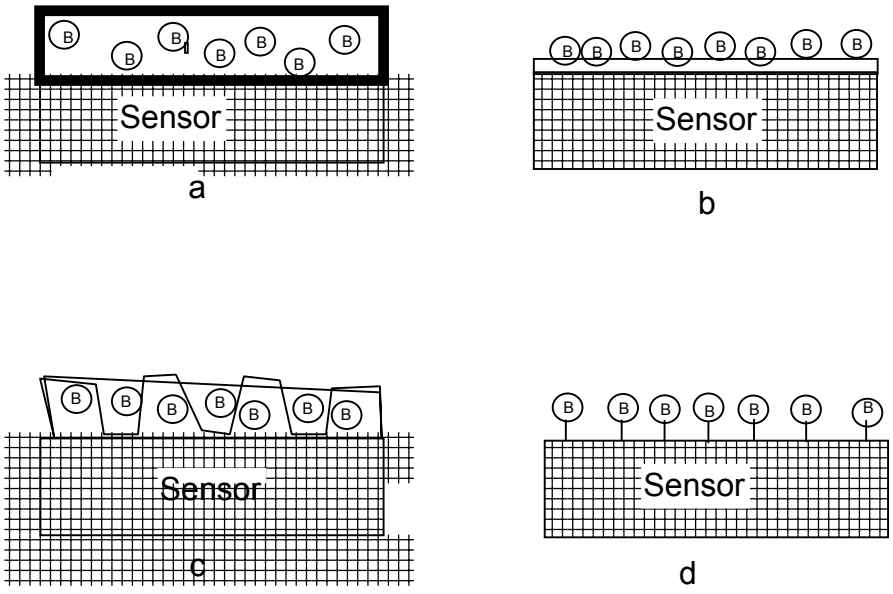


Figure 4.2 Principles of biosensors (a) imembrane entrapment; (b) physical adoption; (c) matrix entrapment and (d) covalent bonding

4.2.1 Physical immobilization

Physical immobilization is based on adsorption, cross linking techniques and electrostatic entrapment. Physical immobilization is including the mixing of biological substance into plastic membrane or solid pastes (e.g., carbon and diamond paste).

4.2.1.1 Biosensors based on plastic membranes

Enzyme adsorption is preferred for the design of plastic membranes based amperometric biosensors. Plastic based biofilms can be prepared by sandwiching a porous cellulose nitrate membrane in a sterile syringe filer holder with a certain volume of the diluted cell broth adsorbed by the membrane by suction [15].

Another technique of physical mobilization on a plastic membrane is the cross-linking technique [16]. The tip of the transducer was cleaned with acetone, then immersed in a solution of cellulose acetate and let to dry at room temperature for two hours. At the end of the procedure, a thin film of cellulose acetate covered the probe tip. The second step was the preparation of an aqueous enzyme solution consisting of 10μL of enzyme 10%, 10μL bovine serum albumine (BSA) 10%, and 6μL glutaraldehyde 5%. Finally the probe is dipped in the solution and left to dry for two hours at room temperature.

4.2.1. 2 Biosensors based on carbon and diamond pastes

One of the most reliable techniques for enzyme immobilization proved to be the mixture of enzymatic solution with the carbon [17-20] and diamond pastes [21]. The utilization of carbon or diamond pastes for physical immobilization of enzymes has the advantages of a reliable construction of the sensor and of ensuring high activity for enzyme. Carbon or diamond paste based biosensor is shown in figure 4.3.

100 mg of carbon or diamond powder is mixed with 20 μL paraffin oil. The plain paste is filled in a plastic tip leaving an empty space of 3-4 mm in the top part to be filled with

modified carbon/diamond paste (containing the different enzymes). 100µL of enzyme solution (1 mg enzyme in the optimum buffer of a certain pH) are mixed with 100 mg graphite or diamond powder and 40µL paraffin oil of IR purity. The diameters of all biosensors were 3 mm. Electric contacts were obtained by inserting silver wires into the diamond paste. The biosensors tips were gently rubbed on fine abrasive paper to produce a flat surface. The surface of the biosensors were wetted with de-ionized water and then polished with an alumina paper (polished strips 30144-011, Orion) before use. The biosensors were stored dry at 4^0C, when not in use. 0.1 mol/L KCl solution was used as electrolyte for these biosensors [2,14].

