CHOLESTEROL OXIDASE BIOSENSORS BASED ON POLYMER NETWORKS OF CHITOSAN/ALGINIC ACID AND CHITOSAN/p(TOLUENESULFONICACID)

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ABSTRACT

CHOLESTEROL OXIDASE BIOSENSORS BASED ON POLYMER NETWORKS OF CHITOSAN/ALGINIC ACID AND CHITOSAN/p(TOLUENESULFONICACID)

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By mixing different stoichiometric ratios of chitosan with alginic acid (AA) and chitosan with p(toluenesulfonicacid) (PTSA), two new polymer networks were prepared. FT-IR spectroscopy results show the protonation of chitosan by AA and PTSA. Elemental analysis (EA) results show the composition of the networks. Thermal gravimetry analysis (TGA) and differential scanning calorimetry (DSC) results were used to characterize the thermal stability of the networks. Then, cholesterol oxidase (ChOx) enzyme were immobilized in these networks and checked for potential use of these enzyme entrapped polymer networks (EEPN) for enzyme immobilization.

Additionally, the maximum reaction rate (Vmax) and Michaelis-Menten constant (Km) were evaluated for immobilized ChOx in these two polymer networks. Also, temperature and pH optimization, operational stability, shelf-life and the proton conductivity of these networks were investigated.

Keywords: chitosan, alginic acid, p(toluenesulfonicacid), cholesterol oxidase, enzyme immobilization.

ÖZ

CHITOSAN/ALGİNİK ASİT VE CHITOSAN/p(TOLUENESULFONİKASİT) İLE ELDE EDİLEN POLİMER AĞLARININ KOLESTEROL OKSİDAZ BİYOSENSÖRÜ OLARAK KULLANILMASI

Yapar, Elif

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Şubat 2012, 71 pages

Bu çalışmada chitosan farklı stokiometrik oranlarda alginik asit (AA) ve p(toluenesulfonikasit) (PTSA) ile karıştırılarak iki yeni polimer ağı sentezlendi. FT-IR spektroskopisi sonuçları ile chitosanın AA ve PTSA tarafından protonlandığı, ısısal ağırlık ölçüm analizi (TGA) ve diferansiyel taramalı kalorimetre (DSC) ile polimer ağlarının ısısal dayanıklılığı ve elemental analiz (EA) ile polimer ağlarının kimyasal içerikleri karakterize edildi. Bu ağlara kolesterol oksidaz enzimi tutuklanarak enzim tutuklanmış polimer ağları (EEPN) elde edildi ve enzim tutuklaması işlemine uygunlukları gözlemlendi.

Farklı iki ağda tutuklanmış kolesterol oksidaz enziminin maksimum reaksiyon hızı (Vmax) ve Michaelis-Menten sabiti (Km) ayrı ayrı hesaplandı. Ayrıca, uygun sıcaklık ve pH değerleri, kararlılığı, raf ömrü ve proton iletkenlikleri yine iki farklı ağ için tayin edildi.

Anahtar Kelimeler: chitosan, alginik asit, p(toluenesulfonikasit), kolesterol oksidaz, enzim tutuklanması.

To My Family

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF SCHEMES

SCHEMES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ChOx: Cholesterol oxidase

HRP: Horseradish peroxidase

AA: Alginic acid

PTSA: p-toluenesulfonic acid

GAA: Glycial acetic acid

EEPN: Enzyme entrapped polymer network

Vmax: Maximum reaction rate

Km: Michaelis-Menten constant

DTA: Differential thermal analysis

EA: Elemental analysis

DSC: Differential scanning calorimetry

HTPCs: High temperature proton conductors

CHAPTER I

INTRODUCTION

1.1 Enzymes

An enzyme is a biocatalyst that increase the rate of reaction by decreasing the energy of activation. There are hundreds of different kinds of enzymes, and each one reacts only with one specific substrate. Almost all enzymes are proteins, so a knowledge of protein structure is prepequisite to any understanding of enzymes.

1.1.1 Structure of Proteins

All proteins consist of series of amino acid units (Figure 1.1) in the specific sequence determined by the structure of the genetic material of the cell. Some proteins are composed of these amino acid building blocks entirely and are termed simple proteins. Others, called conjugated proteins, contain extra material, which is firmly bound to one or more of the amino acid units. Enzymes may be either simple or conjugated proteins.

