CONSTRUCTION OF A CHOLINE OXIDASE BIOSENSOR

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ABSTRACT

CONSTRUCTION OF A CHOLINE OXIDASE BIOSENSOR

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Choline is indispensable for a number of fundamental processes in the body. Besides being the precursor of the acetylcholine, an important neurotransmitter, choline is found in the cell membrane structure combining with fatty acids, phosphate and glycerol. Its deficiency may result in nervous system disorders, fatty acid build up in the liver, along with increased cholesterol levels, high blood pressure and memory loss. Thus, rapid detection methods are required for the determination of choline in biological fluids.

In this study a choline oxidase biosensor was constructed for the determination of choline. During construction of the biosensor, glucose oxidase was used as a model enzyme, before choline oxidase used. The Teflon (PTFE) membrane of the oxygen electrode was grafted with 2-hydroxyethyl methacrylate (HEMA, 15%, v/v) in the presence of ferrous ammonium sulphate (FAS, 0.1%, w/v) by gamma irradiation and ethyleneglycol dimethacrylate (EGDMA, 0.15 %, v/v) was used as a crosslinker in a series of membranes. HEMA-grafted membranes were activated with epichlorohydrin or glutaraldehyde to maintain covalent immobilization of enzyme. The enzyme activity was measured with an oxygen electrode unit based on oxygen consumption upon substrate addition.

Membranes were characterized in terms of grafting conditions and mechanical properties. Membranes, gamma irradiated in a solution of HEMA (15%) and FAS (0.1%) for 24 h, were found to be suitable for use in the further studies. Mechanical test results revealed that HEMA grafting made Teflon membrane more flexible and the presence of EGDMA made the grafted membrane stiffer. During optimization stage, it was found that the immobilized enzyme amount was not sufficient to obtain enzyme activity. Thus, the membrane preparation stage was modified to obtain thinner membranes. The immobilized glucose oxidase and choline oxidase contents on thin HEMA grafted membranes were determined by Bradford and Lowry methods. The influence of EGDMA presence and the epichlorohydrin activation duration on enzyme activity studies revealed that the membrane should be prepared in the absence of EGDMA and 30 min activation duration is appropriate for epichlorohydrin coupling. The study on the influence of membrane activation procedures revealed that the membranes activated with glutaraldehyde had a higher

specific activity than the membranes activated with epichlorohydrin. Upon stretching membrane on the electrode directly rather than placing in the sample unit, the response of the enzyme immobilized sensor improved with high specific activity. The optimum choline oxidase concentration was found to be 2 mg/mL considering the effect of immobilization concentration on enzyme activity. With the choline oxidase biosensor, the linear working range was determined as 0.052-0.348 mM, with a 40 ± 5 μM minimum detection limit. The response of the sensor decreased linearly upon successive measurements.

Keywords: Choline, choline oxidase, Teflon membrane, grafting

ÖZ

BİR KOLİN OKSİDAZ BİYOSENSÖRÜNÜN TASARLANMASI

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Kolinin vücutta gerçekleşen pek çok olayda vazgeçilmez önemi vardır. Kolin önemli nörotransmitterlerden asetilkolinin öncüsü olmasının yanısıra yağ asitleri, fosfat ve gliserolle birleşerek hücre çeperinin yapısında yer alır. Kolin eksikliği sinir sistemi rahatsızlıklarına, yağ asitlerinin karaciğerde birikmesine, kolesterol düzeyinin yükselmesine, yüksek tansiyona ve hafıza kaybına neden olabilmektedir. Bu nedenle biyolojik sıvılardaki kolinin hızlı bir şekilde tayin edilmesi önem taşımaktadır.

Bu çalışmada, kolin tayini için bir kolin oksidaz biyosensörü hazırlanmıştır. Biyosensörün hazırlanmasında kolin oksidazdan önce glikoz oksidaz bir model enzim olarak kullanılmıştır. Oksijen elektrodunda kullanılmakta olan Teflon (politetrafloroetilen) membran ferröz amonyum sülfat (FAS, 0.1%, w/v) varlığında gama ışımasına maruz bırakılarak 2-hidroksietil metakrilat (HEMA, 15%, v/v) ile graft edilmiş ve bazı örneklerde çapraz bağlayıcı olarak etilen glycol dimetilakrilat (EGDMA, 0.15%, w/v) kullanılmıştır. HEMA graft edilen membranlar, enzimin kovalent bağlanmasını sağlamak için glutaraldehit veya epiklorohidrin ile aktive edilir. Enzim aktivitesi sübstrat eklenmesi ile gerçekleşen oksijen tüketimini temel alarak oksijen elektrodu ile ölçülür.

Membranların karakterizasyonu çalışmalarında graft edilme koşulları ve mekanik özellikleri incelenmiştir. Sonraki çalışmalarda HEMA (15%) ve FAS (0.1%) çözeltisi içerisinde 24 saat gama ışımasına maruz bırakılarak hazırlanan membranların kullanılmasının uygun olduğu saptanmıştır. Mekanik test sonuçları HEMA ile graft edilmesinin Teflon membranlara esneklik kazandırdığı ve EGDMA kullanılmasınında graft edilen membranları daha sertleştirdiğini göstermiştir. Membranların optimizasyonu aşamasında membranların yüzeyine bağlanan enzim miktarının aktivite ölçümleri için yeterli olmadığı belirlenmiştir. Bu nedenle daha ince bir membran elde etmek için membran yapım aşamasında değişiklikler yapılmıştır. Yüzeye bağlanmış olan glikoz oksidaz ve kolin oksidaz enzimlerinin miktarı Bradford ve Lowry yöntemleri ile tayin edilmiştir. EGDMA ve epiklorohidrin aktivasyonu süresinin enzim aktivitesine etkisi incelenmiş ve membranların EGDMA kullanılmadan ve 30 dakika epiklorohidrine maruz bırakılarak hazırlanması gerektiği saptanmıştır. Yüzeye glutaraldehid ile bağlanan

enzimlerin spesifik aktivitesinin epiklorohidrin ile bağlananlara göre daha yüksek olduğu saptanmıştır. Yapılan çalışmalarda elektrot üzerine doğrudan yerleştirilen membranların spesifik aktivitesinin oksijen elektrodu içine serbestçe bırakılan membranlardan daha yüksek olduğu belirlenmiştir. Yüzeye enzim bağlama aşamasında optimum kolin oksidaz konsantrasyonunun 2 mg/mL olduğu saptanmıştır. Tasarlanan kolin oksidaz biyosensörünün geniş bir doğrusal çalışma aralığı olduğu (0.052-0.348 mM) belirlenmiştir. Ölçüm sayısı arttıkça sensörün tepkisinde doğrusal bir düşüş olmuştur.

Anahtar kelimeler: kolin, kolin oksidaz, Teflon membran, graft etmek

Dedicated to my parents

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BOD	: Biochemical oxygen demand
BSA	: Bovine Serum Albumin
ChO	: Choline oxidase
EGDMA	: Ethylene glycol dimetylacrylate
Epi	: Epichlorohydrin
FAS	: Ferrous ammonium sulpfate
FET	: Field effect transistor
Glu	: Glutaraldehyde
GOD	: Glucose oxidase
HEMA	: 2- hydroxyethhly methacrylate
IR	: Infrared
PB	: Phosphate buffer
PBS	: Phosphate buffer saline
POD	: Peroxidase
PTFE	: Polytetrafluoroethylene (Teflon)
PVA-SbQ	: poly(vinylalcohol) bearing styrylpyridinium groups
TNBS	: 2,4,6-Trinitrobenzenesulfonic acid

CHAPTER 1

INTRODUCTION

1.1. Biosensors

Biosensors are compact analytical devices having a biologically derived recognition element either intimately connected to or integrated within a transducer to produce a signal, which is proportional to the concentration of a specific chemical or set of chemicals [Gerard et al., 2002] (Figure 1.1). This signal is then converted to concentration of analyte by the data processor.

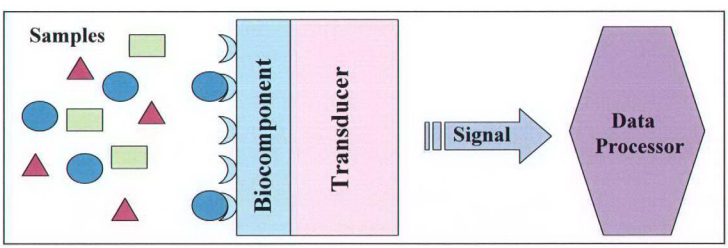


Figure 1.1. Schematic of a simple biosensor r

The biological recognition element is highly specific for the target analyte and does not detect other analytes. Biosensors are of growing importance due to their rapid and specific detection of analytes. Biosensors have applications in medicine, the pharmaceutical industry, the environment, the food and process industries, as well as in security and defense.

Biosensors have advantages such as reliability, sensitivity, accuracy, ease of handling, and low cost over the conventional detection methods [Liang et al., 2000].

Biosensors can be classified according to transducer type and biocomponent type (Figure 1.2).

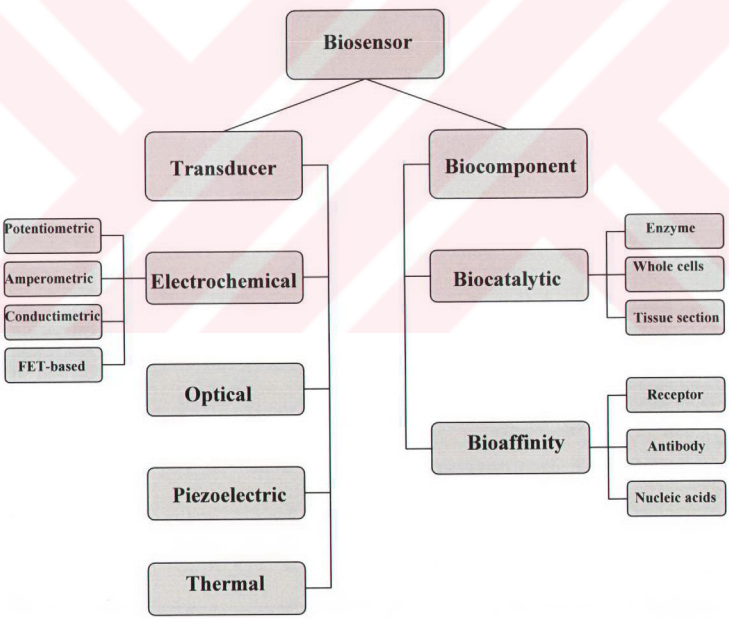


Figure 1.2. Scheme of biosensor types

1.1.1. Transducers

The transducer component of the biosensor converts the chemical or electrical response to a measurable signal. Transducers can be classified into various types according to transduction modes [Eggins, 1996].

1.1.1.1. Electrochemical Transducers

Biosensors based on electrochemical transducers are economic, present fast response and could be used to analyze a large number of samples with its automation possibility [Luong et al., 1988]. The electrochemical biosensors can be subdivided into three types [Eggins, 1996 and 2002] :

a) Potentiometric Biosensors

Potentiometric biosensors are based on the measurement of a potential between two electrodes at zero current. Potentiometric biosensors relate electrical potentials to the concentration of analyte by using an ion selective or a gas sensing electrode [Lewenstam et al., 1994; Domansky et al., 1998].

Urea was the first analyte to be determined with a pH linked potentiometric biosensor [Kuan and Guilbault, 1987]. Also, penicillin and glucose could be determined with pH-linked potentiometric biosensors, and creatinine, phenylalanine, adenosine and aspartame could be determined with ammonia-linked potentiometric biosensors.

b) Conductimetric Biosensors

This type of biosensors are based on the measurement of the change in the conductivity of the medium caused by changes in the number of ions, the charge on the ions, the dissociation of the ions or the mobility of the ions upon any reaction. Conductimetric biosensors are usually nonspecific and have a poor signal/noise ratio, and therefore have been little used [Mello and Kubota, 2002]. In the literature, there are examples of conductimetric biosensors, designed for the determination of urea [Senillou et al., 1999] and for the detection of foodborne pathogens [Muhammad-Tahir and Alocilja, 2003].

c) Amperometric Biosensors

A large variety of redox reactions could be used in the construction of amperometric biosensors and a main advantage is that they can be miniaturized.

An increasing potential is applied to the reaction cell until oxidation of the analyte occurs and the current rises sharply giving a peak current. The height of the peak current is directly proportional to the concentration of the analyte. If the appropriate oxidation potential is known, the potential can be set directly to measure the current. This mode is known as amperometric. This type of biosensors are fast, and are more sensitive, precise and accurate than the potentiometric ones [Mello and Kubota, 2002].

Some amperometric biosensors are based on the measurement of oxygen [Clark et al., 1962; Updike and Hicks, 1967; Vrbova et al., 1993; Peteu et al., 1996;

Doretti et al., 1996] or hydrogen peroxide [Marty et al., 1989; Doretti et al., 1999; Ricci et al., 2003].

i) Oxygen Electrodes

When a voltage of –0.7 V is applied between the platinum cathode and the silver anode, a current proportional to the oxygen concentration is produced. The following reactions occur (Equation 1.1):

$$\text{Ag anode} \quad 4\,Ag + 4\,Cl^- \longrightarrow 4\,AgCl + 4\,e^-$$

$$\text{Pt cathode} \quad O_2 + 4\,H^+ + 4\,e^- \longrightarrow 2\,H_2O \qquad \textbf{(1.1)}$$

The concentration of the analyte is then proportional to the decrease in current upon oxygen consumption by oxidase-substrate reaction. The passage of oxygen from bulk solution to the cathode is through a Teflon (PTFE) membrane. With its high dissociation energy C-F bond, PTFE shows a high thermal stability, a high chemical resistance, a low coefficient of friction and a low surface energy [Jun and Quinji, 1998]. PTFE membranes are used in oxygen electrodes due to their selective permeability only to oxygen, while they do not allow the passage of the analyte.

ii) Hydrogen Peroxide Electrodes

The production of hydrogen peroxide by oxidase-substrate is measured directly by applying a potential of +0.68 V to the platinum electrode, relative to the silver/silver chloride electrode and causing the following reactions to take place (Equation 1.2):

$$\text{Pt anode} \quad H_2O_2 \longrightarrow O_2 + 2\,H^+ + 2\,e^-$$

$$\text{Ag cathode} \quad 2\,AgCl + 2\,e^- \longrightarrow 2\,Ag + 2\,Cl^- \qquad \textbf{(1.2)}$$

d) Field Effect Transistors (FET)-based Biosensors

It is possible to use transistors as electrical signal amplifiers coupled to the type of electrical transducers reported above to achieve miniaturization. This method has mainly been used with potentiometric sensors. PHFET biosensors were formed by coupling the transistor to the pH electrode, [Korpan et al., 2000; Simonian et al., 2001; Dzyadevych et al., 2002]. ISFET bioensors [Munoz et al., 1997; Soldatkin et al., 2002] were formed by coupling transistors to ion selective electrodes. Although FET-based biosensors are temperature sensitive, they are important in biosensor development with their low cost, stable output and requirement of very small amounts of biological material. In addition, several analytes can be monitored simultaneously by FET-based biosensors.

1.1.1.2. Optical Biosensors

The optical biosensors are based on methods such as UV-Vis absorption, bio/chemiluminescence [Tsafack et al.,2000], fluorescence/phosphorescence [Vedrine et al., 2003], reflectance [Brynda et al., 2002], scattering and refractive index, caused by the interaction of biological recognition element with the target analyte. Optical sensors designed for oxygen, carbon dioxide and pH using acid-base indicators [Seitz, 1988], then these biosensors have been extended for the construction of fluorescent and luminescent optical fibers which contain an immobilized biocomponent at one end and excitation-detection components at the other end [Mello and Kubota, 2002]. Chemiluminescent substances as luminol in

combination with oxidoreductases are used in optical biosensors for direct measurement of hydrogen peroxide [Tsafack et al., 2000; Marquette and Blum, 2003]. Nowadays, optic biosensors are receiving considerable attention due to their being miniaturizable and low cost. However, the interference from ambient light and the high energy source requirement are the major drawbacks of these biosensors.

1.1.1.3. Piezoelectric Biosensors

These devices based on the generation of electric currents from a vibrating crystal. The mass of the material observed on the surface affects the frequency of vibration [Eggins, 1996]. The quartz crystal is used in piezoelectric biosensors [Tajima et al., 1998; Makower et al., 2003]. The surface acoustic wave devices are a related system and are applied more in immunosensors by immobilization of antigen or antibody on the surface a crystal [O'Sullivan et al., 1999]. The piezoelecric transducers have fast response and stable output.

1.1.1.4. Thermal Biosensors

Biosensors with thermal transducers are based on the monitoring of the energy exchanged, in the heat form, over time, upon chemical reactions catalyzed by enzymes or microorganisms. There are a number of examples of thermal transducers used in the food industry for the determination of important compounds, including ascorbic acid, glucose [Ramanathan et al., 2001], lactate, galactose, ethanol, sucrose, penicillin G, cephalosporin and oxalic acid.

1.1.2. Bioactive Components

The type of the biocomponent determines the degree of selectivity or the specificity of the biosensor. It may catalyze a reaction involving the substrate (biocatalytic sensors) or it may bind selectively to the substrate (bioaffinity biosensors).

1.1.2.1. Biocatalytic Sensors

Biocatalytic sensors can contain enzyme(s), whole cells (microorganisms), organelles and plant or animal tissue sections.

The most common and well developed recognition systems, enzymes, can be used with potentiometric, amperometric, optoelectric, calorimetric and piezoelectric transducers by the immobilization of the enzyme system onto a transducer. Especially, oxidases offer the advantages of being stable and in some situations do not require coenzymes or cofactors [Phadke, 1992; Davis et al., 1995]. Glucose biosensor is of special importance in monitoring the glucose level in the blood, and is based on the oxidation of glucose to gluconic acid by glucose oxidase, which is made the biocomponent of the glucose biosensor. In the literature, there are a number of examples of enzyme biosensors based on amperometric transduction mode [Marty et al., 1989; Vrbova et al., 1993; Peteu et al., 1996; Doretti et al., 1999; Curulli et al., 2001; Portaccio et al., 2002; Kök et al., 2002; Razola et al., 2003].

Biosensors that contain microorganisms, plant or animal tissue, as biocomponents, have the advantage of avoiding the tedious procedures of extraction

and purification, which are essential when the enzyme is used as an active component. The major drawback of using whole cells is the diffusion of the substrate and products through the cell wall resulting in a slow response as compared to enzyme-based sensors [Rainina et al., 1996]. The diffusional problem can be obviated by using permeabilized cells. Microorganism based biosensors are more tolerant of pH and temperature changes and have longer lifetimes, however, they have less selectivity and longer response times [Eggins, 1996]. Microbial biosensors are used in various analyses such as those for free fatty acids [Schmidt et al., 1996], for waste water [Jing and Mattaiasson, 2002], for p-nitrophenol [Mulchandani et al., 2002], for sucrose [Rotariu et al., 2002], and for the quality control of milk in food industry [Verma and Singh., 2003].

Mitochondria, as a biocatalytic component, improves sensor response and selectivity while the entire tissue lacks the necessary properties [Eggins, 1996].

1.1.2.2. Bioaffinity Sensors

The affinity-based biosensors may be chemoreceptors [Siegel et al., 2003], antibodies [Gizeli et al., 1997; Killard et al., 2000; Sarkar et al., 2002] or nucleic acids [Wang and Krull, 2002; Baeumner et al., 2003; Pedano and Rivas, 2003; Evtugyn et al., 2003]. The biosensors that contain bioaffinity components undergo selective interaction with a given ligand to form a stable complex.

The binding of a ligand to the receptor initiates an amplified physiological response (ei. ion channel opening, second messenger system, activation of enzymes).

They can be used with labelled materials, such as radioactively and fluorescent labelled ligands or the label could be on the receptor [Leech, 1994].

Antibody containing biosensors (immunosensors) are very selective even between different strains of the same material and bind more specifically and powerfully to the corresponding antigen than enzymes do to their substrates. However, they do not have any catalytic effects [Eggins, 1996]. The physicochemical change upon antigen-antibody binding does not generate an electrochemically detectable signal, therefore, either the antigen or the antibody should be labelled with a fluorescent compound, electrochemically active substrates, radionuclides or avidin-biotin complexes [Wilchek and Bayer, 1988; Fitzpatrick et al., 2000].

The development of DNA hybridization biosensors function in a similar fashion but have the advantage of bringing about sequence-specific information for clinical and environmental investigations. These biosensors are based on the immobilization of single-stranded DNA sequences and the generation of an electrical or optical signal upon its hybridization with the complementary strand [Ziegler and Göpel, 1998].

1.1.3. Immobilization Techniques

Biological transducers can be immobilized on a solid support or carrier through a variety of techniques, including adsorption, entrapment, crosslinking and covalent attachment. The immobilization matrix could function purely as a support or could be involved in the signal production and transduction mechanism. The selection of an

appropriate immobilization technique depends on the nature of the biological element, type of the transducer used, physicochemical properties of the analyte and operating conditions for the biosensor [Luong et al., 1988]. The most crucial criterion in enzyme immobilization is to maximize the advantages of enzyme catalysis.

1.1.3.1. Adsorption

Physical adsorption of the biocomponent based on van der Waals attractive forces is the oldest and simplest immobilization method. This method does not require chemical modification of the biological components and the regeneration of the matrix membrane or the support is possible. The disadvantage of this method is the risk of the loss of adsorbed biological components upon changes in pH, ionic strength or temperature during measurements. The adsorption technique was used to immobilize glucose oxidase on conducting polymers for glucose detection [Ramanathan et al., 1996] and to immobilize recombinant horseradish peroxidase directly on polycrystalline gold [Ferapontova et al., 2001].

1.1.3.2. Entrapment

The method involves the formation of a highly crosslinked network of a polymer in the presence of a biocomponents. In the entrapment method, significant barriers are created on the path of the solutes and these inhibit the diffusion of the analyte resulting in decreased reaction rate, and response. The entrapment technique was used in a large number of sensors including urea determination to immobilize

urease and glutamate dehydrogenase in polypyrrole/polyvinyl sulphonate films [Gambhir et al., 2001]. Acetylcholinesterase and choline oxidase were immobilized in photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinium (PVA-SbQ) by this method [Marty et al., 1989], and these enzymes were immobilized on the working electrode surface using gel entrapment [Lenigk et al., 2000].