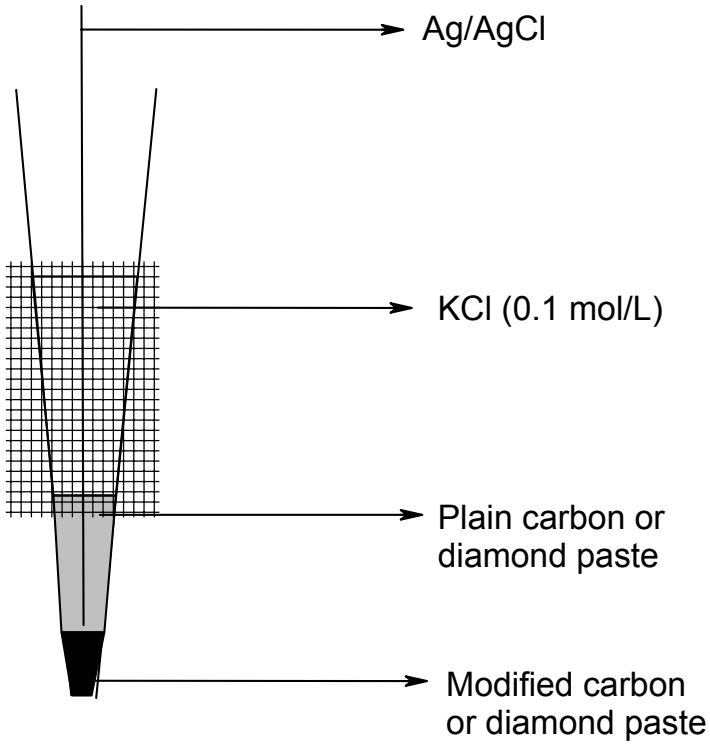


Figure 4.3 Design of carbon or diamond paste based biosensor

4.2. 2 Chemical immobilization

4.2.2.1 Plastic based amperometric biosensors

Chemical immobilization is based on the covalent bonding of the enzyme to a matrix. This type of immobilization may have decrease as a result of the enzyme activity as well as the sensitivity of the electrodes. Sometimes, the covalent bond of the enzyme to the matrix is done at a temperature higher than the temperature of the body (37^{o}) and part of the activity of enzyme is lost.

The most utilized matrix for covalent immobilization of an enzyme is nylon that must have special treatment before enzyme bounding as follows: the nylon meshes need to be cut into disks that are dipped in methanol, rinsed with water and dried in an air steam prior to use. For enzyme immobilization [21] the following solution were added to each disk: 2.0 µL glutaraldehyde (2.5%), 2.5 µL bovine serum albumin (BSA) (1% w/v), and 5.0 µL of enzyme (1 mg enzyme dissolved in a certain buffer). The mixture was homogenized on the surface of the disk. Gelification of glutaraldehyde and the protein was carried out at room temperature for 30 min. Disk was immersed in a certain buffer solution to inactivate the remaining carboxyaldehyde groups.

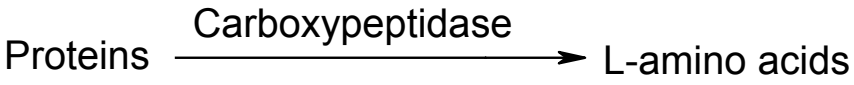
Acrylate polymer was recommended as plastic matrix for enzyme immobilization. A copolymer of 25% glycidyl and 75% methacrylate (25G75M) was chosen as the basic material to provide a polymer with a reactive epoxide group for immobilization. The responsible group of enzyme immobilization is the glycidyl group. 2 mL of 1 mg/mL enzyme were incubated in 100 mg of the copolymer [22].