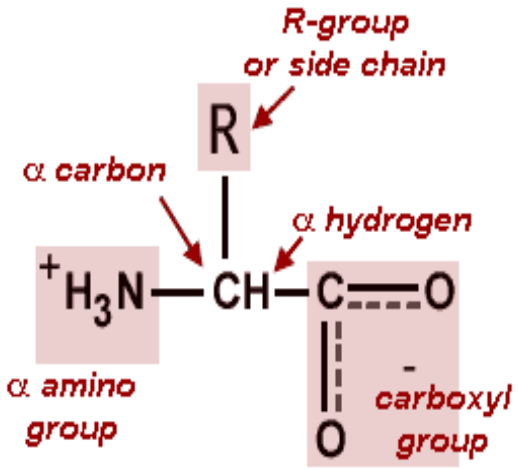


Figure 1.1 General structure of amino acids

Two distinct types are known: fibrous and globular proteins:

1- Fibrous, long and thin, proteins are insoluble in water and consist of amino acid units which their C-N bond can not rotate so (Figure 1.2) giving to the peptide unit NH-CO rigidity (Manz, et al. 2004). The neighbouring α-carbon atoms of rigid NH-CO, can rotate within steric constrains allowing to folding of proteins (Manz, et al. 2004). These types of protein tend to be structural.

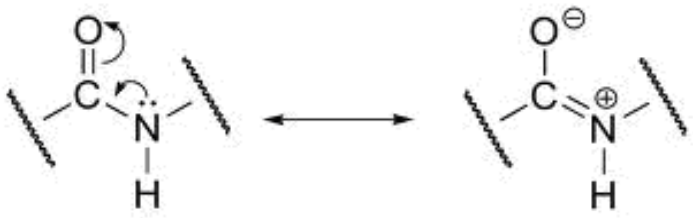


Figure 1.2 Double bond character of the C-N bond

2- Globular, spherical like a ball, proteins are water soluble and used for transport. They have very characteristic grooves and peaks on their surface so have compact structure.

Regular, repeating three-dimensional features constitute the secondary structure. This is largely uninterrupted in fibrous, structural proteins, but disrupted at many points in globular, functional proteins, including enzymes.

In living organisms, common 20 different amino acids found as a building blocks of peptides/proteins (Figure 1.3). They have properties of both bases and acids because of containing an amino group ($-NH_2$) and a carboxyl group (-COOH) in the same molecule.

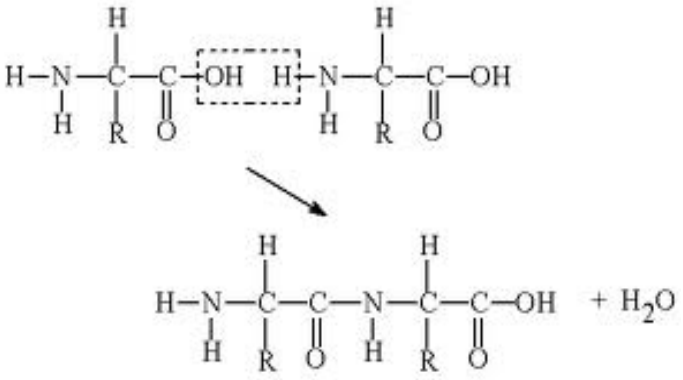


Figure 1.3 Peptide bond formation

Except the α-amino groups and α-carboxyl groups, N- and C-terminal respectively, contributing to the acid-base properties (Figure 1.4), all α-amino and α-carboxyl groups are not to ionize.

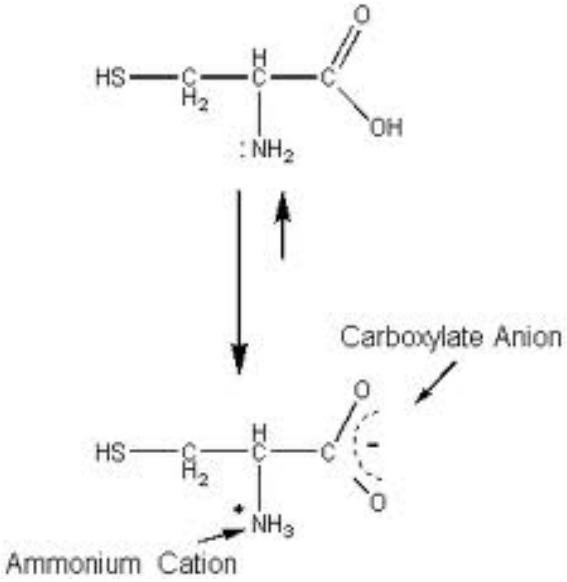


Figure 1.4 Acid-base properties of proteins

At acidic pH's the amino acid is charged positively; on the other hand, at basic pH's it becomes negatively charged. This is because of the carboxyl group has a pKa between 1.8 and 2.5, whereas the amino group has a pKa between 8.7 and 10.7. Hence,

total electrical charge present depends on the degree of dissociation of ionizable groups and thus on pH.