1.1.3.3. Crosslinking

This method uses bifunctional agents or high energy radiation such as gamma rays to bind the biocomponent to solid support or within itself and is also used to stabilize adsorbed enzymes [Eggins, 1996]. Some of the most commonly used bifuncional reagents include glutaraldehyde, carbodiimide, trichlorotriazine, 3-metoxidiphenyl methane-4,4' diisocyanate [Barlett et al., 1997; Zhang et al., 2000]. It is relatively easy to achieve but the indiscriminative bonding could lead to low activity yields due to covalent interaction at the active site or by restrictions imposed by the crosslinks. Crosslinking with glutaraldehyde using BSA was used to immobilize glucose oxidase and mutarotase on polycarbonate track-etched membrane [Mutlu et al., 1997] and to immobilize alcohol dehydrogenase on graphite powder [Santos et al., 2003]. In addition, crosslinking with glutaraldehyde was used to immobilize choline oxidase, butyrylcholinesterase, acetylcholinesterase onto the nonconducting polymer modified electrodes [Curulli et al., 2001].

1.1.3.4. Covalent Bonding

Covalent bonding is used to achieve immobilization of biological components to a carrier membrane or directly onto the surface of the transducer. Some functional groups which are not essential for the catalytic activity of an enzyme can be used to covalently bond to the transducer or the membrane. Functional groups that could be employed include the amino groups from lysine, carboxyl groups from aspartate and glutamate, sulphydryl groups from cysteine and phenolic hydroxyl groups from tyrosine [Mello and Kubota, 2002]. The advantage of the technique is that the enzyme will not be released during use because the binding is irreversible. There is very low diffusional resistance, but the matrix can not be regenerated to employ fresh bioactive component. Glutaraldehyde and cyclohexyl isocyanide [Vrbova et al., 1993], epichlorohydrin [Kök et al., 2002] and poly(o-amino benzoic acid) [Ramanathan et al., 2000] are some of the coupling agents used in covalent bonding. The covalent bonding technique was used to immobilize glucose in a poly(vinyl alcohol) cryogel membrane [Doretti et al., 1998] and onto a carbon sol-gel silicate composite surface [Yang et al., 2003].

Grafting technique assumed a relevant role in the concept of immobilization. By grafting upon chemical treatments or gamma irradiation, the membranes that are unable to bind enzymes become good supports to immobilize the biocomponents. Enzymes can be bound to the grafted support through covalent bonding with coupling agents. The membrane prepared by thermally-induced grafting of acrylic acid with the ozone-preactivated poly(vinyllidene fluoride) [Ying et al., 2002] and the nylon membrane chemically grafted with butyl methacrylate or

glycidyl methacrylate [Portaccio et al., 2002] were used in glucose oxidase immobilization via covalent bonding. In addition, Teflon membrane grafted with acrylic acid followed by 2-hydroxyethyl methacrylate by gamma irradiation was used to immobilize β-galactosidase to obtain a catalytic membrane [Mohy Eldin et al., 1998].

1.1.3.5. Modified Transducers

The surface of the transducer is modified through incorporation of the biocatalyst within the bulk of a carbon composite matrix. The modified transducers offer several advantages such as being proximity of the biocatalytic and sensing sites, possibility to incorporate other components (cofactors), an easy renewing (refreshing) of the surface. Since embedding in a matrix is required, biosensors with these features can be achieved by using the carbon paste electrodes [Mullor et al., 1996; Boujtita and Murr, 2000; Razola et al., 2003] or screen-printed biosensors [Capanessi et al., 2000; Ricci et al., 2003].

1.1.4. Performance Factors

Performance requirements should be considered to develop a new biosensor. Among the most important requirements are high selectivity, high sensitivity, broad linear working range, accuracy, response time, recovery time, repeated use or working life time.

1.1.4.1. Selectivity

The most important characteristic of the sensors is the ability to discriminate between different substances. Selectivity is principally a function of the biocomponents. This property is essential to avoid interference by solutes with very similar chemical structures to the analyte.

1.1.4.2. Sensitivity (Linear Working) Range

It is vital to know the concentration range of substrate over which the response is linear so that a calibration curve could be employed. The lower limit of this range is the detection limit a value which defines the sensitivity of the sensor. The detection limit generally needs to be better than 10^{-5} M.

1.1.4.3. Accuracy

The sensor must also be capable of measurements with an accuracy close to the expected value. The systemic errors must be below certain limits. Using biological selective elements, systemic errors could be observed, as one sample batch can differ from another in terms of purity, age, activity. Therefore, sufficient controls and standards must be used to enable repeatable results of sufficient accuracy to be obtained over an extended period of time.

1.1.4.4. Response Time

The time required to allow the system to come to equilibrium and yield a result. If the time is too long it can materially affect the reliability, and the usefulness of the method for repetitive routine analyses. In biosensors, the response times can vary from a few seconds to a few minutes (upto 5 min).

1.1.4.5. Recovery Time

This is the time for the sensor to be ready to analyse the next sample. The resulting recovery time may be immediate or it may be that after one measurement the sensor system has to rest to resume its base equilibrium before it can be used with the next sample. For an effective sensor the recovery time must not be more than few minutes.

1.1.4.6. Working Lifetime

Lifetime of the biosensor is determined by the stability of the selective bioactive material. One of the major drawbacks with biosensors is that the biological component usually has a fairly limited lifetime before it needs replacing. For biological materials lifetime can be as short as a few days, although it is often several months or more.

1.1.5. Application Areas of Biosensors

1.1.5.1. Food and Process Industry

Biosensors are used in the food industry to analyze the carbohydrates (fructose [Boujtita and Murr, 2000; Paredes et al., 1997], lactose [Amarita et al., 1997; Sekine and Hall, 1998], starch [Marconi et al., 1998]), and alcohols [Katrlik et al., 1998; Tobalina et al., 1999], carboxylic acids [Maines et al., 2000], amino acids [Curulli et al., 1998; Sarkar et al., 1999], biogenic amines, inorganic and organic compounds, contaminants (antioxidants, pesticides [Dong and Tsai, 2001], hormones, etc.) and additives. In the process industry, monitoring a range of direct reactants and products could result in improved product quality, increased product yields and control on tolerance of variations in quality of raw material [Badea et al., 2003], and therefore biosensors have a role to play here, too.

1.1.5.2. Environmental Monitoring

There is an enormous range of potential analytes in air, water, soils, and other environmental media. Such analytes include biochemical oxygen demand (BOD) [Yang et al., 1997], acidity, salinity, nitrate [Lorenzen et al., 1998], phosphate, calcium and fluoride concentrations. Pesticides [Kök et al., 2002], fertilizers and both industrial and domestic wastes generally require extensive analyses with expensive equipment.

1.1.5.3. Health Care

Health care is a major area of application of biosensors. Measurements of blood, gases, ions and metabolites are needed to follow a patient's metabolic state. Biosensors are constructed for the determination glucose [Shichiri et al., 1986; Marquette and Blum, 2003], lactate [Mascini et al., 1985], oxalate, urea [Hirose et al., 1983], cholestrol, creatinine [Ho et al., 1999], bilirubin, acetylcholine [Larsson et al., 1998] and choline [Garguilo and Michael, 1995; Burmeister et al., 2003].

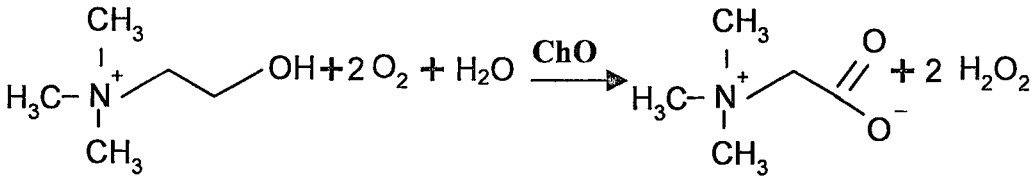
1.2. Choline

Choline is indispensable for a number of fundamental processes in the body. It plays an important role in the biochemistry of most living cells which use choline for the production of phosphotidylcholine, an important constituent of lipid membranes [Garguilo and Michael, 1995]. Choline from food or supplements is absorbed into the blood stream. From the blood stream, it passes selectively across the blood-brain barrier into the central nervous system where it is incorporated as phophotidylcholine into the membranes of brain cells. Phosphatidylcholine, a basic component of lecithin, helps the emulsification of fats and cholestrol in the body by helping form smaller globules in the blood and also by helping the transport of fats through the smaller vasculature and in and out of the cells. Choline is also the precursor and metabolite of the important neurotransmitter acetylcholine in both the peripheral and central nervous system of mammals [Garguilo and Michael, 1995]. Choline as being the precursor of the acetylcholine, involved in the transmission of brain impulses

between nerves, muscles and organs. Many symptoms of Alzheimer's disease are believed to result from decreased levels of acetylcholine in the brain. Choline or lecithin are probably most helpful in the early stages of short term memory loss [Rosenberg and Davis, 1982; Levy et al., 1982; Sitaram et al., 1978]. The determination of choline in the biological materials (human bile, serum, amniotic fluid, brain extracts and pharmaceutical products) is therefore very important. Time consuming and expensive chromatographic methods can be used for its determination, but the improved biosensor technology offer simple, rapid and reliable detection alternatives [Gülce et al., 2003].

1.3. Choline Oxidase

Choline oxidase is a cytosolic enzme that catalyzes the oxidation of choline to betaine and hydrogen peroxide in the presence of oxygen (Equation 1.3). The enzyme, found in *Arthrobacter sp.* and *Alcaligenes sp.* bacteria, is a monomer of 66 kDa and contains covalently bound FAD.



Choline Betaine **(1.3)**

1.4. Choline Biosensors

There are a few examples in the literature about the construction of a choline biosensor through use of choline oxidase immobilization. In most of the other cases, choline oxidase was immobilized along with other enzymes in the construction of a biosensor.

An amperometric choline biosensor was constructed by Doretti et al. [1994]. To carry out the measurements using Clark-type oxygen or hydrogen peroxide electrodes, choline oxidase was immobilized on poly(2-hydroxyethylmethacrylate) membranes which were obtained by gamma radiation induced polymerization at low temperature. The reported linear range for choline was 10-200 $\mu mol.l^{-1}$ with oxygen probe and 5-250 $\mu mol.l^{-1}$ for hydrogen peroxide based probe. When the sensor was stored either in buffer or in dry state at 4°C, the electrode response was maintained over a period of three months.

Peteu et al. [1996] constructed a microbiosensor for the determination of choline (also for glucose, and galactose). In general, the appropriate enzyme was immobilized on the Clark-type electrode tip in a polyacrylamide matrix and then coated with a polyurethane membrane. However, for choline determination the electrode was not coated with polyurethane. Even then, choline microbiosensors were much less sensitive, remained operational for only 2 weeks and had a more limited linear range (approximately 1 mM) than the glucose and galactose sensors.

Leca et al. [1995] designed a choline electrode based on direct coating of the transducer tip. The photocrosslinkable polymer, PVA-SbQ was mixed with choline oxidase and a minute amount of this solution was directly deposited on a platinum

electrode, dried, and then photopolymerized. It was reported that the sensitivity was high (close to 22 mA 1 mol^{-1}) the response time was as short as 30 s as a result of the direct coating performed.

A choline biosensor was constructed by Gülce et al. [2003] by attaching choline oxidase onto a polvinylferrocenium-coated platinum surface of the electrode. The response measurements were based on the change in current upon hydrogen peroxide oxidation. The minimum detectable substrate concentration was $4.0.10^{-6}$ M and the upper limit of the linear range was 1.2 mM choline concentration.

Karube et al. [1979] constructed a biosensor to determine phosphotidyl choline in serum. Amperometric measurements were carried out with phospholipase D and choline oxidase co-immobilized on different types of membrane (octyl-Sepharose, porous glass, polystyrene and collagen). The highest specific activity (300 $mU.g^{-1}$ carrier) of the immobilized enzymes was observed for octyl-Sepharose membrane due to the hydrohobic nature of the membrane.

Vrbova et al. [1993] designed a choline biosensor for the determination of phospholipase D activity in rape seeds. Choline oxidase and catalase were co-immobilized on a nylon net via gluataraldehyde and cyclohexyl isocyanide and then the enzyme immobilized membrane was fixed on a Clark-type oxygen electrode. The constructed choline biosensor has a linear response between $3.34.10^{-3}$–$1.67.10^{-1}$ mM and the biosensor was stable for 600 analyses over 18 months without any detectable decrease in activity.

Razola et al. [2003] constructed a choline biosensor based on determination of hydrogen peroxide to detect choline released from phosphatidylcholine by phosphalipase D in isolated rat salivary gland cells. Choline oxidase was retained on

a horseradish peroxidase immobilized solid carbon paste electrode. Before application of the biosensor, the choline oxidase measurements were performed in 0.1 M phosphate buffer (pH 7.4) and it was observed that the response was linear between $5.0.10^{-7}$–$7.0.10^{-5}$ M, with a detection limit of $1.0.10^{-7}$ M.

Garguilo and Michael [1995] used a choline microsensor for monitoring choline in the extracellular fluid of the brain. The microsensor was constructed by immobilizing horseradish peroxidase and choline oxidase within a cross-linkable redox polymer deposited onto carbon fiber electrodes. The microsensor could detect 10 μM choline at 37°C.

Lenigk et al. [2000] co-immobilized choline oxidase and acetylcholinesterase on the working electrode surface of a three electrode system (Ag/AgCl reference, platinum counter and platinum) using gel entrapment in poly(carbamoyl)sulfonate hydrogel. The sensor was designed for the investigation and comparison of anti-Alzheimer medications based on the inhibition of acetylcholinesterase.

Doretti et al. [1999] designed a two-enzyme biosensor by covalent co-immobilization of choline oxidase and butyrylcholinesterase on methacrylate-vinylene carbonate copolymer. The substrate determination was carried out with a hydrogen peroxide electrode. The constructed butyrylthiocholine sensor has low detection limit (2 μM) and a linear range (5-100 μM).

Marty et al. [1989] constructed a choline and acetylcholine sensor using the same PVA-SbQ photocrosslinkable polymer to entrap choline oxidase and/or acetylcholinesterase. The choline sensor gave a linear calibration for the range $2.5.10^{-6}$–$1.5.10^{-4}$ M choline, and acetylcholine sensor gave a linear range $2.0.10^{-5}$–$7.5.10^{-4}$ M acetylcholine.

Curulli et al. [2001] reported the synthesis of different nonconducting electrochemical polymers on the surface of a platinum electrode to obtain sensitive amperometric biosensors for choline, butyrylcholine, and acetylcholine. Co-immobilization of the enzymes (choline oxidase and acetylcholinesterase or butyrylcholinesterase) was achieved with bovine serum albumin and glutaraldehyde. The optimized biosensors (butyrylcholine and acetylcholine) were used for rapid quantitative assay of paraoxon which inhibits cholinesterase and the limit of detection was found as 0.1 ppb.

Kök et al. [2002] constructed an acetylcholinesterase-choline oxidase biosensor for pesticide aldicarb determination. The enzymes were co-immobilized on poly(2-hydroxyethyl methacrylate) membranes via entrapment or hybrid immobilization with epichlorohydrin and Cibacron blue. It was reported that the hybrid immobilized enzymes responded to aldicarb presence more quickly than the entrapped ones. The lower aldicarb concentration could be detected by hybrid immobilized enzymes (12 ppb aldicarb) compared to entrapped enzymes (23 ppb aldicarb).

Besides, choline biosensors constructions using chemiluminescent technique were reported in the literature. Tsafack et al. [2000] designed a chemiluminescent choline biosensor based on the non-covalent immobilization of choline oxidase histidine modified horseradish peroxidase on derivatized Sepharose beads subsequently entrapped in PVA-SbQ photopolymer. The choline oxidase activity studies were based on hydrogen peroxide detection and the working range was found as 0.35 pmol-10 nmol, with 0.5 pmol detection limit. Later, a biochip for the concominant detection of glucose, lactate and choline was developed by Marquette

and Blum [2003] based on luminol/hydrogen peroxide electrochemiluminescence and enabled the detection of choline in the range 2 μM-0.2 mM and the ranges found for glucose and lactose were 20 μM-2 mM and 2 μM-0.2 mM, respectively.

1.5. Aim

The goal of this study was to construct an amperometric choline oxidase biosensor to determine choline in biological fluids with minimal diffusional restriction. Choline oxidase was covalently immobilized onto HEMA-grafted Teflon membranes. HEMA grafting was achieved by gamma irradiation of HEMA and Teflon membrane. Immobilized choline oxidase studies were then carried out in an oxygen electrode unit and was based on oxygen depletion in the reaction medium. Teflon membrane was the one used in the oxygen electrode to maintain selective oxygen permeability, while not allowing the passage of other substances. The primary reason for using Teflon as a site for immobilizing choline oxidase directly on it was to improve diffusional restrictions which is the main problem in biosensor construction. By stretching the choline oxidase immobilized membrane directly onto the electrode instead of the original untreated Teflon membrane of the oxygen electrode, the oxidation of choline would occur nearer the sensing transducer and the response level would be increased, because of minimized diffusional limitations. The effect of activation procedure, the location of the catalytic membrane and the immobilization enzyme concentration on immobilized enzyme activity were carried out in the oxygen electrode. Optimization for the appropriate working conditions with high enzyme activity (glutaraldehyde coupling, stretching electrode on the

electrode, 2 mg/mL choline oxidase immobilization concentration), the performance of the choline oxidase biosensor was studied in terms of reusability, linear working range and sensitivity.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Teflon (PTFE) membrane (12.5 μm thick, 25 mm wide) was purchased from Hansatech Instruments Ltd. (Helmut Saur, Germany). Infrared studies (section 3.1.1) revealed that the Teflon membrane was modified and did not behave as a typical Teflon.

2-Hydroxyethyl methacrylate (HEMA) was from Sigma Chemical Co. (USA), was distilled under vacuum and stored at +4°C until use. The crosslinker, ethylene glycol dimethacrylate (EGDMA), was from Sigma (USA) and purified by extraction with a NaCl-NaOH solution (20% NaCl, 5% NaOH). After extraction, upper phase was taken to be washed with distilled water and the water was removed by $CaCl_2$ before use. Ferrous ammonium sulphate (FAS) ($FeSO_4(NH_4)_2SO_4.6H_2O$) was purchased from British Drug Houses Ltd. (England) and was of Analar purity.

The coupling agents, epichlorohydrin (1-chloro-2,3-epoxypropane) and glutaraldehyde (50% aqueous solution) were purchased from Sigma (USA).

The buffers were prepared using potasssium dihydrogen phosphate and boric acid which were obtained from Merck (Germany).

Fluorescamine, Coomasie brillant blue G, picryl sulfonic acid (TNBS) and o-dianisidine were purchased from Sigma Chemical Co. (USA). Folin-Ciocalteau's Phenol reagent was obtained from Merck (Germany).

The enzymes; choline oxidase (E.C. 1.1.3.17, from *Alcaligenes* species), glucose oxidase (E.C. 1.1.3.4, from *Aspergilus niger*, Type VII-S) and peroxidase (E.C. 1.11.1.7, from *Horseradish*, Type II) were purchased from Sigma Chemical Co. (USA). The substrates; choline (Ch) (chloride salt) and β-D-glucose were obtained from Sigma Chemical Co. (USA).

2.2. Methods

2.2.1. Preparation of HEMA-Grafted Teflon Membranes

A Teflon (PTFE) membrane of the oxygen electrode (S4 membrane, Helmut Saur, Germany) was used as a solid support to perform the grafting process on. The role of hydrophobic Teflon membrane was also to maintain oxygen transport towards an oxygen electrode unit. Preparation of HEMA-grafted Teflon membranes was carried out according to the method adapted from Mohy Eldin et al. [1999].

Membrane preparation process was optimized before the thin coated membranes were obtained. In the optimization stage, membranes were prepared by placing Teflon membranes (2.5 x 2.5 cm^2) in a solution of 2-hydroxyethylmethacrylate (HEMA) (15%, v/v) and ferrous ammonium sulphate (FAS) (0.1%, w/v) in test tubes and then polymerizing HEMA on the Teflon membranes by initiation with gamma irradiation (irradiation source Co^{60}, the average dose rate at the core of the radiation chamber 0.15 kGy/h) (Gamma Cell 220, Canada). The membranes with HEMA graft were washed with distilled water while mildly scrubbing to remove the excess polymer from the Teflon membranes. Membranes were then stored in distilled water at +4° C.

In the later stages of the study, to obtain thinner membranes, membranes were exposed to gamma irradiation in a system which consisted of two glass plates separated by a spacer (Figure 2.1). Teflon membrane was placed against one of the glass plates and a Teflon plate was placed against the other. Two glasses were then brought together with the rubber spacer in between and then the edges of the system

were sealed with silicone. Then HEMA (15%, v/v) and FAS (0.1%, w/v) solution was poured in the space between the glasses. The system was irradiated by gamma rays as before. In the preparation of a series of samples EGDMA (0.15%, v/v) was added to serve used as a crosslinker.