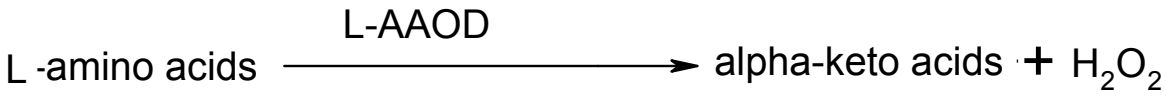
4.2.2.2 Carbon paste based amperometric biosensors

The chemical modified carbon paste was prepared as follows: 0.4 g graphite powder were mixed with 400 µL (0.03 mol/L) solution of 1-ethyl-3-3-(3-dimethylaminopropyl) carbodiimid (EDAC) and heated to 700^{o}C for 60 s in a muffle furnace. After cooling down to ambient temperature, 10 µL polyethylanimine (PEI) and 10 µL of glutaraldehyde were added. 100 µL solution containing 1 mg enzyme was allowed to react at 4^{o}C for 2 hours and then it was mixed with 40 µL of paraffin oil to produce the modified pastes [23].

The design of multienzymatic sensors improved the reliability of analytical information by increasing the sensitivity of biosensors. The second enzyme is added because:

1. the products obtained in the first enzymatic reaction cannot be determined directly by a transducer, e.g., it is necessary to use a bienzymatic sensor for the determination of proteins, when the following reactions are taking place under the influence of carboxypeptidase and L-amino acid oxidase (L-AAOD) [24]:





2. the selectivity and sensitivity of the reaction of product recognition by the transducer is improved, e.g., Horse radish peroxidase (HRP) is added to catalyze the redox transformation of H_2O_2.

4.3. Response characteristics of amperometric biosensors

Amperometric biosensors function is based on the direct relation of a recorded current, I and the enantiomer concentration. The current is developed due to the electron or mass transfer under the influences of the controlled applied potential between the working and reference electrodes. All the response characteristics (response of the electrode, limit of detection, linear concentration range, ionic strength and activity coefficients, response time and the temperature) are the same like for amperometric electrodes in chapter 3. In this section, particular response characteristics of the biosensors such as pH range, life time (t_L) and Michael-Menten constant (K_M) will be discussed.

4.3.1 pH range

The pH value plays the main role in the biochemical reaction at the electrode-solution interface. The enzymes are working in a specific pH range. Accordingly, it is very important to optimize the pH to find the suitable enzyme catalytic activity of the substrate reaction.

4.3.2. Life time (t_L)

Life time can be defined as the storage or operation time necessary for the sensitivity, with the linear concentration range, to decrease by a factor of 10% (t_{L10}) or 50% (t_{L50}) [25]. The mode of assessment of lifetime should be specified by reference to initial sensitivity, upper limit of detection of the linear concentration range for the calibration curves, accuracy and reproducibility. The biosensor should have a prolonged lifetime; it should be easily replaceable if necessary and not be expensive if it has to be replaced. In

cases where the sensors to be partially inserted in the subcutaneous tissue, in a needle-like fashion, a lifetime of several days if not weeks, could be accepted [1]. It is obvious, a totally implantable device would require a much longer lifetime.

4.3.3. Michaelis-Menten constant (K_M)

Assume a scheme involves a single substrate, S, which combines with enzyme, E, to give the intermediate enzyme-substrate complex, ES. This complex then undergoes a reaction to produce the product, P, the overall scheme being summarized by:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant for complex decomposition into product. The Michaelis-Menten constant (K_M) can be represented by the following equation [11]:

$$K_M = \frac{k_{-1} + k_2}{k_1} \tag{4.1}$$

where k_1 and k_{-1} are the forward and backward rate constants for complex formation and k_2 is the rate constant for complex decomposition into product. Michaelis-Menten constant (K_M) represents the analyte concentration yielding a response equal to half of its maximum value for the current or potential measured for infinite analyte concentration [25]. When the apparent K_M is much larger than its value for soluble enzyme, it means either that a significant substrate diffusion barrier is present between the sample and reaction layer, or that the rate of reaction to the co-substrate with the enzyme is increased. As for enzyme solution kinetics, the apparent K_M is usually computed using Lineweaver-

Burk reciprocal plots, $\frac{1}{\Delta I}$ versus $\frac{1}{c}$, where $\Delta I = I - I_b$, I and I_b are the value of the current recorded for the analyte and the blank solutions respectively, and c is the concentration of the analyte.