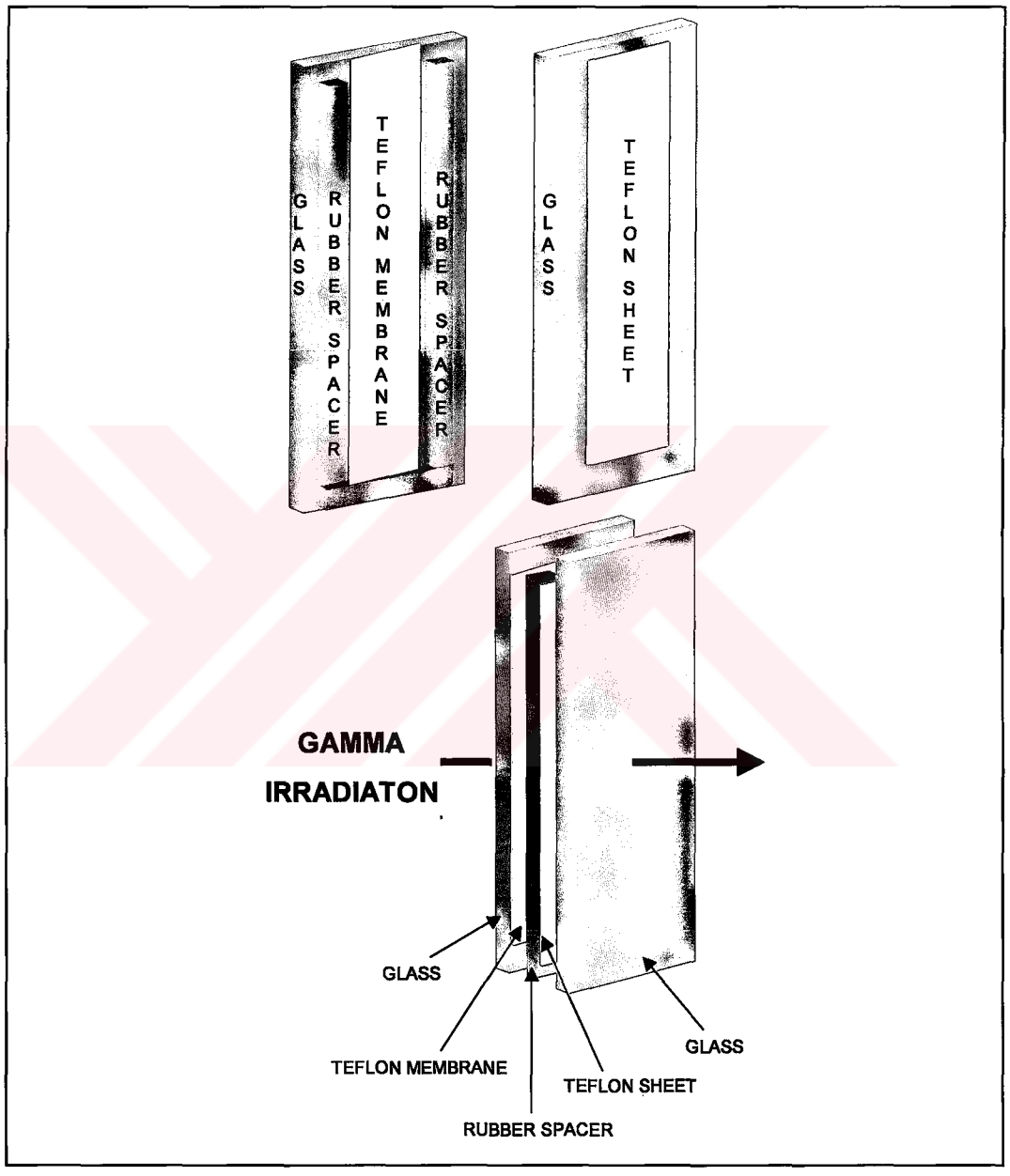


Figure 2.1. Membrane preparation in a vertical system

To prevent mechanical damage to the product, membranes were rinsed in a beaker with continuous stirring without scrubbing and then stored in distilled water at +4°C until use.

The scheme for grafting HEMA on Teflon membrane is presented in Figure 2.2. Free radicals were formed on Teflon membrane, on HEMA monomers and the hydroxyl radicals from radiolysis of water upon gamma irradiation (Figure 2.2a-c). Radicals of Teflon, the hydroxyl and the HEMA radicals attack the C=C bond of HEMA and try to form HEMA chains (pHEMA). Thus, chains growing from the Teflon surface and also free in the medium are formed. FAS was used to prevent formation of HEMA polymerizations initiated by the hydroxyl radicals. Fe^{+2} ions available in FAS react with free hydroxyl radicals obtained from radiolysis of water and convert them into hydroxyl ions, which are unable to initiate free radical reactions (Equation 2.1).

$$OH^{\cdot} + Fe^{+2} \longrightarrow Fe^{+3} + OH^{-} \qquad (2.1)$$

Thus, in the presence of FAS more HEMA monomer units are expected to be available to react with the free radicals formed on the Teflon membrane upon gamma irradiation and HEMA grafting on Teflon membrane was achieved (Figure 2.2d). HEMA polymers could also form in the medium initiated by HEMA radicals and have no interaction with Teflon. However, the probability of HEMA grafting on Teflon should be higher than the formation of HEMA homopolymers in the medium due to that the large surface area of Teflon exposed more to the gamma rays compared to the HEMA monomers in the medium.

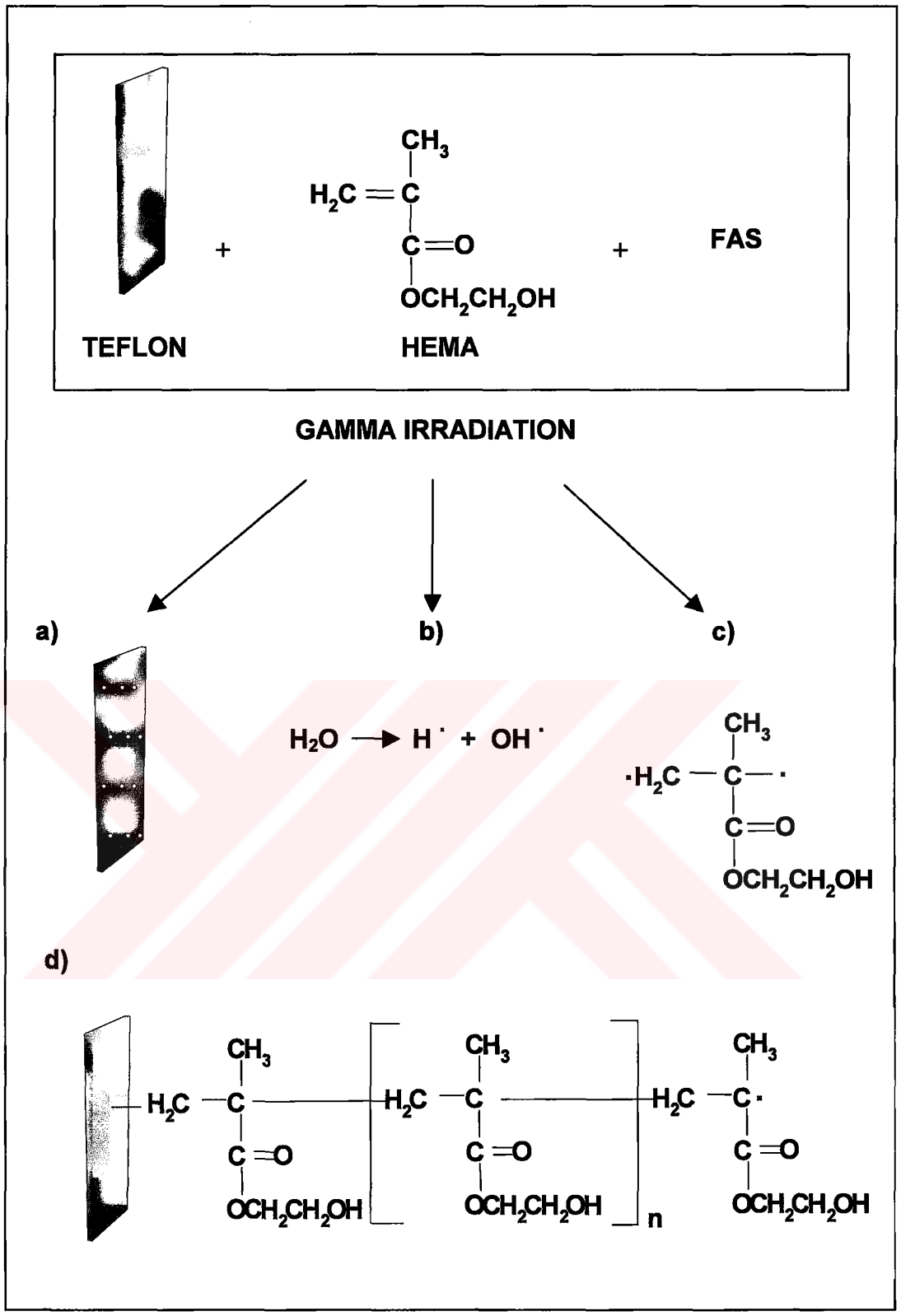


Figure 2.2. The grafting of HEMA on Teflon membranes by gamma irradiation.

2.2.2. Characterization of Membranes

2.2.2.1. Influence of Gamma Irradiation Duration and FAS Concentration

Influence of gamma irradiation duration and FAS concentration on HEMA grafting were studied during the optimization stage and the optimal conditions determined here were used in the following studies.

To investigate the effect of gamma irradiation duration, Teflon membranes in HEMA (15%, v/v) and FAS (0.1%, w/v) were exposed to gamma irradiation for various lengths of time (30 min to 24 h) while keeping the other parameters constant. As a control to check the effect of gamma radiation on Teflon membrane in the absence of the monomer, one Teflon membrane was exposed to gamma for 24 h in distilled water.

The effect of FAS concentration on HEMA grafting was investigated by irradiating the membranes for 3 h in media with different FAS concentrations (0.1-0.8%, w/v). Membranes were dried completely under vacuum for about 2 days and then studied with an Infrared Spectrophotometer (PU9716, Philips, Scientific and Analytical Equipments, UK). The Teflon used through this thesis was surface treated when purchased and this Teflon was used as a reference to detect the presence of HEMA on membrane surface.

2.2.2.2. Mechanical Properties of Membrane

Tensile testing was carried out on HEMA-grafted membranes prepared in the vertical system with and without any crosslinker and also on the membranes treated further (activated with epichlorohydrin or glutaraldehyde). The membranes (1 x 4 cm^2) were tested in a wet state with MTS Mechanical Tester (MTLQ, Stable Micro Systems, England) at room temperature with a 0.2 mm/s test speed. Tensile strength, Young's Modulus and toughness were determined.

2.2.2.3. Examination of Membrane Topography by Scanning Electron Microscopy (SEM)

The thin membranes prepared under optimized conditions in the vertical system were vacuum dried until complete dryness, fixed on stubs and coated with gold under vacuum before examining with a scanning electron microscope (SEM) (JSM-6400, Japan).

2.2.3. Immobilization of Protein and Enzyme on Membrane through Covalent Bonding

2.2.3.1. Activation of HEMA-Grafted Teflon Membranes

Methods of activation of membranes with epichlorohydrin and glutaraldehyde were adapted from Kök [2001].

a) Activation with Epichlorohydrin

Epichlorohydrin, a short chain epoxide compound was used to activate the hydroxyl group of HEMA grafted on the Teflon membrane to introduce functional epoxy groups for covalent immobilization of enzymes and proteins. The activation scheme is presented in Figure 2.3. The HEMA-grafted Teflon membranes (2.5 x 2.5 cm^2 or 1 x 2.5 cm^2) were placed in a solution containing distilled water (1 mL), NaOH (4.3 mL, 2.0 M) and epichlorohydrin (0.1 mL) and were maintained at 40°C for 2 h while continuously agitating. The membranes were washed with 30% acetone and then with distilled water to remove unreacted epichlorohydrin. After the optimization stage, the 30% acetone wash step was omitted and the thin membranes were washed with excess distilled water instead. The epichlorohydrin activation duration was decreased to 30 min from 2 h as a result of the enzyme activity studies (section 3.6.2.1).

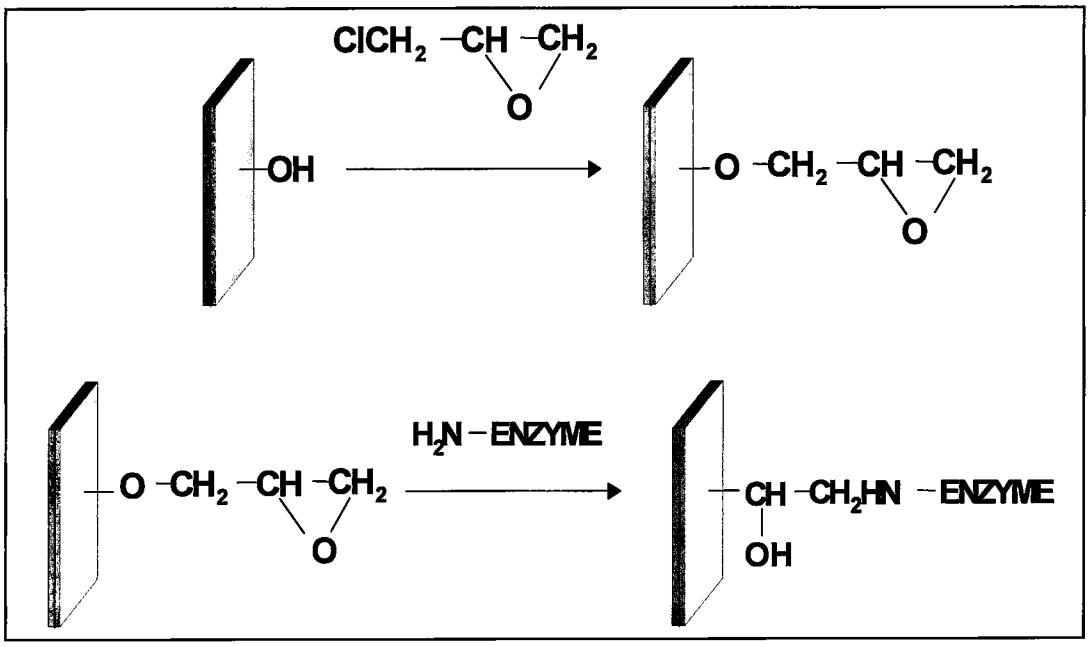


Figure 2.3. Activation of HEMA-grafted Teflon membrane by epichlorohydrin and covalent immobilization of protein/enzyme

b) Activation with Aminohexyl Glutaraldehyde

Scheme for the activation of HEMA-grafted Teflon membrane with aminohexyl glutaraldehyde is presented in Figure 2.4. HEMA-grafted membranes were placed in polyethylene bottles containing $NaIO_4$ (4 mL, 0.2 M) and gently shaken for 2 h at room temperature to achieve the aldehyde formation (Figure 2.4a). The membrane was washed with distilled water and placed in a hexadiamine solution (4 mL, 2.0 M, pH 5.0) and gently shaken for 6 h at room temperature (Figure 2.4b). After washing with PB (50 mL, 0.1 M, pH 8.5) the membrane was placed in a glutaraldehyde solution (4 mL, 2.5%) and gently shaken for 10 min at room temperature (Figure 2.4c). Unbound glutaraldehyde was washed-off with PB

(100 mL, 0.1 M, pH 8.5). To eliminate the interference of glutaraldehyde observed during protein determination studies (section 3.4) the amount of PB (0.1 M, pH 8.5) was increased to 250 mL during the wash step after glutaraldehyde coupling.

2.2.3.2. Immobilization of Protein or Enzyme

BSA and glucose oxidase were immobilized onto activated membranes as the model protein and the target enzyme before choline oxidase was used.

During the optimization stage HEMA grafted membranes were activated with one of the coupling agents indicated in section 2.2.3.1, membrane were placed in BSA solution (2 mg/mL) or glucose oxidase solution (2 mg/mL) both prepared in borate buffer (0.2 M, pH 9.0) and gently shaken overnight at 4°C. Free protein/enzyme was washed off with PB (0.1 M, pH 8.5).

The thin HEMA grafted membranes obtained in vertical system, were also activated with epichlorohydrin or glutaraldehyde and using the above procedure. The activated membranes were placed in glucose oxidase (2 mg/mL) or choline oxidase (2 mg/mL) solution prepared in borate buffer (0.2 M, pH 9.0) and gently shaken overnight at room temperature. The membranes were then washed with PBS (0.1 M, 0.9% NaCl, pH 7.0) instead of PB (0.1 M, pH 8.5) to remove enzyme not immobilized with covalent bonding.

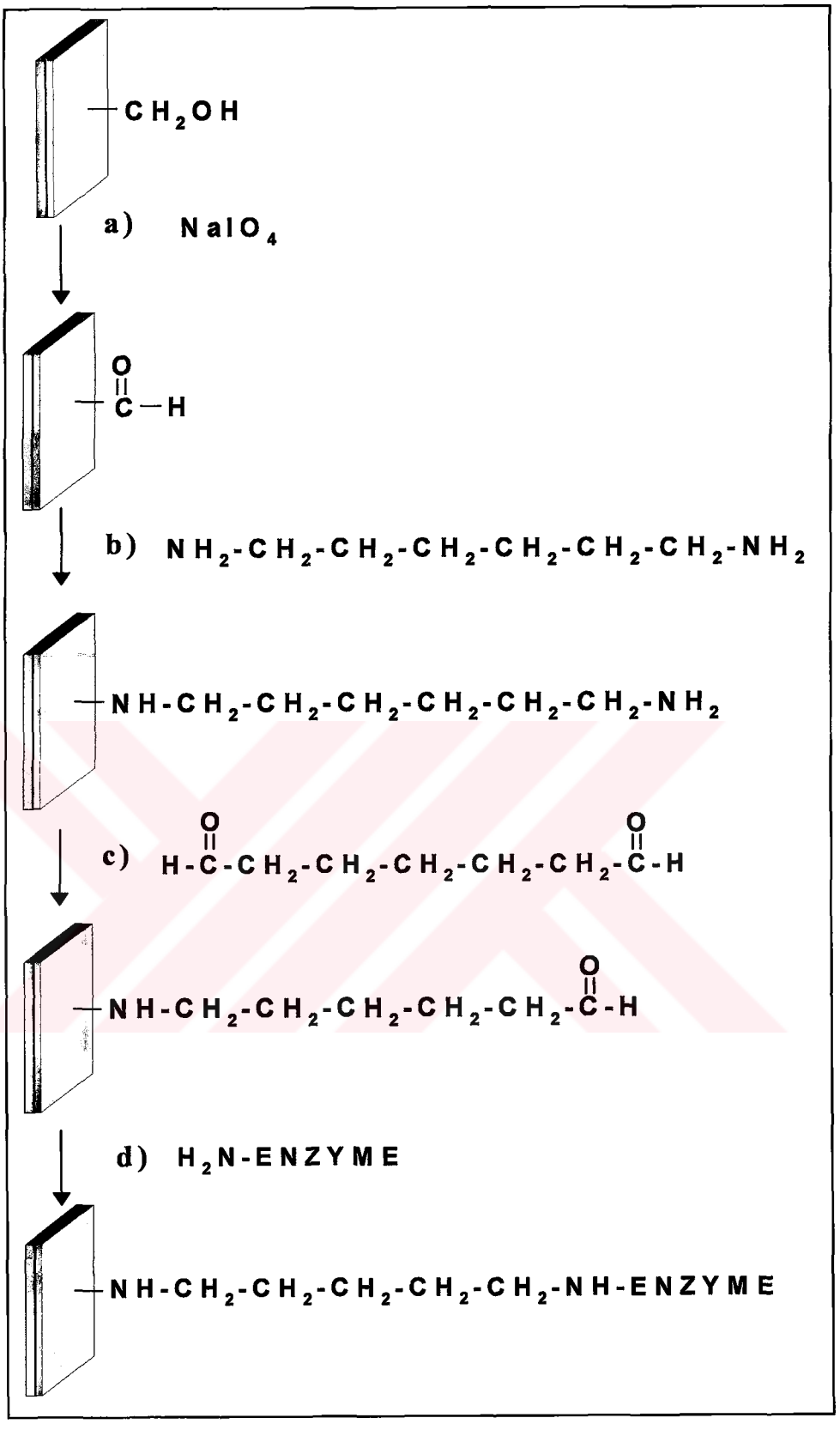


Figure 2.4. Activation of HEMA-grafted Teflon membrane by glutaraldehyde (a-c) and covalent immobilization of protein/enzyme (d).

2.2.4. Qualitative Detection of Immobilized BSA

At the optimization stage, immobilized BSA on the membranes were detected qualitatively using Fluorescamine and TNBS staining methods.

a) Fluorescamine Staining

Fluorescamine reacts with the primary amino groups of terminal amino acids to form highly fluorescent moieties [Udenfriend et al., 1972].

After protein immobilization, membrane was washed with borate buffer (0.2 M, pH 9). Following the wash step, membrane was placed onto a glass slide in a petri plate, air-dried for 15 min and then one drop of fluorescamine solution (0.1%, in acetonitrile) was added onto the membrane. Membrane was examined under a fluorescence microscope (Olympus IX70, with inverted reflected light fluorescence observation attachment) using 330-385 nm and 450-480 nm filters.

b) TNBS Staining

The membrane was placed in a petri plate containing borate buffer (3 mL, 0.2 M, pH 9.5) and 6 drops of aqueous TNBS (5% aqueous solution) was added [Turkova, 1993]. The color was allowed to develop for 2 h at room temperature. The membrane was examined with fluorescence microscope (Olympus IX70, with inverted reflected light fluorescence observation attachment) using the 330-385 nm filter.

2.2.5. Quantitative Determination of Immobilized Protein or Enzyme Amount

2.2.5.1. Amino Group Detection Method

The amount of immobilized protein was determined directly through amino acid quantification by the usage of 2,4,6-trinitrobenzene sulfonic acid (TNBS) treatment [Kök, 2001]. In this method the proteins on the membrane had to be initially be cleaved to their amino acids by acid hydrolysis. At the optimization stage before hydrolysis, the membranes, were washed with distilled water to remove the buffer, and then washed with acetone-water solutions (from 20% to 80%) and finally with pure acetone. The membranes were completely dried under vacuum for 24 h and were placed in tubes containing HCl (6 M, 4mL). The membranes in the tubes were evacuated by standing in vacuum oven for 1 h. Then, the tubes were inserted in liquid nitrogen and their necks were sealed with heat. For hydrolysis, membranes were incubated in an oven at 110°C for 24 h. After cooling the tubes, NaOH (6 M, 4 mL) was added for neutralization. The particles left after hydrolysis were removed by centrifugation and filtration through Millipore filter (MF-Millipore Membrane Filter, 0.45 μm filter pore size). Thus, the amino acid solution was ready to carry out TNBS assay.

Aliquots of amino acid solutions (250 μL) were placed into the test tubes and then sodium bicarbonate buffer (250 μL, 0.5 M, pH 8.5) and aqueous TNBS (250 μL, 0.1%) were added. After mixing, the samples were incubated at 40°C for 2 h. Then, sodium dodecyl sulfate (SDS, 250 μL, 10%) and HCl (125 μL, 1 M) were added and mixed gently. The absorbance was measured at 335 nm. Fresh, unused

Teflon membrane was used as blank. Glycine standards (250 μL, 25-500 μM) were used to construct a calibration curve (Appendix C) with distilled water as blank.

2.2.5.2. Lowry Method

The peptide bonds of a protein forms a complex with copper II ions in an alkaline medium. Then, Folin reagent is reduced by the tyrosine and tryptophan residues present in copper–treated protein, and gives molybdenum/tungsten blue color which can be determined spectrophotometrically [Lowry et al, 1951].

Duplicate aliquots of diluted samples (0.5 mL) (initial, remaining and wash solutions) were put into the test tubes. After addition of alkaline copper reagent (2.5 mL) (Appendix A) into the test tubes, the samples were vortexed and allowed to stand for 10 min. Folin-Phenol reagent (250 μL, 1N) (Appendix A) was added rapidly and each duplicate was mixed immediately within 8 s. The samples were allowed to stand for 30 min at room temperature and then the color intensity of each sample was measured spectrophotometrically at 660 nm against the blank, distilled water. For a calibration curve, BSA standards (0.05-0.2 mg/mL) were used (Appendix C). Duplicate aliquots of each standard (0.5 mL) were placed into the test tubes and the procedure indicated above was carried out using distilled water as blank.

In the studies following the optimization stage, borate buffer was used as blank instead of distilled water and glucose oxidase (0.02-0.3 mg/mL) (Appendix C) or choline oxidase (0.02-0.3 mg/mL) (Appendix C) was used as standard for calibration curve instead of BSA.

The immobilized protein content was calculated through mass balance according to the Equation 2.2.

$$P_{Im} = P_{In} - (P_r + P_w) \quad \text{where } P_{Im}: \text{immobilized protein}$$

P_{In} : input protein

P_r : protein remaining after immobilization

P_w : protein in the wash **(2.2)**

2.2.5.3. Bradford Method

The anionic dye Coomassie brillant blue G-250 dye specifically binds to proteins at arginine, tryptophan, tyrosine, histidine and phenylalanine residues and protein-dye complex formed has an absorbance maximum at 595 nm [Bradford, 1976].

Duplicate 0.5 mL aliquots of diluted samples (initial, remaining and wash solutions) were put into the test tubes. After addition of Bradford reagent (5 mL) (Appendix B) into test tubes, the samples were vortexed and allowed to stand for 10 min at room temperature. The absorbance of each sample was measured spectrophotometrically at 595 nm. Borate buffer was used as blank. The calibration curve was plotted using glucose oxidase standards (5-100 μg/mL) (Appendix C). Immobilized enzyme content was calculated from the mass balance as in section 2.2.5.2.

2.2.6. Measurement of Enzyme Activity

2.2.6.1. Activity Measurement of Immobilized Glucose Oxidase Activity with o-Dianisidine

Glucose oxidase activity assay was carried out according to the following reactions (Equations 2.3 and 2.4).

$$\text{D-Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + H_2O_2 \qquad \textbf{(2.3)}$$

$$H_2O_2 + \text{o-Dianisidine}_{(\text{reduced})} \xrightarrow{\text{POD}} \text{o-Dianisidine}_{(\text{oxidized})} + H_2O \qquad \textbf{(2.4)}$$

The assay mixture was prepared by dissolving peroxidase (6 mg) and o-dianisidine (6.6 mg) in PB (100 mL, 0.1 M, pH 6). Glucose oxidase (0.1 mL) and glucose solution (0.1 mL) were added into the assay mixture (2.3 mL). After incubation for 30 min at 30°C, the absorbance of each sample in the test tube was measured at 530 nm. A standard curve was drawn by using free glucose oxidase (2 mg/mL) solutions with different glucose concentrations (0.5 mM-10 mM). For glucose oxidase immobilized membranes obtained at the optimization stage, 0.1 mL PB was put into test tube with a membrane instead of 0.1 mL free glucose oxidase solution. PB (0.1 mL) was used as a blank instead of glucose solution [adapted from Arıca, 1992].

2.2.6.2. Amperometric Measurement of Free and Immobilized Glucose Oxidase Activity

The activity studies were carried out with oxygen electrode unit (Figure 2.5) (Helmut Saur, Germany) according to Kök [2001].

Upon the application of a voltage (600-700 mV) across the two electrodes, the platinum electrode becomes negative and the silver one positive. The oxygen in the medium passes freely through the Teflon membrane, it is reduced to hydrogen peroxide at the platinum surface, and this leads to the generation of electrical current (Figure 2.6). The electrical current is converted to a voltage output and recorded by a flatbed pen-recorder (SCI-TEC Instruments, Netherlands).

Free glucose oxidase (10 μL, 10 mg/mL) and immobilized glucose oxidase activity studies were carried out in the oxygen electrode unit.

Enzyme immobilized HEMA-grafted Teflon membranes were introduced to oxygen detection unit in two different ways:

a. placing as an unattached roll or sheet in the sample unit (Figure 2.5a)
b. stretching on the electrode unit (Figure 2.5b)

During a typical electrode preparation, a Teflon (PTFE) membrane is stretched over the paper spacer which is placed over the platinum cathode of the electrode and moistened with electrolyte to provide electrical continuity between the platinum and the silver electrodes. When the electrode is fitted into the oxygen electrode unit, the residual current of the electrode is removed by means of consumption of oxygen upon the addition $Na_2S_2O_4$ into the medium. The dithionite reacts with dissolved O_2 in the sample unit according to the Equation 2.5.

$$Na_2S_2O_4 + O_2 + H_2O \longrightarrow NaHSO_4 + NaHSO_3 \quad \textbf{(2.5)}$$

After adjustment to zero with dithionite, the sample unit was rinsed several times with distilled water and the calibration of the oxygen electrode was made with air-saturated water at the desired temperature by adjustment of the control box and the recorder.

For the activity assay of enzyme immobilized membranes stretched on the electrode, enzyme immobilized membrane was used directly without an additional untreated Teflon membrane covering the electrode surface. The residual current of the electrode was removed by bubbling nitrogen gas into the sample unit through substrate injection port. Nitrogen was used instead of $Na_2S_2O_4$ to eliminate possible damaging effect of the dithionite on enzyme activity. The calibration step was carried out with air saturated distilled water.

Glucose oxidase activity measurements were carried out in phosphate buffer (PB, 0.1 M, pH 6.0, 1.5 mL) which was aerated for 30 min before use.

Different glucose concentrations (10 µL, 0.066-6.6 mM) were introduced to the sample unit with Hamilton syringe through the sample injection port and the oxygen in the medium was depleted due to the presence of free or immobilized glucose oxidase according to the reaction presented in the Equation 2.6.

$$\text{Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \quad \textbf{(2.6)}$$

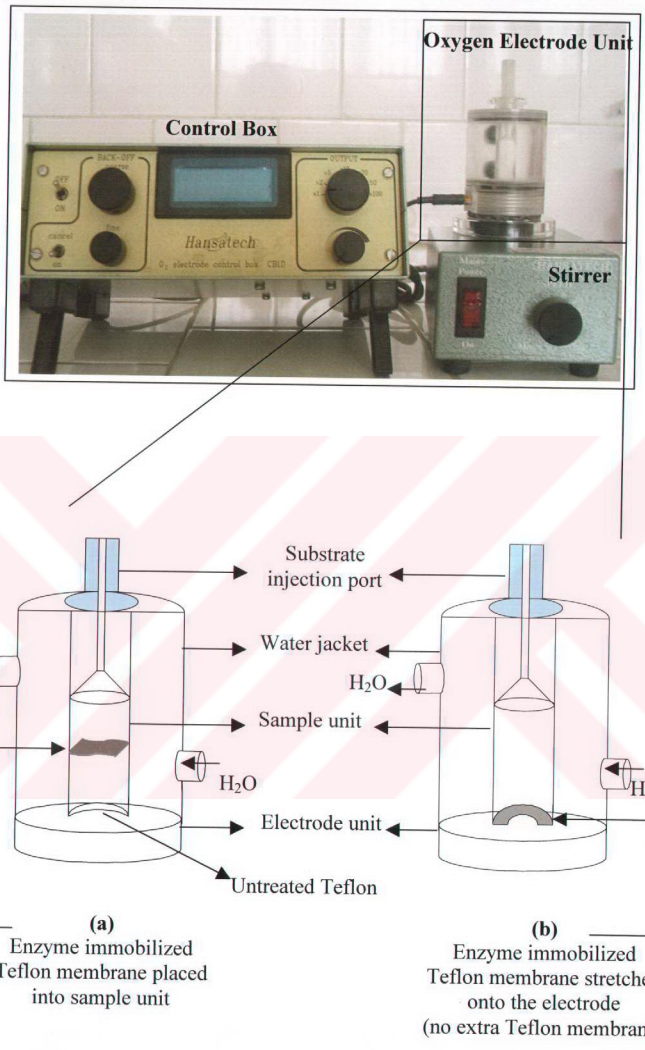


Figure 2.5. Schematic presentation of oxygen electrode unit

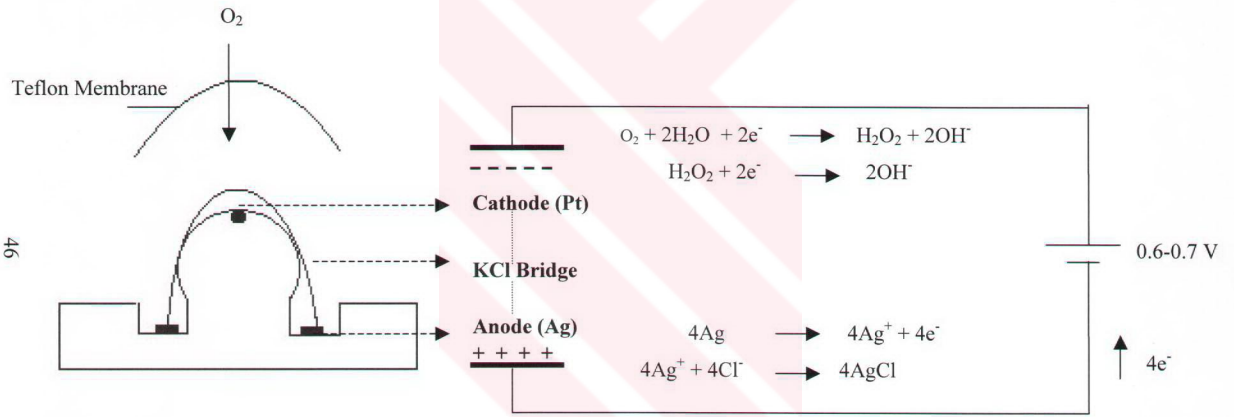


Figure 2.6. Electrochemical reactions carried out in electrode unit

Upon the application of substrate, oxygen depletion in the medium due to oxidase enzyme activity leads to a change in the signal and this is recorded by a chart recorder in the form of mV vs time plots. From the slopes of these plots obtained at each substrate concentration, an enzyme activity graph was constructed as "oxidation rate (mV/s) vs substrate concentration (mM)" and then V_{max} and K_m values were determined from Eadie-Hofstee plots.

2.2.6.2.1. Effect of Crosslinker and Epichlorohydrin Activation Duration on Enzyme Activity

The influence of EGDMA presence and the epichlorohydrin activation duration on enzyme activity was studied with oxygen electrode by placing glucose oxidase immobilized membranes in the sample unit. The preparation and the activation conditions for the membranes were indicated in Table 2.1.

Table 2.1. The membrane preparation conditions and epichlorohydrin activation duration

Membrane Preparation Condition	in 15% HEMA and gamma irradiated for 24 h			
	With EGDMA (0.15%,v/v)		Without EGDMA	
Epichlorohydrin activation duration	30 min	2 h	30 min	2 h

2.2.6.2.2. Effect of Activation Procedure Type on Enzyme Activity

As was stated in section 2.2.3., glucose oxidase was immobilized on HEMA-grafted Teflon membranes via two different activation procedures: a) glutaraldehyde, and b) epichlorohydrin. Immobilized membranes were tested in the oxygen electrode unit by placing in the sample unit to determine the effect of activation method on enzyme activity.

2.2.6.2.3. Effect of Membrane Location in the Oxygen Electrode on Membrane Performance

Glucose oxidase was immobilized on HEMA-grafted Teflon membranes via glutaraldehyde. Then, the activity assays were carried out by using membrane at two different locations of the oxygen detection unit (Figure 2.5). Membranes were set into the oxygen electrode as placing into sample unit or stretching over the electrode and then the activities ($mV.s^{-1}.\mu g^{-1}$) of the immobilized membranes were determined.

2.2.6.3. Amperometric Measurement of Free Choline Oxidase and Immobilized Choline Oxidase Activity

Choline oxidase activity studies were carried out in the oxygen electrode unit using choline as substrate. In the presence of choline oxidase, oxygen available in the medium was depleted according to the reaction shown in Equation 2.7. by application of choline. Before free choline oxidase activity measurements, the

oxygen electrode preparations were performed according to the approaches described in section 2.2.6.2. for free glucose oxidase activity.

$$\text{Choline} + H_2O + O_2 \xrightarrow{\text{ChO}} \text{Betaine} + H_2O_2 \qquad (2.7)$$

Activity studies of choline oxidase immobilized membranes were carried out by stretching the membrane over electrode and the electrode preparations were done following the steps described in section 2.2.6.2.

Activity measurements of free and immobilized choline oxidase were determined in PB (0.1 M, pH 7.0, 1.5 mL, aerated for 30 min before use) at different choline concentrations (10 µL, 0.104–3.480 mM). From the change in the signal upon addition of choline, mV vs time plots were obtained at each choline concentration and were used to construct enzyme activity graph.

2.2.6.3.1. Effect of Immobilization Concentration on the Activity of Choline Oxidase

During the immobilization step, glutaraldehyde activated, HEMA grafted Teflon membranes were placed in two different choline oxidase concentrations (2 mg/mL and 5 mg/mL). The effect of immobilization concentration on enzyme activity was determined with the oxygen electrode.

2.2.6.3.2. Reusability

Choline oxidase immobilized membranes (activated with glutaraldehyde and prepared with 2 mg/mL choline oxidase concentration according to section 2.2.3.2) were tested by measuring the activity for 45 successive runs within 8 h using choline (0.348 mM) as the substrate to determine the reusability of the membranes.

2.2.6.3.3. Linear Working Range

Linear working range of choline oxidase immobilized membrane (activated with glutaraldehyde and prepared with 2 mg/mL choline oxidase concentration according to section 2.2.3.2) was studied using choline concentrations (0.026-3.480 mM range). Linear range was determined by plotting delta oxidation rate ($mv.s^{-1}$)/delta choline concentration (mM) versus choline concentration (mM).

CHAPTER 3

RESULTS AND DISCUSSION

The goal of this thesis was the construction of a Teflon based biosensor for choline determination with substantially decreased diffusional restriction. HEMA was grafted on a Teflon membrane that was used as the support to immobilize choline oxidase via covalent bonding with epichlorohydrin or glutaraldehyde. The characterization of HEMA grafted membrane was carried out by Infrared Spectrophotometry and by the determination of mechanical properties. To determine the presence of immobilized protein on the membrane surface qualitative and quantitative analyses were carried out. The effects of the crosslinker, epichlorohydrin activation duration, activation procedure and the membrane location in the electrode chamber on the immobilized enzyme activity were studied in an oxygen electrode unit using glucose oxidase as the model enzyme. Following the determination of optimum immobilization choline oxidase concentration, the performance of the choline oxidase biosensor was evaluated by establishing the linear working range and reusability.

3.1. Characterization of Membranes

3.1.1. Influence of Gamma Irradiation Duration and FAS Concentration

At the optimization stage, the effect of gamma irradiation duration and FAS concentration on the extent of grafting of HEMA on the Teflon membrane was studied with IR Specrophotometry through determination of the functional groups formed on the Teflon membrane upon grafting with HEMA.

The Teflon membrane used in this study was the commercial oxygen permeable membrane of the oxygen electrode unit and appeared to be not pure PTFE, and to detect the indication of HEMA grafting on Teflon surface, this membrane was used as the reference.

Infrared spectra of the membranes are presented in Figure 3.1. When the IR spectrum of HEMA grafted membranes were compared with that of the untreated Teflon membrane, appearance of a peak around 3000 cm^{-1} and an increase in the intensity of the peak around 1700 cm^{-1} were observed in the spectrum of the membranes in the grafting medium gamma irradiated for 3 h and more. However, these changes could not be observed in the spectra of 30 min and 2 h gamma irradiated Teflon membranes.

In addition, to be able to analyze quantitatively, certain peak heights were measured and their ratios were calculated and the results are presented in Table 3.1. Taking the 2300 cm^{-1} peak as a reference because of its being present in all the spectra with significant height and studying the peaks, 2500 cm^{-1} and 2800 cm^{-1} changing with gamma exposure duration, it was not possible to detect a significant

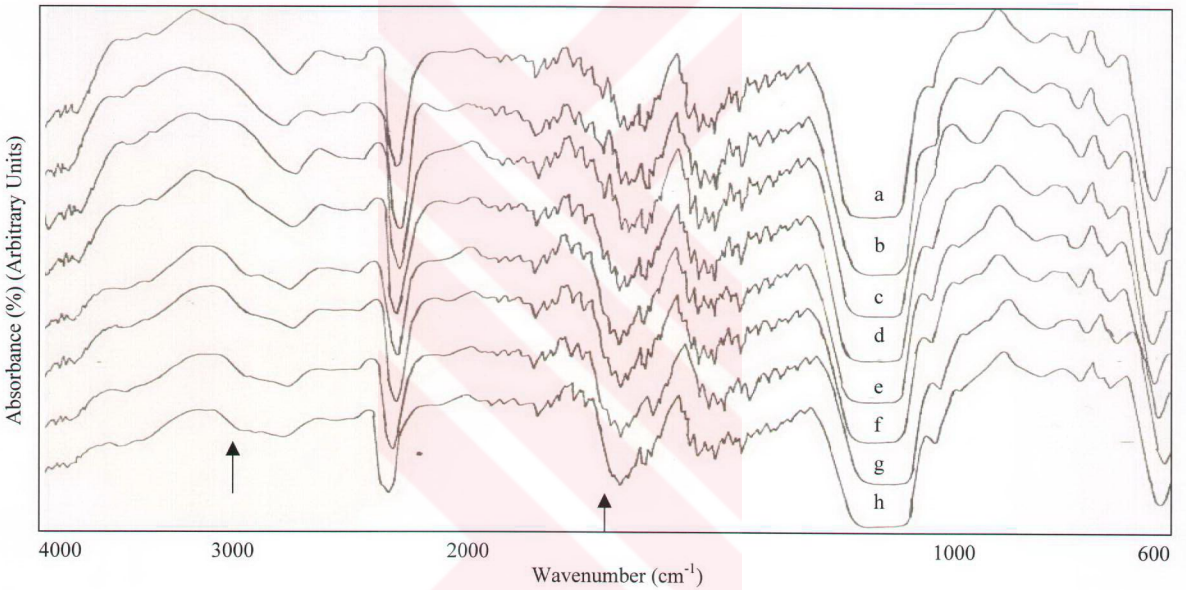


Figure 3.1. IR Spectra of Teflon gamma irradiated (in distilled water) for 24 h (a), untreated Teflon (b), Teflon membranes gamma irradiated (in HEMA solution) for 30 min (c), 2 h (d), 3 h (e), 4 h (f), 8 h (g), 24 h (h). The changes in the spectra of the Teflon membranes upon grafting were indicated by arrows at 3000 cm^{-1} and 1700 cm^{-1}.

difference between the untreated and gamma irradiated membranes (in HEMA solution).

When 3000 cm^{-1} peak is compared, however, a distinct difference (3 fold increase in ratio, a/d) could be seen in the peak height ratios between untreated and the membranes gamma irradiated for 3 h or more (in HEMA solution). A significant difference could also be observed by taking the 3000 cm^{-1}/2800 cm^{-1} peak ratios. Both evaluations support the appearance of a peak around 3000 cm^{-1}, indicative of HEMA grafting.

Table 3.1. IR data of untreated Teflon, Teflon membranes gamma irradiated (in HEMA solution) for different duration

Samples		Peak Heights and Ratios							
		A (cm)	b (cm)	c (cm)	d (cm)	a/d	b/d	c/d	a/b
UT		0.4	2.2	0.7	7.4	0.1	0.3	0.1	0.2
(in HEMA solution)	30 min	0.4	2.4	1.0	6.9	0.1	0.3	0.1	0.2
	2 h	1.0	2.2	0.9	6.5	0.2	0.3	0.1	0.4
	3 h	1.4	1.8	0.7	5.4	0.3	0.3	0.1	0.8
	4 h	1.4	2.0	0.7	5.8	0.3	0.3	0.1	0.7
	8 h	1.3	1.8	0.6	5.2	0.3	0.3	0.1	0.7
	24 h	1.2	1.5	0.5	4.7	0.3	0.3	0.1	0.8

The peak heights at: (a) ~3000 cm^{-1}, (b) ~2800 cm^{-1}, (c) ~2500 cm^{-1}, (d) ~2300 cm^{-1}

The compounds containing C=O groups have absorptions at around 1700 cm^{-1} while the alcohols absorb around 3000 cm^{-1} as well as other locations. These changes (around 1700 cm^{-1} and 3000 cm^{-1}) could indicate HEMA grafting on Teflon membrane since HEMA has both the OH and the C=O groups. The influence of gamma exposure becomes more distinct as the exposure duration is increased from 3 to 24 h.

The most appropriate gamma irradiation duration for HEMA grafting appears to be at least 3 h and 24 h was used in the following studies. Teflon membrane was irradiated for 24 h in distilled water and its IR spectrum was checked to see the effect of gamma irradiation. No significant differences could be determined in the spectra of this membrane and that of the untreated membrane proving that the changes are caused by the presence of HEMA.

FAS was used to prevent homopolymerization of HEMA initiated by hydroxyl radicals and to increase the number of HEMA chains attached to the membrane surface by attachment of more monomer to the Teflon membrane [Mohy Eldin et al.,1998]. From the spectra of Teflon membranes grafted with HEMA in different FAS concentration (0.1-0.8%) solutions, it was observed that there was no significant difference between IR of membranes. The quantitative data, peak heights and their ratios were measured and the results are presented in Table 3.2.