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4.4. Selectivity of the amperometric biosensors and immunosensors.

Selectivity is related to the accuracy and precision of enantiomer measurements in presence of the interfering species. Enzymes are used in amperometric biosensors to catalyze only the reaction of one of the enantiomers. Accordingly, the selectivity of the biosensor is higher than for the other types of electrodes, e.g., EPME. The amperometric selectivity coefficient can be determined similar to the one for amperometric electrodes using the mixed and separate solution methods as described in chapter 3. Equations (3.7) and (3.8) may used for the calculation of the selectivity coefficient, $K_{i,j}^{amp}$, based on mixed and separate solutions method, respectively.

4.5. Chronoamperometry

Chronoamperometry is a potential step technique in which the potential of the working electrode is changed instantaneously from initial to final value and the current-time response recorded. A certain potential-time profile (Figure 4.4) is applied to the working electrode. E_1 should be a potential at which neither reduction of analyte species nor any electrode reactions occur. At zero time, the potential is instantaneously changed to a new value, E_2, at which the reduction of analyte species occurs at diffusion controlled rate. A typical current-time profile for chronoamperometry is shown in Figure 4.5. The form of

this response can be understood by considering the concentration profiles for analyte and its reduced species (products) during the measurement.

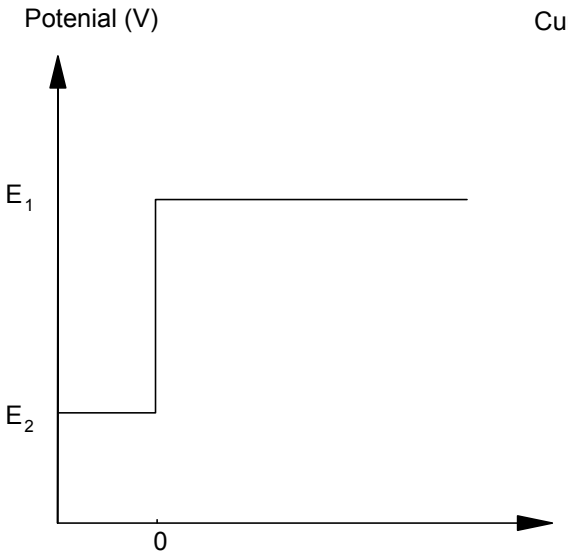

Figure 4.4 Potential-time profile

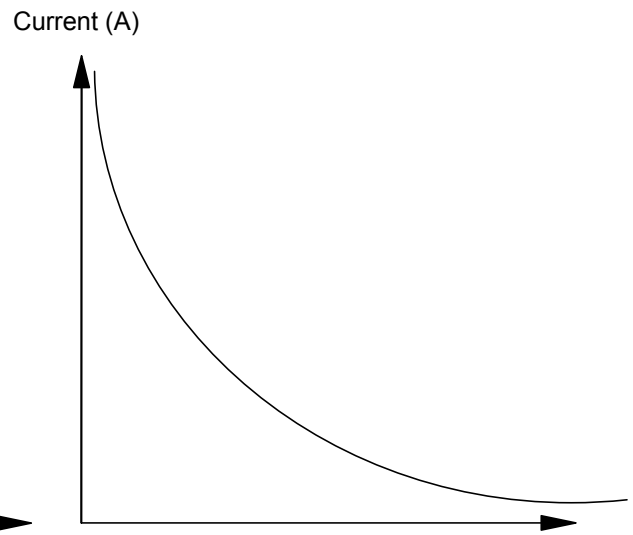

Figure 4.5 Current-time response

If the potential is stepped from a value significantly positive of the redox potential of the couple to one sufficiently negative, then a short time after the step the concentration of analyte species will have changed from its initial value only at points very close to the electrode surface. The concentration profile will be very steep, and over the subsequent time period, diffusion will cause the concentration profiles to relax towards their steady-state by extending into solution and becoming less steep. The current will decrease with time because current is a function of the concatenation of analyte species at the electrode surface.

4.6 Direct amperometry

The direct amperometric method is applied for analysis using amperometric biosensors, and has been discussed in Chapter 3.

4.7. References

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