Table 3.2. IR data of untreated Teflon and Teflon membranes prepared with different FAS concentration and 3 h gamma irradiation

Samples	Peak Heights and Ratios							
	a(cm)	b(cm)	c(cm)	d(cm)	a/d	b/d	c/d	a/b
UT	0.5	2.5	0.9	7.1	0.1	0.4	0.1	0.2
0.1%FAS	1.4	1.8	0.7	5.4	0.3	0.3	0.1	0.8
0.2% FAS	1.2	2.1	0.8	6.2	0.2	0.3	0.1	0.6
0.4% FAS	1.5	1.7	0.5	5.4	0.3	0.3	0.0	0.9
0.8% FAS	1.4	1.8	0.6	5.5	0.3	0.3	0.1	0.8

The peak heights at: (a) ~3000 cm^{-1}, (b) ~2800 cm^{-1}, (c) ~2500 cm^{-1}, (d) ~2300 cm^{-1}

When 2300 cm^{-1} peak is taken as a reference and studying the peaks changing with treatment conditions, 2800 cm^{-1}, 2500 cm^{-1} and 3000 cm^{-1} peaks, no significant difference could observed in the IR of membranes with different FAS concentration. It was therefore deduced that FAS concentration did not affect the product formed on the membrane upon HEMA grafting. Higher FAS concentration decreases grafted branch length but increases the number of branches, and therefore makes the membrane less active due to formation of hydrogen bonds between the functional groups of enzyme and the grafted branches. To obtain a more active membrane, the membrane prepared with 0.1% FAS concentration was used in the later studies. Upon using lower FAS concentration, the length of the polymer chains on the surface of the Teflon would be increased and so the interaction of the immobilized enzyme (on HEMA grafted Teflon) with the HEMA branches and the Teflon support would be minimimized.

3.1.2. Mechanical Properties of Membranes

Since the enzyme immobilized Teflon membrane would be stretched onto the electrode, the membranes need to be sufficiently elastic and it should not deform easily upon direct contact with the magnet of the oxygen electrode unit while the reaction medium is stirred.

In order to investigate the effect of the crosslinker on the mechanical properties of the membrane, tensile testing was carried out on thin membranes prepared with and without EGDMA. The effect of gamma irradiation and membrane activation type were also investigated. The test results were obtained in the form of a

"stress vs strain" graph (a representative graph is presented in Figure 3.2). The Young's Modulus of the membrane was calculated using the initial slope of the graph and the toughness of the membrane was obtained as the area under the stress-strain curve.

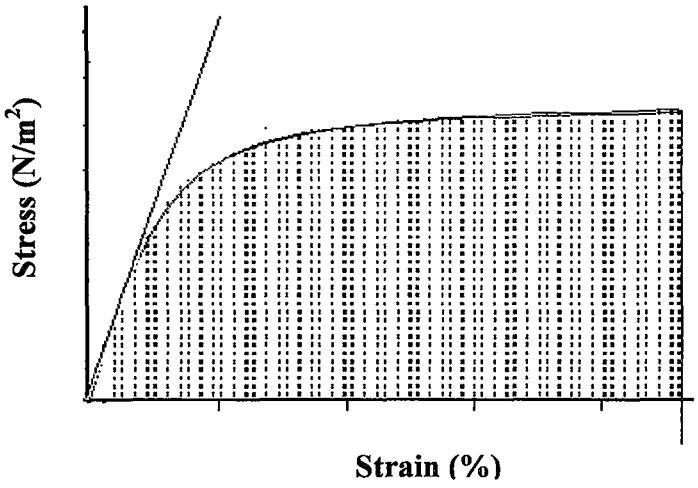


Figure 3.2. A representative stress vs strain curve

The Young's Modulus and the toughness values of the samples are presented in Figures 3.3 and 3.4.

It can be seen (in Figure 3.3) that gamma irradiation makes the membranes harder (high elastic (Young's) modulus). Upon HEMA grafting the membranes become substantially softer (low elastic modulus) compared to the untreated and gamma irradiated (in distilled water) Teflon. The presence of EGDMA, a crosslinker, made the membranes stiffer (higher elastic modulus), as expected. The thickness of the membrane was another important parameter influencing mechanical properties. It was observed that the thinner membranes (0.2-0.3 mm) did not deform as easily as

the thick membranes (0.5-0.55 mm). Based on the results of the mechanical testing, it was decided to use the thin membranes without EGDMA in the later studies.

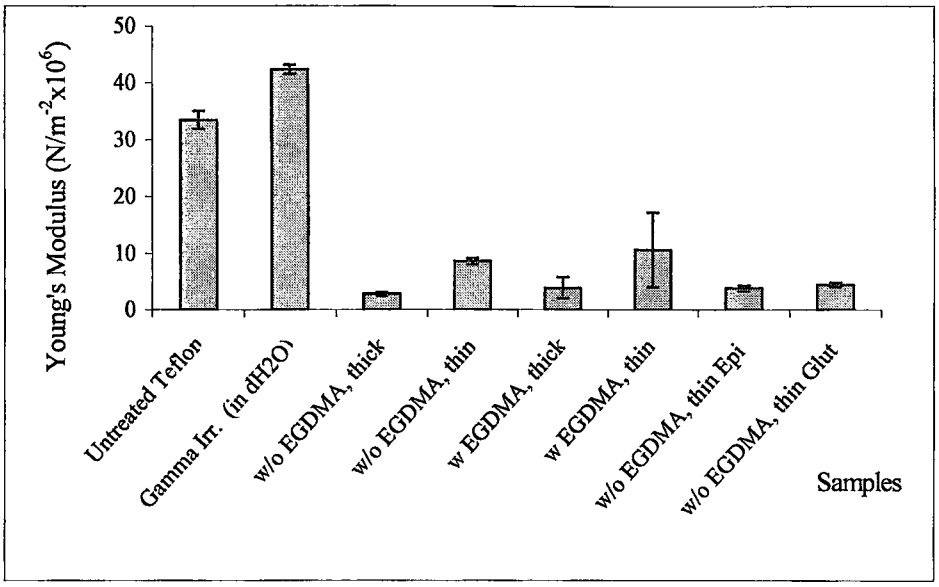


Figure 3.3. Young's Modulus values of different samples

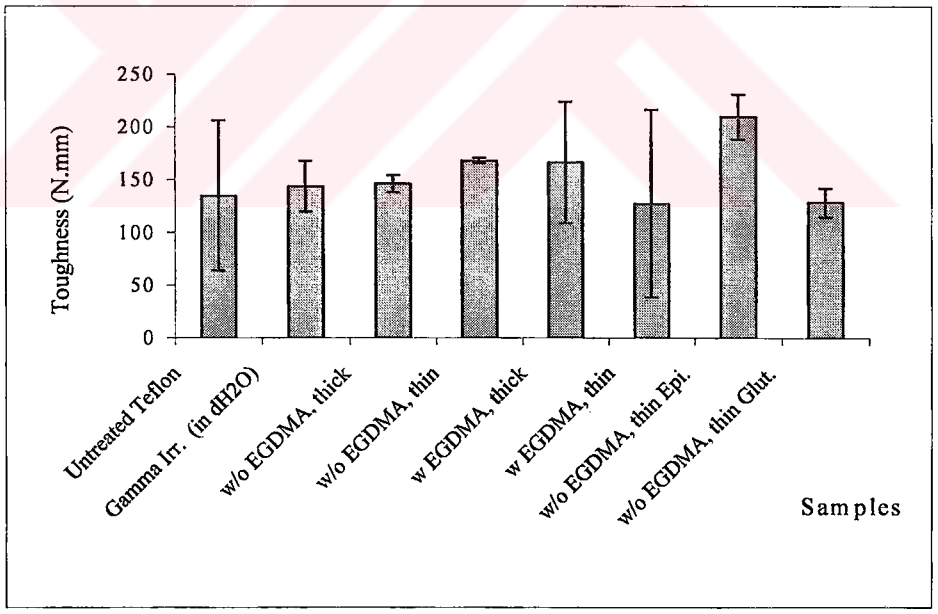


Figure 3.4. Toughness values of different samples

The differences in the toughness study was not as significant as in the Young's modulus (Figure 3.4). The results showed that activation via epichlorohydrin makes the HEMA grafted Teflon membranes tougher, compared to the rest. Thus, Epi-activated membrane appeared to be the best in terms of toughness.

3.1.3. Examination of Membrane Topography by SEM

The change in the membrane topography in successive preparation steps was studied by SEM. Compared to the SEM of the untreated Teflon membrane (Figure 3.5) with the SEM of HEMA grafted membrane (Figure 3.6), the presence of the HEMA coating as a granular structure could be observed. During use of the membrane in the electrode HEMA need to be grafted only on one side of the membrane, the side which faces the assay medium. Figure 3.7 shows that it was possible to graft HEMA mainly on one side of the membrane. The thickness of the membrane could be determined from the SEM of the cross-section of the membrane as 350 μm (Figure 3.7a). There was no significant difference upon epichlorohydrin activation (Figure 3.8) but the lace structure on the surface seems to be looser with respect to glutaraldehyde activated membrane (Figure 3.9). There was no change observed on the membrane surface through glutaraldehyde coupling (Figure 3.9). Any indication of enzyme immobilization through epichlorohydrin (Figure 3.10) and glutaraldehyde (Figure 3.11) activation could not be observed with SEM.

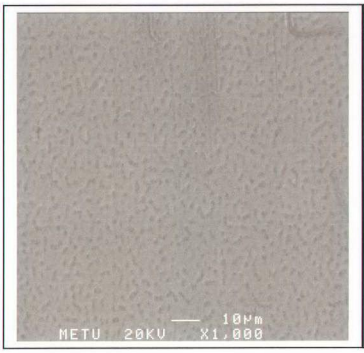


Figure 3.5. SEM of untreated Teflon membrane (x 1000)

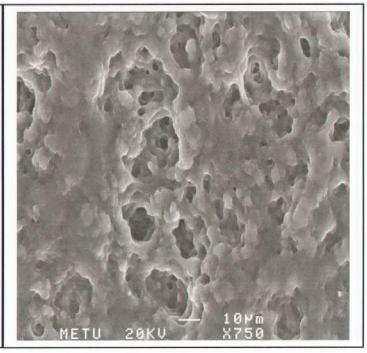


Figure 3.6. SEM of HEMA grafted Teflon membrane (x 750)

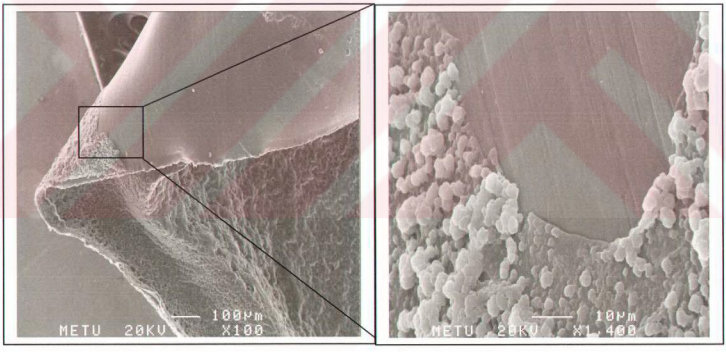


Figure 3.7. SEM of HEMA grafted Teflon membrane

(a) Low magnification (x 100) **(b)** An enlarged view of the back (x 1400)

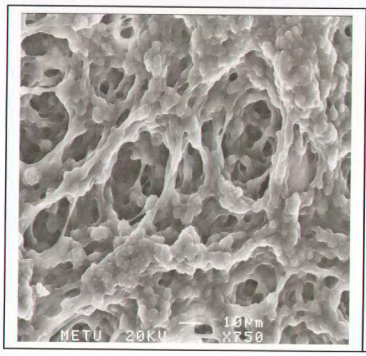


Figure 3.8. SEM of Epi-activated membrane (x 750)

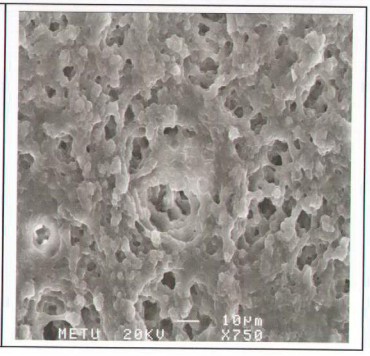


Figure 3.9. SEM of Glu-activated membrane (x 750)

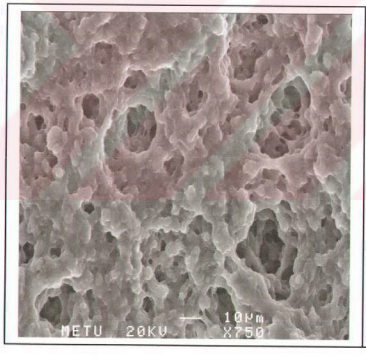


Figure 3.10. SEM of Epi-activated GOD immobilized membrane (x 750)

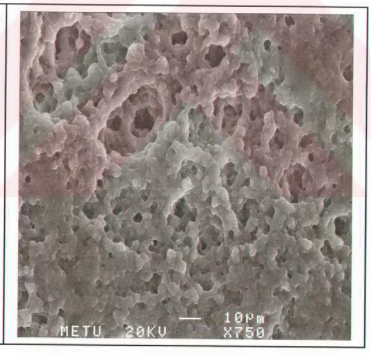


Figure 3.11. SEM of Glu-activated GOD immobilized membrane (x 750)

3.2. Immobilization of Enzymes

3.2.1. Activation with Different Covalent Bonding Methods

Two coupling agents were used to immobilize the enzyme and protein on HEMA grafted Teflon membrane via covalent bonding: epichlorohydrin and glutaraldehyde.

a) Activation with Epichlorohydrin

Thin membranes became easily deformable after the epichlorohydrin activation step and therefore the detected enzyme activity was low. Therefore, epichlorohydrin activation method was modified omitting the 30% acetone wash step and decreasing the activation duration to 30 min. The enzyme activity results revealed that the modified procedure yielded membranes with better activity (in section 3.6.2.1) and lower deformability. However, the Epi-immobilized enzymes had very low activity.

b) Activation with Aminohexyl Glutaraldehyde

Activation of the membranes via glutaraldehyde method did not lead to any damage on the membrane and could be used successfully in enzyme activity studies.

3.3. Qualitative Detection of Immobilized BSA

During the optimization stage, BSA immobilized on the Glu-activated, 24 h irradiated HEMA grafted membranes could not be detected with fluorescamine staining. It was probably due to the low amount of immobilized protein, as supported by the later studies.

The other qualitative detection method, TNBS staining, was used and the membranes at different stages of preparation were examined by fluorescence microscopy. Compared to the untreated Teflon (Figure 3.12), the change on the membrane surface morphology was significant with the even granular appearance after HEMA grafting (Figure 3.13). When the grafted membrane was activated with epichlorohydrin (Figure 3.14) the granular appearance become much more distinct. Upon BSA immobilization the surface granulation was more distinct and the color was darker (with dispersed yellow spots) implying the presence of the protein attached on the surface (Figure 3.15). On the other hand, there was no difference between the Glu-activated and Glu-activated BSA immobilized membranes probably because of the presence of free NH_2 groups of hexadiamine remaining on the membrane after glutaraldehyde activation and acting similarly to the NH_2 of proteins.

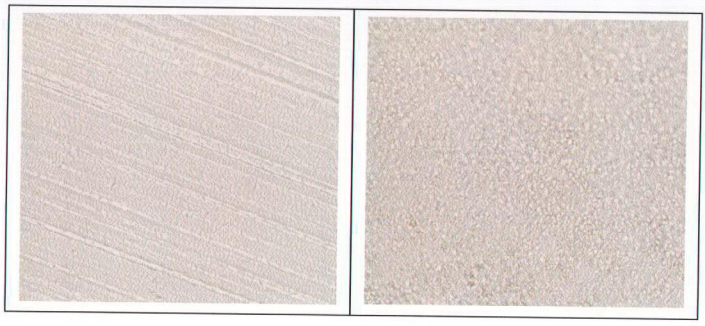


Figure 3.12. Fluorescence micrograph of untreated Teflon membrane stained with TNBS (x 100)

Figure 3.13. Fluorescence micrograph of HEMA grafted Teflon membrane stained with TNBS (x 100)

Figure 3.14. Fluorescence micrograph of Epi-activated membrane stained with TNBS (x 100)

Figure 3.15. Fluorescence micrograph of Epi-activated BSA immobilized membrane stained with TNBS (x 100)

3.4. Quantitative Determination of Immobilized Protein and Enzyme Amount

During the optimization stage, immobilized BSA content on the Glu-activated, 24 h irradiated HEMA grafted membranes could not be determined by Lowry method. Therefore, a much more sensitive method, TNBS assay was carried out after acid hydrolysis. The immobilized BSA amount on these membranes was determined as 14 μg/membrane or 2.3 $\mu g.cm^{-2}$ by TNBS assay. Amino acid quantification method and earlier TNBS staining results (section 3.3) implied that proteins could be immobilized on the membrane but the amount was very low. The results of glucose oxidase activity studies (in section 3.5) during the optimization stage also showed no activity which could be due to the insufficiency of the amount of immobilized enzyme to obtain activity.

The immobilized enzyme content on the thin membranes was determined by Bradford and Lowry methods. The immobilized enzyme content of Epi-activated membranes was determined successfully by Bradford method. However, the immobilized enzyme content on Glu-activated membranes could not be detected properly by the same method. It can due to the probable reaction of glutaraldehyde itself along with the enzyme with the Bradford reagent. Glu-activated membrane's enzyme content was successfully determined by Lowry method. The determined enzyme contents are presented in sections 3.6 and 3.7.

3.5. Measurement Glucose Oxidase Activity during Optimization Stage

During the optimization stage, the activity of the Epi-immobilized glucose oxidase was determined with the o-dianisidine assay (section 2.2.6.1) but no significant activity was observed after a 30 min incubation. The incubation period was therefore extended to 2 h-8 h and 24 h and again no activity was detected while the assay worked with free glucose oxidase. In addition, immobilized glucose oxidase activity was studied in the oxygen electrode unit but no activity could be detected.

During optimization stage the enzyme activity studies and protein determination (quantitative and qualitative) results revealed that the activity of the immobilized enyme could not be detected successfully due to insufficient enzyme content. It could be due to that, the immobilization efficiency decreases due to the removal of some grafted HEMA from the surface of Teflon during washing with scrubbing after grafting by gamma irradiation.

3.6. Measurement of Glucose Oxidase Activity

3.6.1. Free Glucose Oxidase Activity

Measurement of the model enzyme free glucose oxidase activity was carried out in aerated PB (pH 6) in the oxygen electrode unit. The small decline in the signal of the electrode with time in the absence of substrate should be taken into account during activity measurements. If the background signal of the electrode was not negligible, it was subtracted from the obtained experimental value.

The voltage output (mV) vs time (s) plots were obtained from the chart recorder for different substrate concentrations and the slopes of these plots were used to construct "oxidation rate vs substrate concentration" plots (Figure 3.16). K_m and V_{max} values were determined from the Eadie-Hofstee plot (Figure 3.17) the equation of which is presented below:

$$V = -K_m \cdot \frac{V}{[S]} + V_{max}$$

The K_m and V_{max} values determined were 3.334 mM and 15.997 $mV.s^{-1}$, respectively. The specific activity for the free glucose oxidase was calculated as $9.25 \cdot 10^{-2}$ $mV.s^{-1}.\mu g^{-1}$.

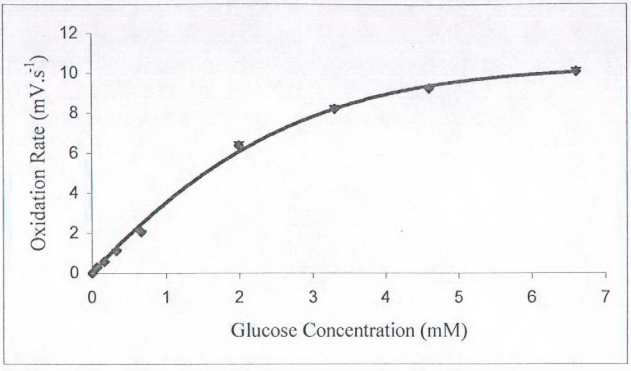


Figure 3.16. Michaelis-Menten plot for free glucose oxidase activity

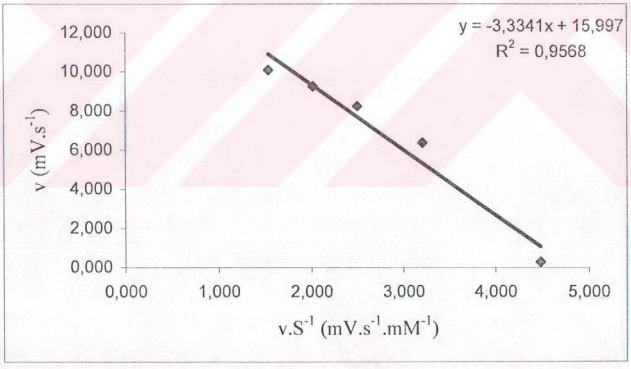


Figure 3.17. Eadie-Hofstee plot for free glucose oxidase activity

3.6.2. Characterization of Immobilized Membranes through Glucose Oxidase Activity Studies

3.6.2.1. Effect of Crosslinker and Epichlorohydrin Activation Duration on Enzyme Activity

In order to investigate the effect of the crosslinker and epichlorohydrin activation duration on enzyme activity, the membranes prepared with and without EGDMA following epichlorohydrin activation for 30 min and 2 h, were used in enzyme activity studies by placing them into the sample unit of the oxygen electrode. The oxidation rate vs glucose concentration plots and Eadie-Hofstee plots were constructed for each membrane and the representative plots are given in Figures 3.18 and 3.19, respectively. K_m and V_{max} values, specific activities and protein amounts are presented in Table 3.3.

Table 3.3. Effect of crosslinker and epichlorohydrin activation duration on kinetic parameters, specific activities and immobilized protein contents

Membrane	V_{max} ($mV.s^{-1}$)	K_m (mM)	Specific Activity ($mV.s^{-1}.\mu g^{-1}$)	Protein Content (µg/membrane)
w/o crosslinker & 30 min Epi	0.623	0.509	5.76×10^{-4}	977
w/o crosslinker & 2 h Epi	0.425	0.584	5.50×10^{-4}	909
w crosslinker & 30 min Epi	0.307	1.044	2.82×10^{-4}	1080
w crosslinker & 2 h Epi	0.578	0.482	3.77×10^{-4}	1107

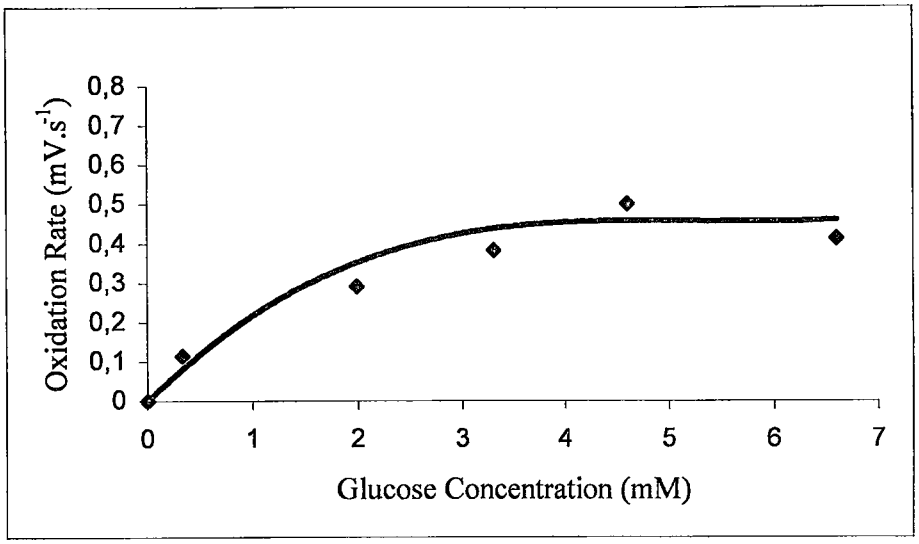


Figure 3.18. Michaelis-Menten plot for the activity of the membrane prepared without crosslinker and 2 h Epi-activated

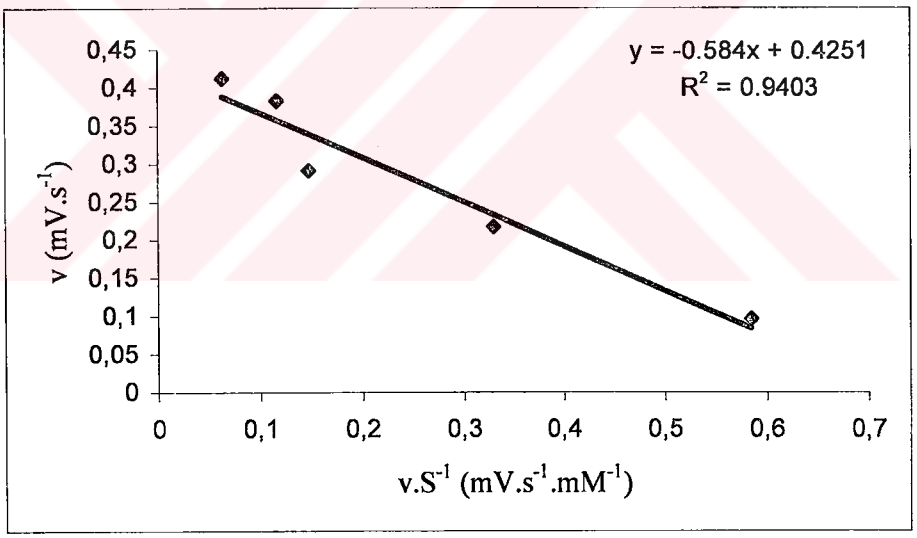


Figure 3.19. Eadie-Hofstee plot for the activity of the membrane prepared without crosslinker and 2 h Epi-activated

Of the membranes prepared without the crosslinker, the 2 h Epi-activated membrane had lower V_{max} value and slightly lower specific activity and higher K_m value compared to the 30 min Epi-activated membrane. This result was expected because the ones activated for 2 h were damaged easily during activity studies. However, the results of the membranes prepared with crosslinker shows that 2 h Epi-activated membrane has higher V_{max} value and specific activity with low K_m value in comparison to the membranes activated for 30 min. This could be due to that the membranes prepared with crosslinker were more resistant to the distruption by epichlorohydrin and therefore with a 2 h activation the immobilization efficiency increased resulting in better enzyme performance. Although the membranes prepared with crosslinker were not damaged easily (as shown by the mechanical tests), the membranes prepared without crosslinker had better enzyme activity with high specific activity even when the amount of enzyme on the membrane was low. The immobilization effficiency was better in the membranes with crosslinker but the specific activity results was low probably due to interaction of the enzyme with EGDMA or partial entrapment of the enzyme inside membrane and not being able to show activity due to conformational restriction.

It can be concluded from the results that the membrane prepared without crosslinker and activated for 30 min has the highest activity. For the further studies, it was decided that EGDMA was not needed and 30 min duration was suitable for epichlorohydrin activation.

3.6.2.2. Effect of the Activation Procedure Type on Enzyme Activity

Activation method performance in enzyme activity was studied by placing the membranes into the sample unit of the oxygen electrode rather than by stretching. The representative plots of the oxidation rate vs substrate concentration plot and Eadie Hofstee plot are presented in Figures 3.20 and 3.21 for Epi-activated membranes, in Figure 3.22 and 3.23 for Glu-activated membranes, respectively. Kinetic parameters, specific activity and protein amounts are presented in Table 3.4

Table 3.4. Effect of the activation method on kinetic parameters, specific activities and immobilized protein contents

Membrane	V_{max} ($mV.s^{-1}$)	K_m (mM)	Specific Activity ($mV.s^{-1}.\mu g^{-1}$)	Protein Content (μg/membrane)
Epichlorohydrin Activated	0.901 ± 0.047	0.596 ± 0.165	9.54×10^{-4} ± 1.25×10^{-4}	866 ± 137
Glutaraldehyde Activated	2.434 ± 0.152	1.002 ± 0.042	4.05×10^{-3} ± 0.65×10^{-3}	536 ± 63

It can be observed that, the V_{max} value was 2.7-fold higher and the specific activity was 4.25-fold higher for Glu-immobilized enzyme than the Epi-immobilized enzyme. The increase in the enzyme activity through glutaraldehyde immobilization is probably due to the better mechanical properties of the membrane because epichlorohydrin damaged the HEMA grafted Teflon membranes and then the membrane was deteriorated during activity studies, which can be seen from the activity results.

Glutaraldehyde coupling was chosen for further studies because of its better performance in enzyme activity studies, even though its enzyme immobilization efficiency was lower.

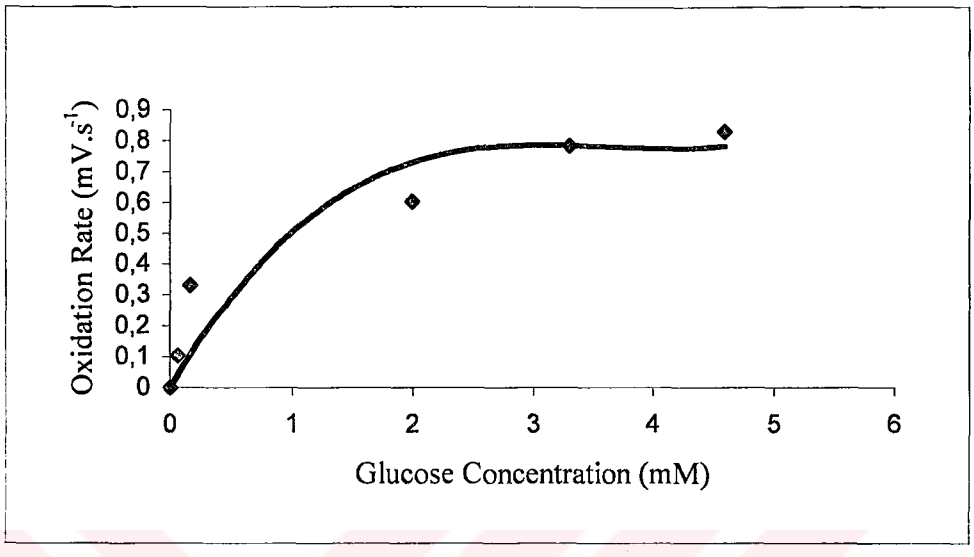


Figure 3.20. Michaelis-Menten plot for the activity of 30 min Epi-activated membrane

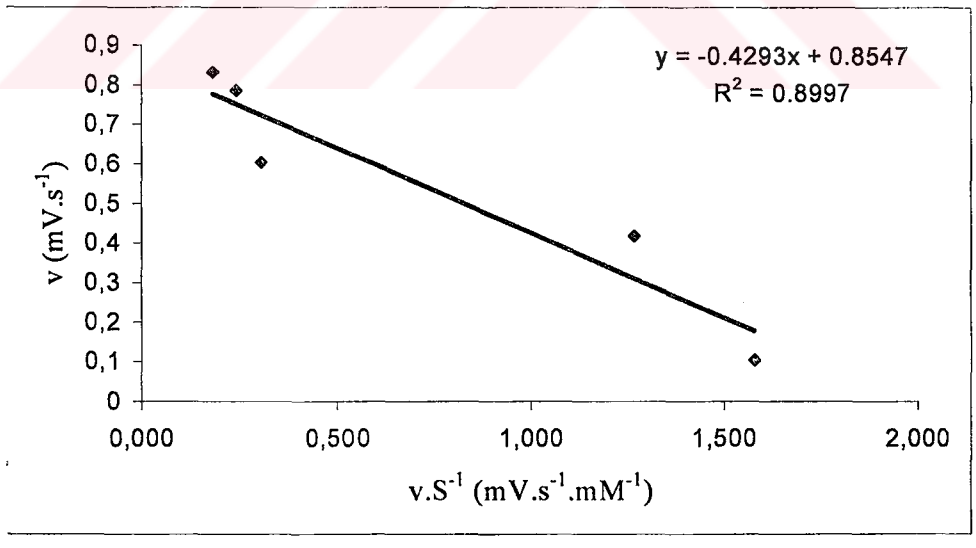


Figure 3.21. Eadie-Hofstee plot for the activity of 30 min Epi-activated membrane

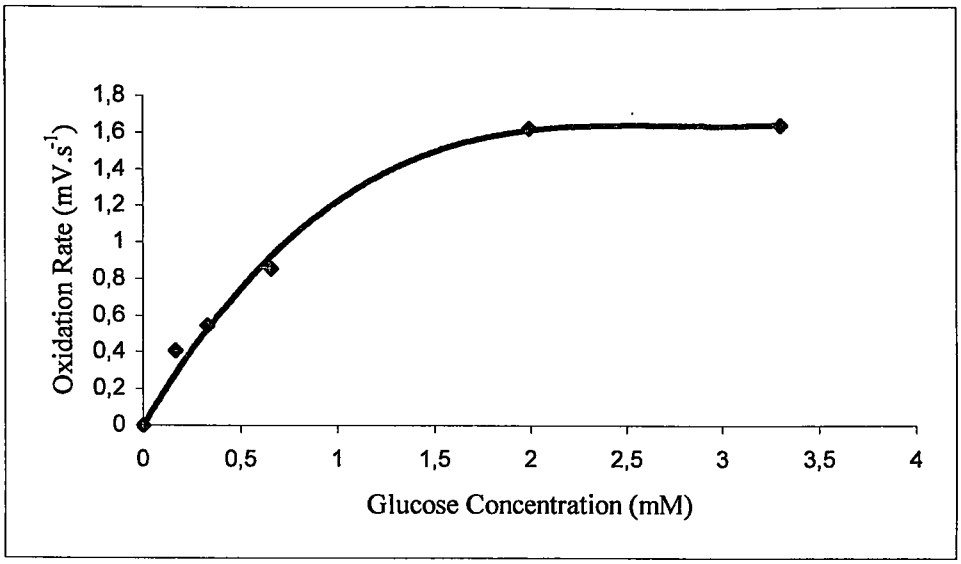


Figure 3.22. Michaelis-Menten plot for the activity of Glu-activated membrane

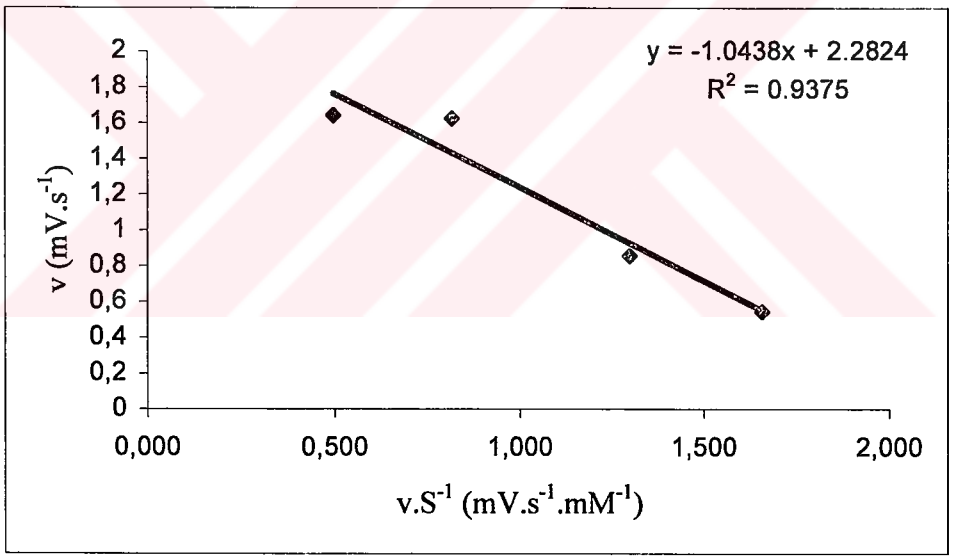


Figure 3.23. Eadie-Hofstee plot for the activity of Glu-activated membrane

3.6.2.3. Effect of Membrane Location in the Oxygen Electrode on Membrane Performance

Ultimately the enzyme immobilized HEMA grafted Teflon membrane would be used by stretching it onto the electrode instead of using a typical Teflon membrane of the oxygen electrode unit.

To compare the activities of the membranes upon stretching directly onto the electrode and simply placed into the sample unit, the Glu-immobilized glucose oxidase membranes were used. The representative oxidation rate vs time plots and Eadie-Hofstee plots are presented in Figures 3.24 and 3.25 for the immobilized membrane simply placed into sample unit, and in Figures 3.26 and 3.27 for immobilized membrane stretched over the electrode. K_m and V_{max} values are presented in Table 3.5 along with specific activity and protein amounts (for the stretched membrane the protein content on the membrane exposed to reaction medium was taken into consideration).

Table 3.5. Effect of membrane location in the oxygen electrode on kinetic parameters, specific activities and immobilized protein contents

Membrane	V_{max} ($mV.s^{-1}$)	K_m (mM)	Specific Activity ($mV.s^{-1}.\mu g^{-1}$)	Protein Content (μg/membrane)
Unstretched	1.401 ± 0.259	0.968 ± 0.098	4.90×10^{-3} ± 0.00	271 ± 54
Stretched	1.823 ± 0.204	2.565 ± 0.229	1.83×10^{-2} ± 0.22×10^{-2}	82 ± 26

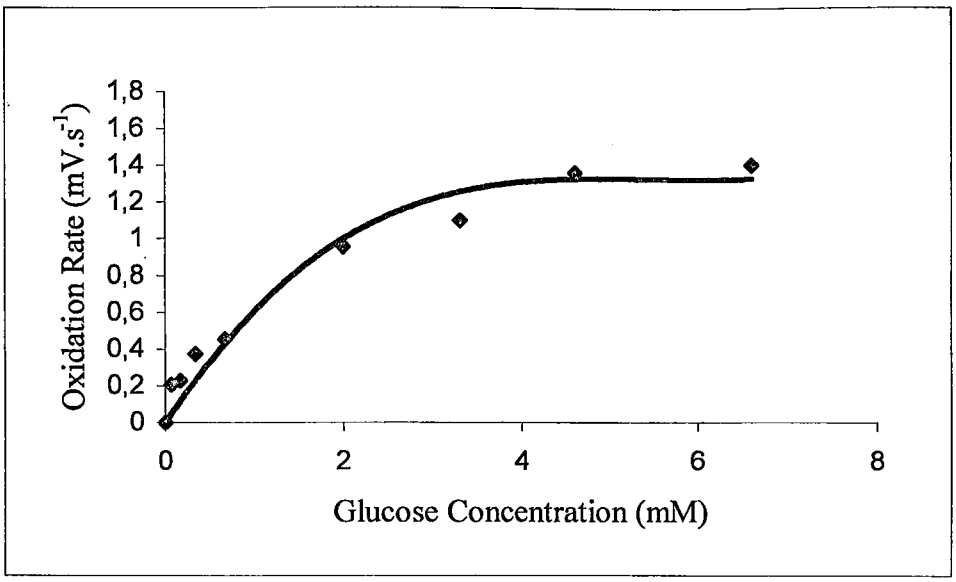


Figure 3.24. Michaelis-Menten plot for the activity of the membrane placed into the sample unit of the electrode

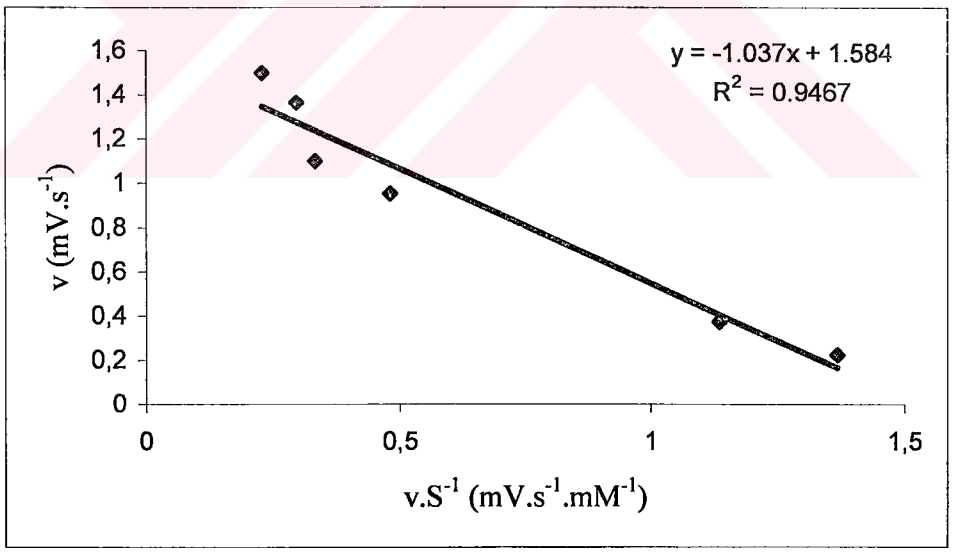


Figure 3.25. Eadie-Hofstee plot for the activity of the membrane placed into the sample unit of the electrode

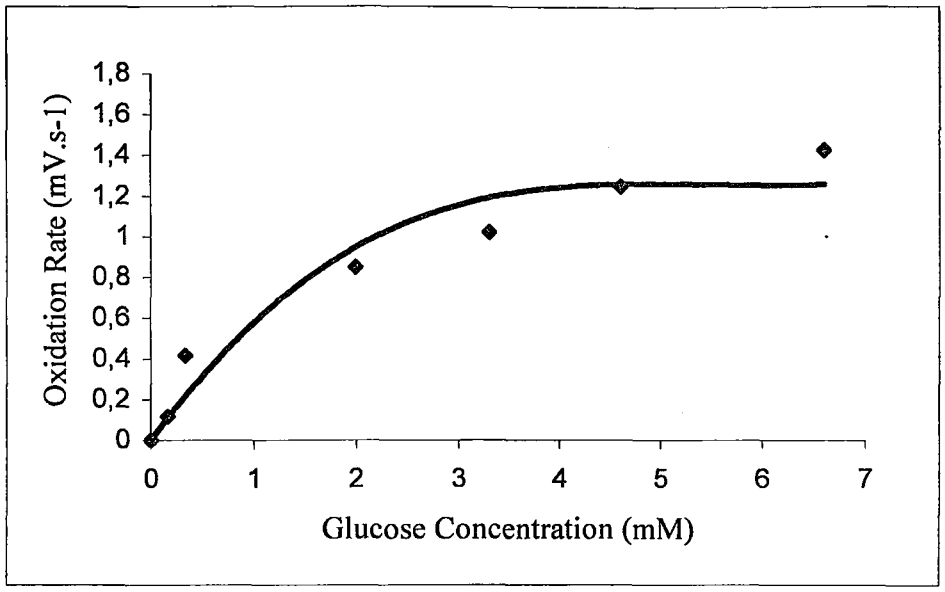


Figure 3.26. Michaelis-Menten plot for the activity of the membrane stretched onto the electrode

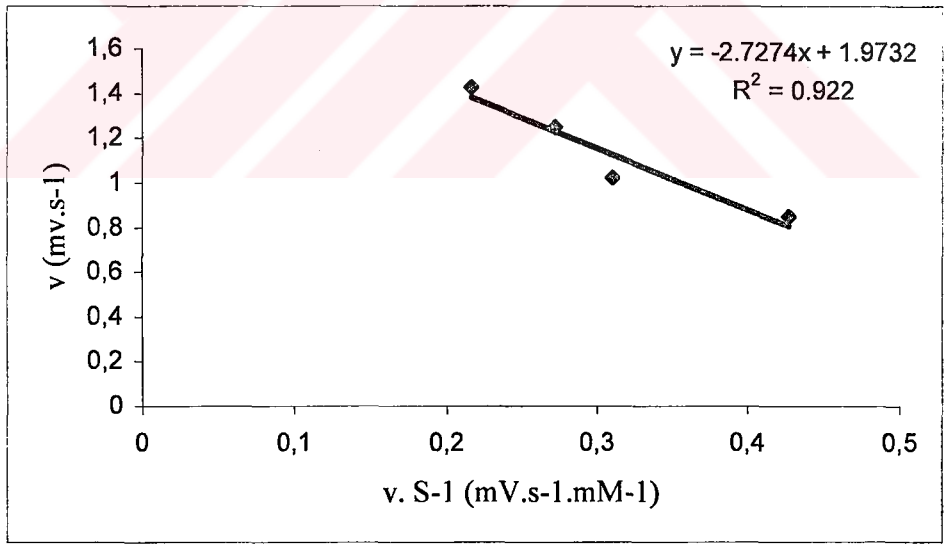


Figure 3.27. Eadie-Hofstee plot for the activity of the membrane stretched onto the electrode

It could be seen from the table that, using the membrane directly on the electrode increased the specific activity by 3.7-fold.

The increase in enzyme activity of the stretched membrane could be due to the improvement of diffusional restriction because the enzyme reaction was carried out directly on the electrode as was originally aimed. The oxygen electrode unit generates a signal due to the oxygen depletion in the medium and by this placement the decrease in oxygen concentration in the medium much more rapid due to decrease in the diffusional restriction. Meanwhile, the increase in K_m value was 2.65-fold higher by using enzyme immobilized membrane instead of original Teflon.

The immobilization process leads a change in the kinetic behaviour due to either conformational alteration within enzyme resulting in deactivation or to restriction in the enzyme mobility. Upon immobilization, the increase in K_m value was expected because of the (diffusional resistance) limited rate of mass transfer of the substrate from the bulk of the solution to the surface of the support material. However, the activity results obtained in this study show that the V_{max} values for the immobilized enzymes (1.401 and 1.823 $mV.s^{-1}$) were lower than that of the free (15.997 $mV.s^{-1}$) but K_m values for the immobilized enzymes (0.968 and 2.565 mM) were lower than that of the free enzymes (3.334 mM). The specific activities were $4.90.10^{-3}$, $1.83.10^{-2}$ and $9.25.10^{-2}$ for placed, stretched and free enzymes, respectively. The apparent value of K_m and V_{max} may differ due to the changes in the properties of the solution in the immediate vicinity of the immobilized enzyme or due to the effects of molecular diffusion within the local environment. K_m value of an enzyme can be reduced when the substrate concentration in the vicinity of enzyme

active site is higher than that measured in the bulk of the solution due to the influence of charge and hydrophobicity of the surface [Chaplin and Bucke, 1990].

A decrease in K_m and V_{max} values similar to our study, was also reported by Fernandes et al.[2003] They immobilized horseradish peroxidase on chemically synthesized polyaniline with glutaraldehyde and found that the K_m value for the immobilized enyme (5.2 $mmol.l^{-1}$) was lower than the K_m value for the free enzyme (9.58 $mmol.l^{-1}$) using pyrogallol as substrate. In addition, V_{max} value was decreased from 1.47 $mmol.min^{-1}$ to 0.96 $mmol.min^{-1}$ upon immobilization. Although it was not as significant as in the present study, a similar K_m decrease was also reported in the study of Gülce et al [2002]. Galactose oxidase was immobilized in polyvinylferrocenium matrix coated on a platinum electrode surface, and the K_m value was found to be 21.7 mM for the immobilized galactose oxidase, while the K_m for the free enzyme was 22.8 mM.

According to the model enzyme activity results, the membrane prepared without crosslinker, activated with glutaraldehyde in stretched form was chosen for the final stage immobilized choline oxidase activity studies.

3.8. Amperometric Measurement of Choline Oxidase Activity

3.7.1. Free Choline Oxidase Activity

Measurement of free choline oxidase activity was carried out in aerated PB at pH 7 in the oxygen electrode unit. The oxidation rates were determined for each choline concentration taking into consideration the background signal.

Oxidation rate vs choline concentration plots were obtained (Figure 3.28). For the free enzyme K_m and V_{max} values were determined from Eadie Hofstee plot (Figure 3.29) as 1.033 mM and 4.773 $mV.s^{-1}$. The specific activity of free choline oxidase was $3.04.10^{-2}$ $mV.s^{-1}.\mu g^{-1}$.

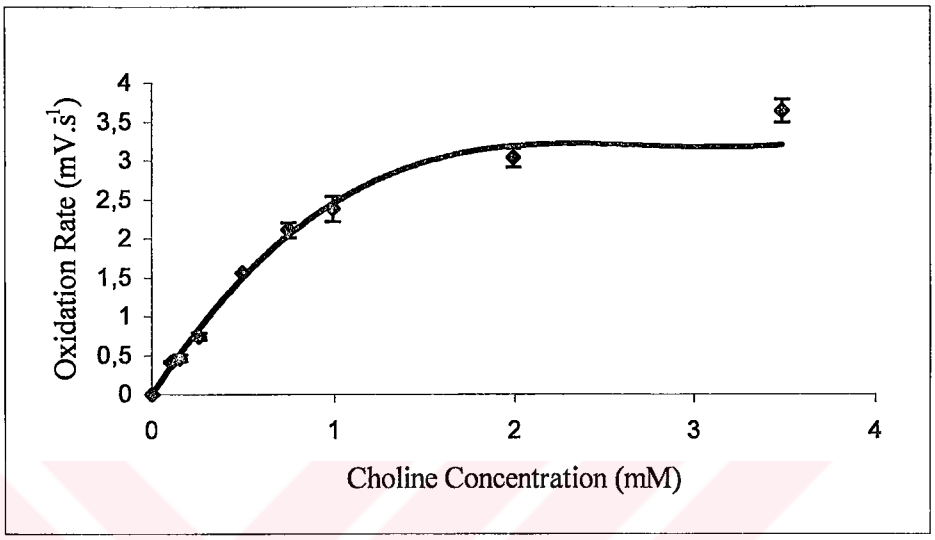


Figure 3.28. Michaelis-Menten plot for free choline oxidase activity

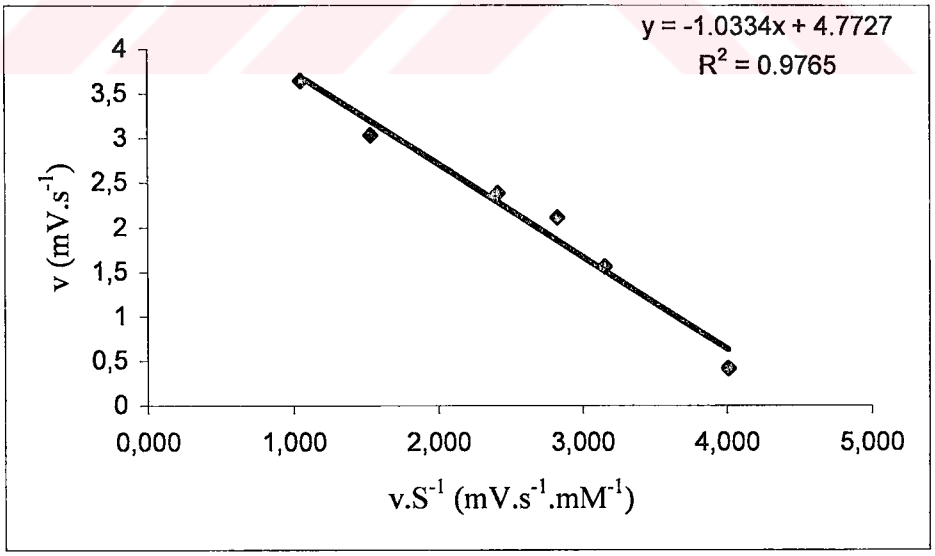


Figure 3.29. Eadie-Hofstee plot for free choline oxidase activity

3.7.2. Effect of Immobilization Concentration on the Activity of Choline Oxidase

The effect of concentration of choline oxidase used during immobilization was investigated using 5 and 2 mg/mL choline oxidase solutions during the enzyme immobilization step. For the determination of K_m and V_{max} values, oxidation rate vs concentration plots (the representative plots in Figures 3.30 and 3.32) and Eadie-Hofstee plots (the representative plots in Figures 3.31 and 3.33) were constructed for the duplicates of each membrane immobilized in different concentrations. The obtained K_m and V_{max} values, specific activity and the protein amounts are given in Table 3.6.

Table 3.6. Effect of choline oxidase concentration on the kinetic parameters, specific activities and immobilized protein contents

Choline oxidase concentration (mg/mL)	V_{max} ($mV.s^{-1}$)	K_m (mM)	Specific Activity ($mV.s^{-1}.\mu g^{-1}$)	Protein Content (μg/membrane)
2	0.243 ± 0.003	0.446 ± 0.024	3.65×10^{-3} $\pm 0.50 \times 10^{-3}$	64 ± 16
5	0.202 ± 0.049	0.595 ± 0.059	1.15×10^{-3} $\pm 0.21 \times 10^{-3}$	158 ± 10

V_{max} and specific activity values were 1.2 fold and 3.2 fold higher for the membrane subjected to immobilization in 2 mg/mL while there was a lower enzyme content on the membrane. It is possible that a portion of the enzyme is probably confined to the region between an upper layer of protein and the polymer membrane and could not easily take part in the reaction with the substrate. K_m value is 1.33-fold higher for the membranes subjected to immobilization in 5 mg/mL. The high amount

of enzyme on the membrane probably leads to a crowding of the enzymes resulting in the diffusional restriction of the substrate and the product reaction.

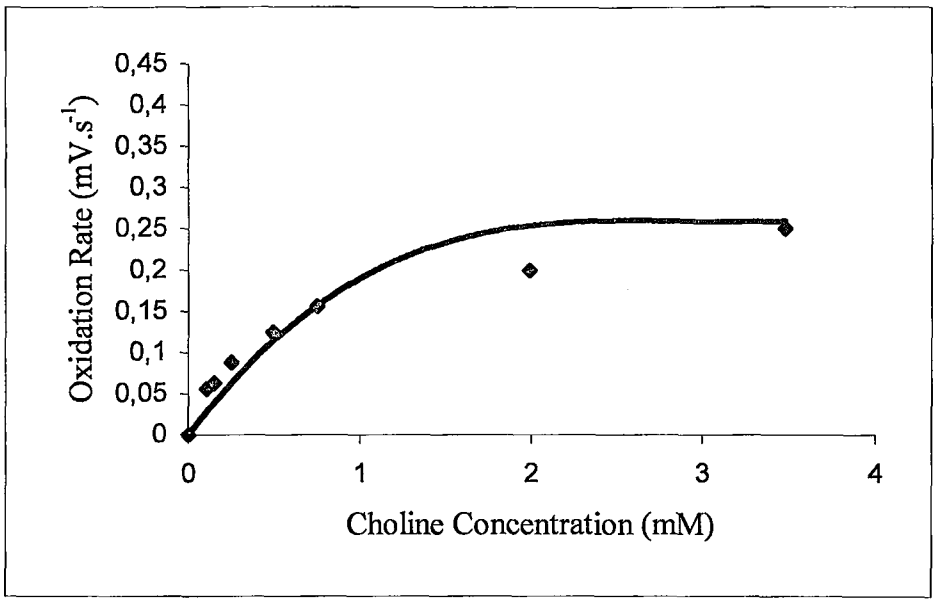


Figure 3.30. Michaelis-Menten plot for the activity of the membrane immobilized in 2 mg/mL choline oxidase solution

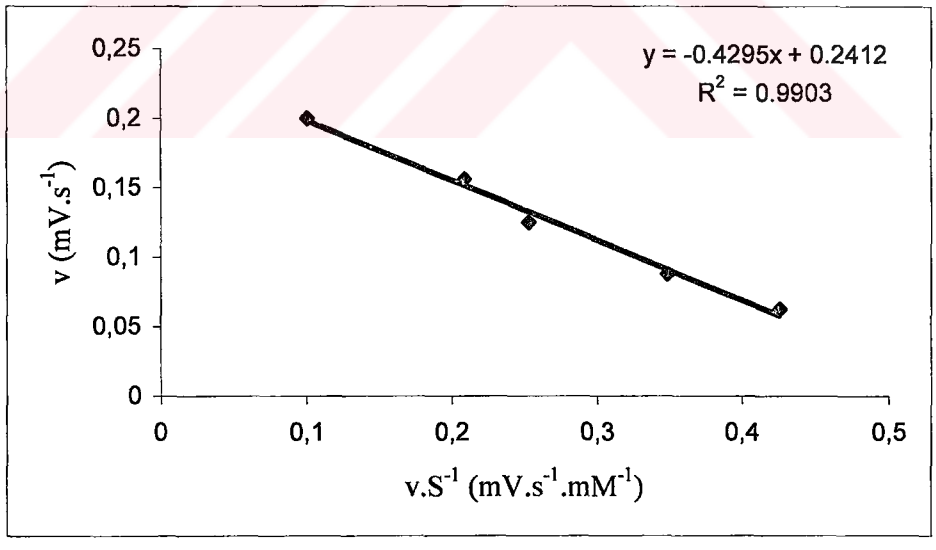


Figure 3.31. Eadie-Hofstee plot for the activity of the membrane immobilized in 2 mg/mL choline oxidase solution

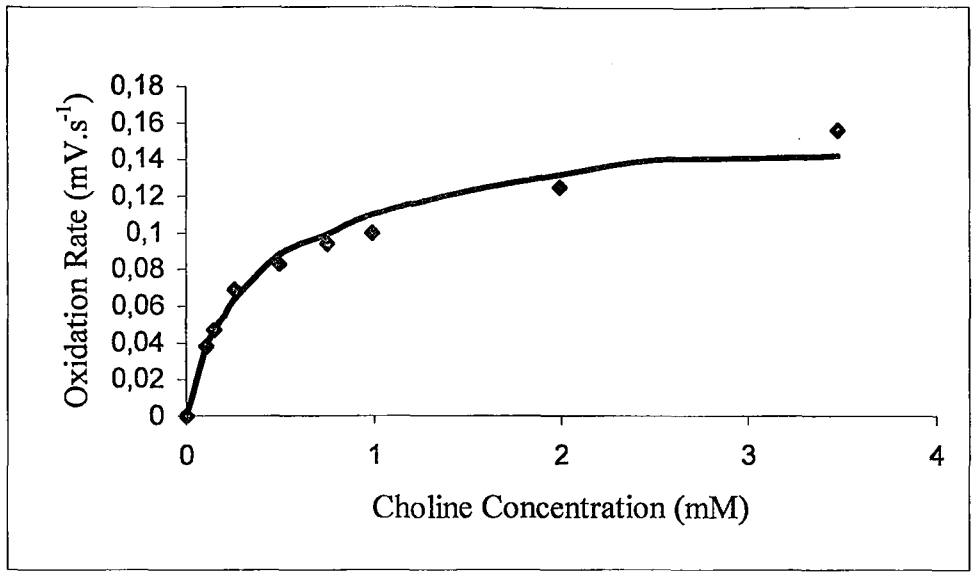


Figure 3.32. Michaelis-Menten plot for the activity of the membrane immobilized in 5 mg/mL choline oxidase solution

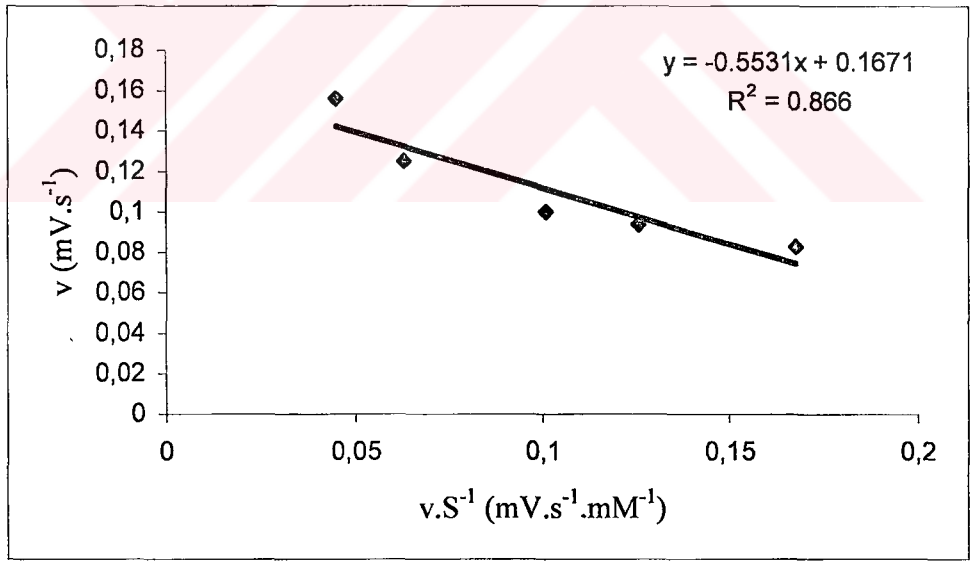


Figure 3.33. Eadie-Hofstee plot for the activity of the membrane immobilized in 5 mg/mL choline oxidase solution

Cremisini et al [1995] immobilized choline oxidase on polymeric membrane with polyazetidine prepolymer and tried different enzyme loadings to find the optimum operating conditions. There was an increase in the sensitivity of the biosensor with an increase in enzyme density up to a certain level, after which a sharp decrease was observed.

3.7.3. Reusability

The activity of the immobilized choline oxidase was measured using a 0.348 mM choline concentration for 45 successive runs conducted in 8 h and the results obtained with two separate runs are given in Figures 3.34 and 3.35. The fluctuations in the signal appeared to be 5% (based on the calculation from these data). The decrease in the activity of choline oxidase biosensor started after 5 measurements and it dropped to 50% of the original response after 30 measurements in quite a linear fashion. The loss in the activity could be due to damage during each wash or due to direct contact with the magnet while stirring the reaction medium. It could also be simple aging due to hydrolysis.

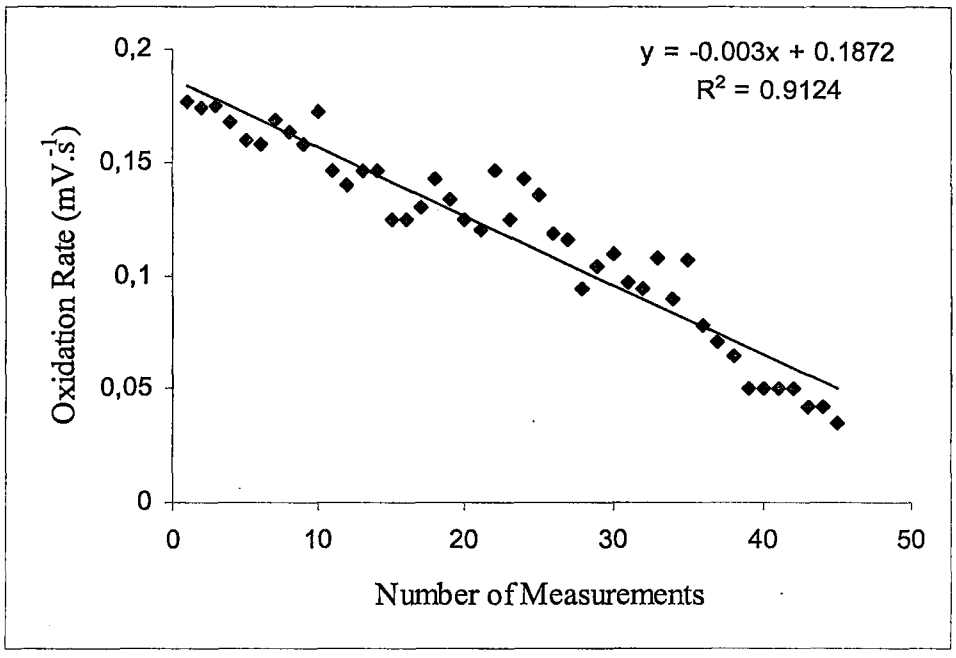


Figure 3.34. Reusability of the Glu- immobilized choline oxidase membrane (Run 1)

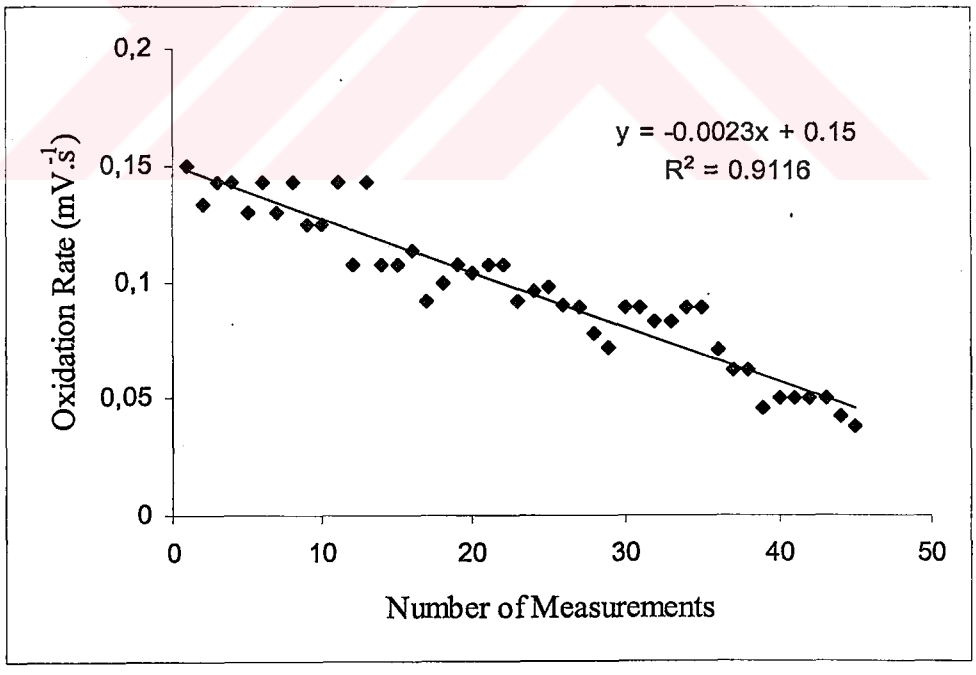


Figure 3.35. Reusability of the Glu- immobilized choline oxidase membrane (Run 2)

3.7.4. Linear Working Range

To determine the linear working range of the biosensor, the oxidation rate vs choline concentration was plotted (Figures 3.36 and 3.37). Plotting Δ (oxidation rate) / Δ (substrate concentration) vs substrate concentration (Figures 3.38 and 3.39), it was observed that the calibration curve was linear in the range 0.052-0.348 mM. Considering the standard deviation (5%) found in the reusability studies, the minimum detection limit (twice the value of standard deviation) was found as 40 ± 5 μM choline concentration for the constructed choline biosensor. The response higher than 10% was due to the presence of the choline in the reaction medium, while the lower values could be due to the fluctuations in response. During linear working range studies the average response time was determined as 22 ± 6 s.

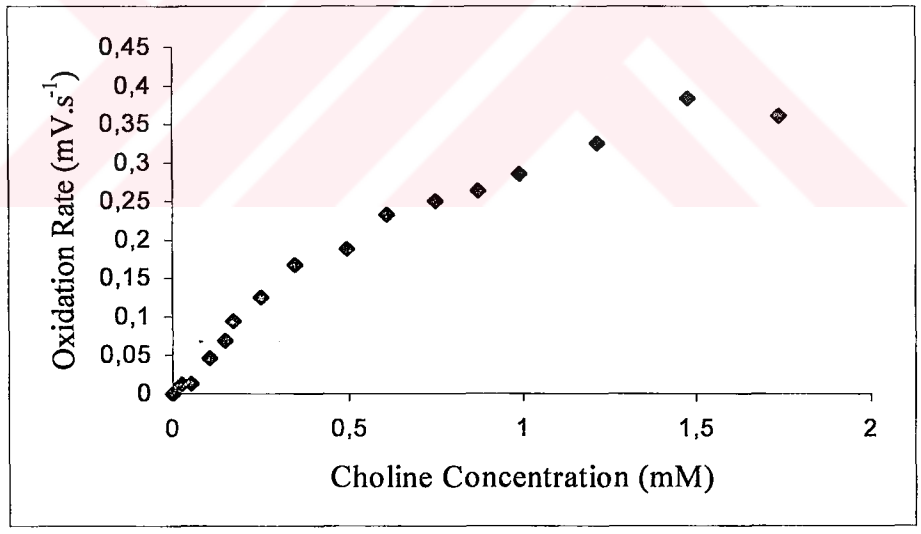


Figure 3.36. The change in the activity of the immobilized choline oxidase with choline concentration (Run 1)

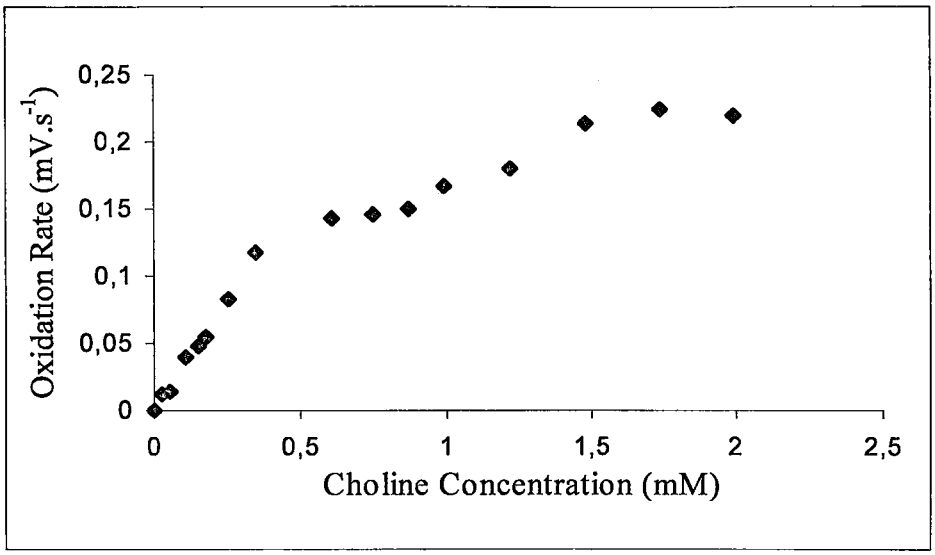


Figure 3.37. The change in the activity of the immobilized choline oxidase with choline concentration (Run 2)

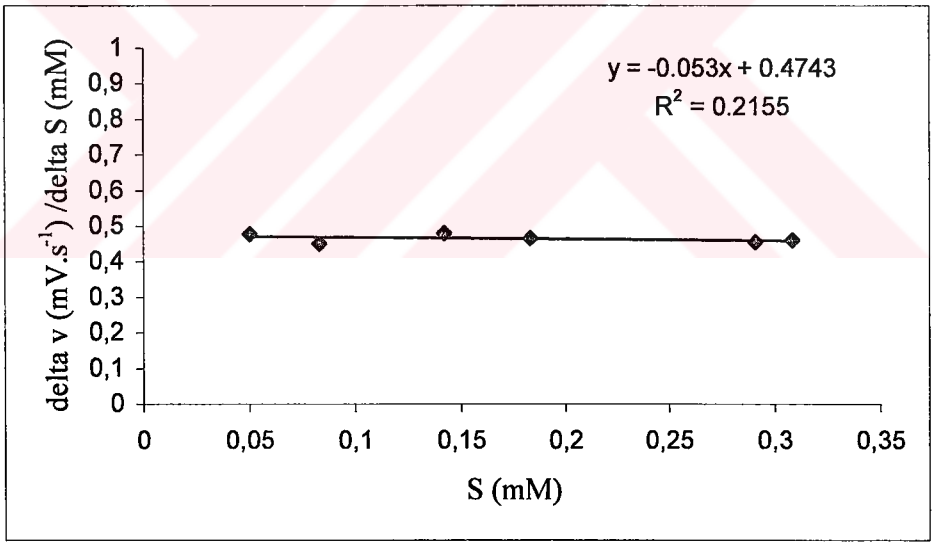


Figure 3.38. Plot of Δ (oxidation rate) / Δ (substrate concentration) against substrate concentration (Run 1)

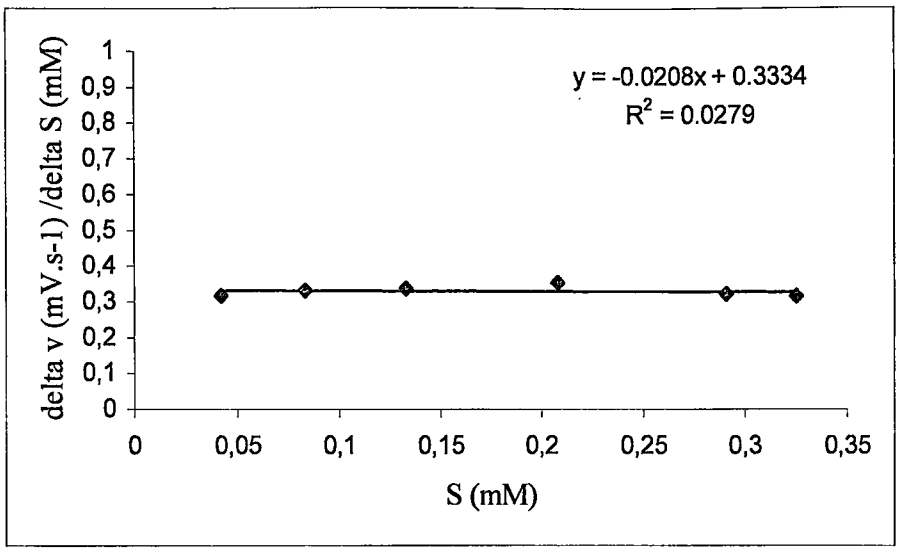


Figure 3.39. Plot of Δ (oxidation rate) / Δ (substrate concentration) against substrate concentration (Run 2)

Ruiz et al [1993] constructed two calibration curves for two different choline oxidase loadings and linearity range was reported as 1-10 mM for 0.30 $U.cm^{-2}$ and 1-13 mM for 0.382 $U.cm^{-2,}$ with a detection limit of 50 μM choline. Although their linear working range was more extended than ours it was in a much higher concentration region than the present study, and their minimum detection limit was 25% higher.

Choline oxidase was entrapped in photocrosslinkable poly(vinyl alcohol) bearing styrylpyridinum [Marty et al., 1989]. Based on hydrogen peroxide detection, the choline biosensor gave a linear response in the range of $2.5.10^{-3}$–0.15 mM choline with an average response time of 2 min. In the other study, choline oxidase

was co-immobilized with catalase on a nylon net via glutaraldehyde and cyclohexyl isocyanide by Vrbova et al [1993]. Using a Clark type oxygen electrode, the range of linear biosensor response to the choline was found as $3.34.10^{-3}$–0.167 mM. Doretti et al in 1994 constructed an amperometric choline biosensor immobilizing choline oxidase on poly (2-hydroxyethyl methacrylate) membranes and obtained linear working ranges of 0.01-0.2 mM and 0.005-0.25 mM choline concentration with an oxygen and an hydrogen peroxide probe, respectively. The reported linear working ranges by Marty et al. [1989], Vrbova et al. [1993], and Doretti et al. [1994] obtained results quite close to that in the present study. The upper and the lower limits of their linear range are lower than ours, but the linear working range reported in this study was more extended (broader) than theirs. In addition, Ricci et al. [2003] immobilized choline oxidase onto Prussian Blue modified, screen printed electrodes with the crosslinking method using glutaraldehyde and BSA. The linear range of the sensor was found as $5.0.10^{-4}$-0.1 mM by hydrogen peroxide detection. The constructed biosensor had a lower detection limit ($5.0.10^{-4}$ mM) and a lower upper limit (0.1 mM) than the current study (0.348 mM) and the linear range found in our study (0.052-0.348 mM) is broader than the linear range reported in Ricci et al. [2003].

In the Ph.D. thesis of Kök [2001], acetylcholine oxidase and choline oxidase were co-immobilized on a pHEMA membrane. Several immobilization methods were tested to achieve immobilization of both the enzymes. It was observed that acetylcholine could bind covalently to the membrane via glutaraldehyde or epichlorohydrin, but immobilization of choline oxidase was not successful. The immobilization of choline oxidase was achieved via complexation with a dye,

Cibacron blue F36A. However, in our study it was observed that choline oxidase could be immobilized via glutaraldehyde or epichlorohydrin.

The specific activity retention after immobilization was 20% for glucose oxidase and 10% for choline oxidase immobilized membranes (stretched on the electrode). This decrease in specific activity was probably due to the influence of covalent immobilization on the enzyme conformation.

CHAPTER 4

CONCLUSIONS

The goal of the study was the construction of an amperometric choline oxidase biosensor for choline determination. The Teflon membrane of the oxygen electrode unit was used as a support for choline oxidase immobilization to improve diffusional restrictions by using it as stretched directly on the electrode. Teflon membranes were grafted with 2-hydroxyethyl methacrylate in the presence of FAS by gamma irradiation. HEMA grafted membranes were activated with epichlorohydrin or glutaraldehyde. Choline oxidase and the model enzyme (GOD) and protein (BSA) were covalently immobilized onto the activated membranes.

SEM analysis showed that HEMA grafting could be maintained mainly on one side of the Teflon membrane. During optimization stage, BSA, a model protein, was immobilized on activated membranes to see the success of immobilization. However, qualitative and quantitative protein determination studies revealed that low amount of protein can be immobilized on the membrane. To check the activity of the enzyme immobilized membrane, glucose oxidase, a model enzyme, was immobilized

covalently on the grafted membrane and the activity was measured with o-dianisidine assay and also with oxygen electrode unit. However, no activity could be detected with each approach. It was concluded that the enzyme amount on the membrane was not sufficient to determine enzyme activity. It was thought the membrane preparation steps should be improved because the reason for the low immobilized enzyme amount could be the removal of HEMA grafted part of the membrane from the Teflon during washing with scrubbing.

Glucose oxidase was immobilized on the activated final thin membranes to be used in activity studies with oxygen electrode to optimize catalytic membrane preparation. The immobilized glucose oxidase activity studies, with oxygen electrode by placing electrode simply into the sample unit, revealed that the membrane activated with epichlorohydrin for 30 min, in the absence of EGDMA has better activity with high specific activity ($5.76.10^{-4}$ $mV.s^{-1}.\mu g^{-1}$) compared to the membranes activated for 2 h with epichlorohydrin and prepared with EGDMA. Also, it was found that membrane coupled with glutaraldehyde has better activity properties with high specific activity ($4.05.10^{-3}$ $mV.s^{-1}.\mu g^{-1}$). It was observed that the activity properties improved upon stretching the membrane over the electrode rather than leaving to more freely in the unit. The specific activity of the immobilized glucose oxidase activity increased about 3.7 fold ($1.83.10^{-2}$ $mV.s^{-1}.\mu g^{-1}$) by stretching on the electrode.

It was found that the membranes exposed to immobilization in 2 mg/mL choline oxidase solution had better activity properties with high specific activity value ($3.65.10^{-3}$ $mV.s^{-1}.\mu g^{-1}$) compared to membranes in 5 mg/mL choline oxidase solution. It was observed that the constructed choline biosensor had a linear working

range as 0.052-0.348 mM, with 40 μM minimum detection limit. Although there was a decrease in biosensor response through several successive measurements, the constructed choline oxidase biosensor can be used in choline determination studies with its broad linear working range.

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APPENDIX A

REAGENTS OF LOWRY METHOD

a. Alkaline Copper Reagent

Alkaline reagent was composed of the reagents:

- Reagent I : 2% Copper sulfate ($CuSO_4.H_2O$)
- Reagent II : 2% Sodium potassium tartrate ($NaKC_4H_4O_6.4H_2O$)
- Reagent A : 2% Sodium carbonate (Na_2CO_3) in 0.1 M sodium hydroxide (NaOH)

Alkaline copper reagent was prepared freshly by mixing the reagents indicated above in order as Reagent I (0.5 mL), Reagent II (0.5 mL) and Reagent A (50 mL).

b. Folin-Ciocalteau Phenol Reagent

Folin-Ciocalteau Phenol Reagent (1 N) was prepared by diluting (1:1) commercially available Folin-Ciocalteau Phenol Reagent (2 N) with distilled water immediately before use.

APPENDIX B

REAGENTS OF BRADFORD METHOD

Bradford Reagent

Coomassie Brilliant blue G (100 mg) was dissolved in ethanol (50 mL, 96%) by mixing for 6 h at room temperature in dark because Coomassie Brilliant blue G is light sensitive. Then, orthophosphoric acid (100 mL, 85%) was added and the reagent was mixed for overnight and kept in dark at room temperature. Bradford stock solution was diluted to 1 L with distilled water and filtered before use.

APPENDIX C

CALIBRATION CURVES

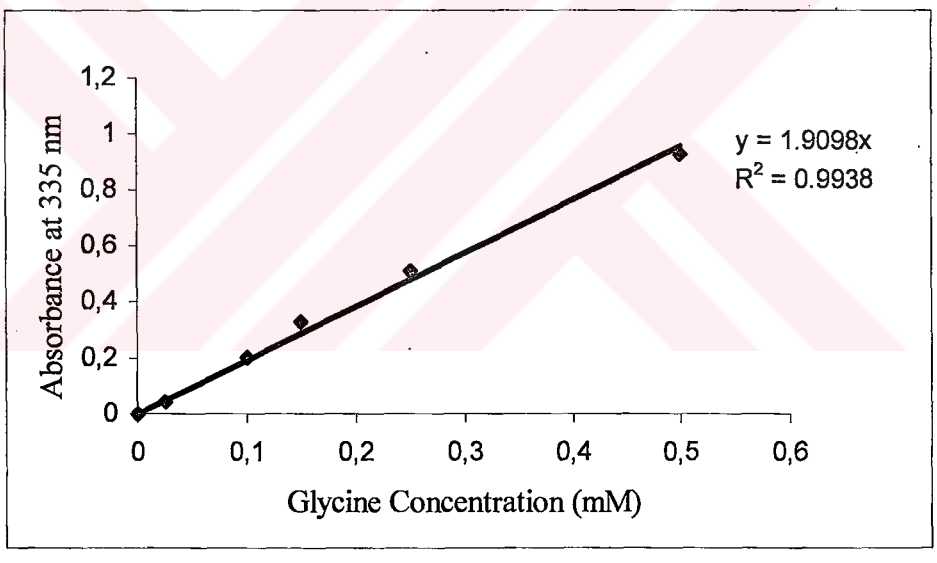

Figure C.1. Calibration curve of glycine by amino group determination

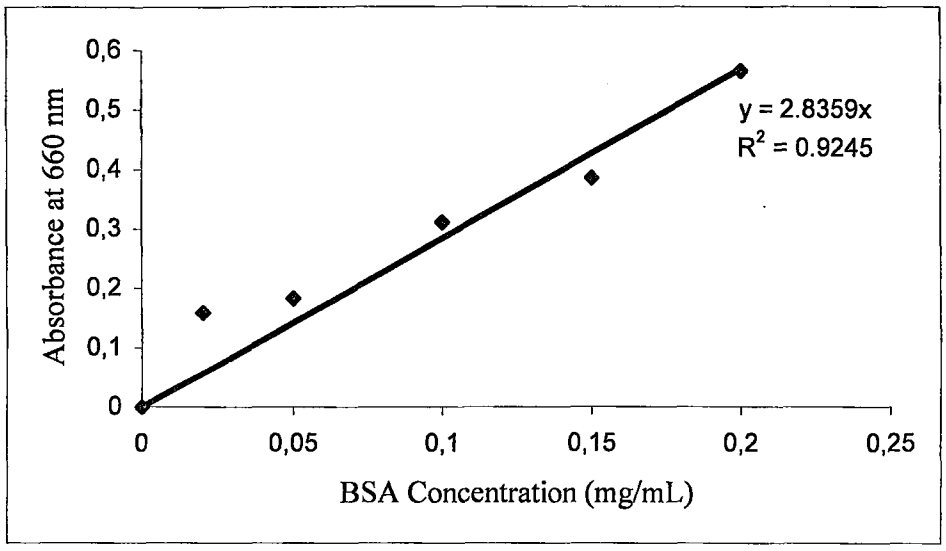

Figure C.2. Calibration curve of BSA by Lowry method

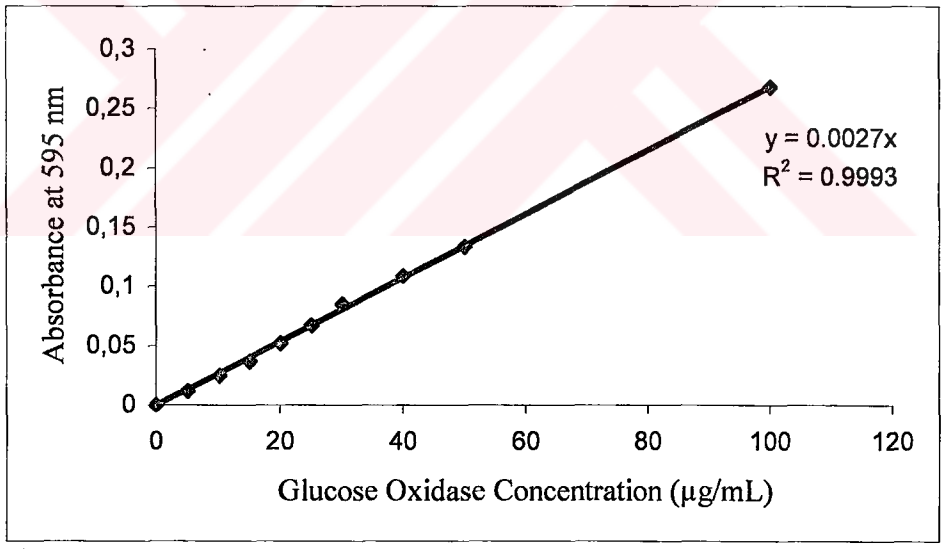

Figure C.3. Calibration curve of glucose oxidase by Bradford method

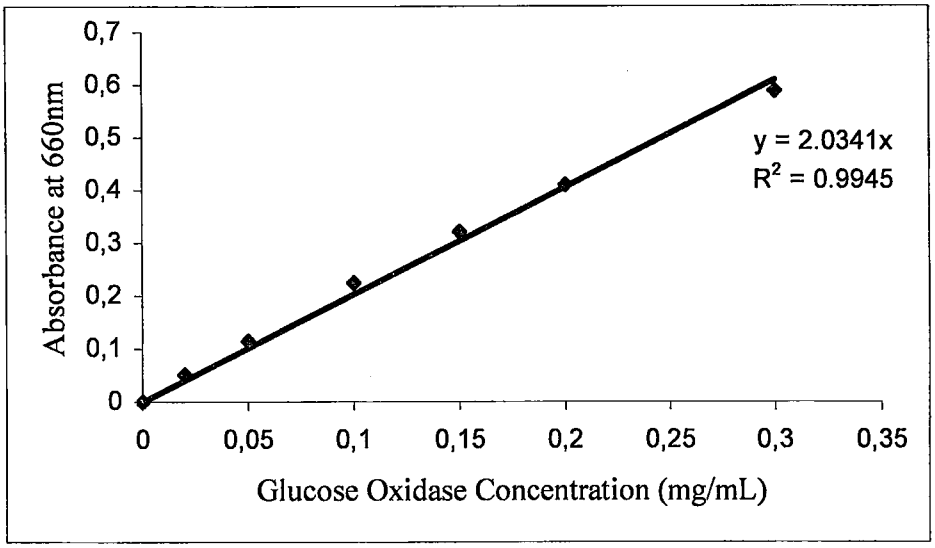

Figure C.4. Calibration curve of glucose oxidase by Lowry method

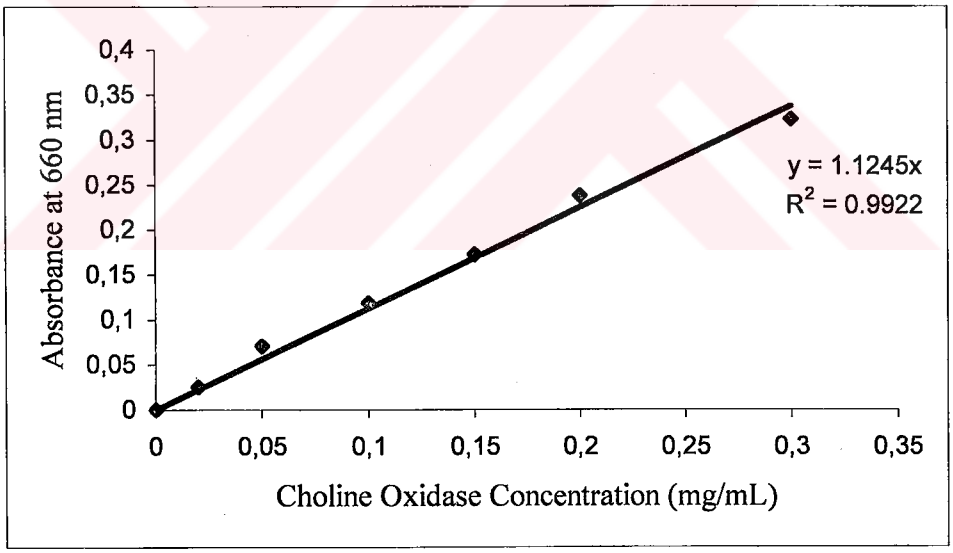

Figure C.5. Calibration curve of choline oxidase by Lowry method