Because of isoelectric point, each enzyme has characteristic small range of optimum pH to be active. Isoelectric point is the pH of molecule having an equal balance between positive and negative charges on it (Palmer and Bonner 2007).

1.1.2 Structure and Properties of Enzymes

Enzymes are biological catalysts that increase the rate of chemical reactions. This is done by decreasing the activation energy (Figure 1.5). The energy needed to form the transition-state from the reactants is activation energy. They combine with the reactions to form more stable transition-state than that involved in the uncatalysed reaction, so of lower energy (Palmer and Bonner 2007).

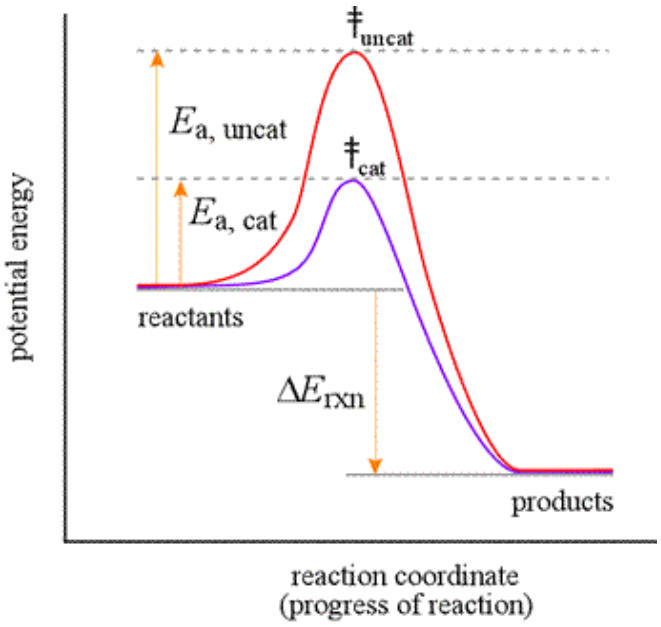


Figure 1.5 Energy of activation of uncatalyzed and catalyzed reactions

It has no overall thermodynamic effect : the amount of free energy liberated or taken up when a reaction has been completed will be the same whether a catalyst is present or not.

Many enzymes require cofactors to exert their catalytic. This active complex is the haloenzyme. Therefore, haloenzyme is an example of apoenzyme (protein portion) plus the cofactor.

Holoenzyme = Apoenzyme + Cofactor

To summarize diagrammatically and as a figure (Figure 1.6);

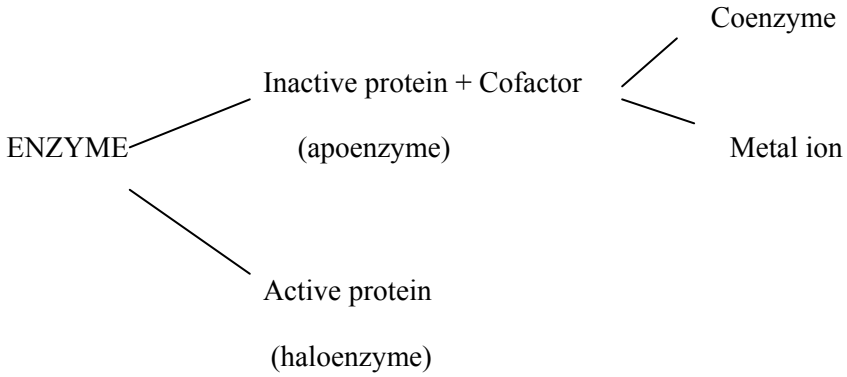


Figure 1.6 Active protein, haloenzyme, apoenzyme and cofactor relationship

1.1.3 The Naming and Classification of Enzymes

Except the proteolytic enzymes, usually end with ‘’-in’’, all enzyme names ending in ‘’-ase’’.

1.1.3.1 The Enzyme Commission’s Classification

The enzyme commission classified enzymes on the basis of the reaction catalysed. There are six main classes of enzymes. Each enzyme was assigned a four elements code number (Palmer and Bonner 2007).

(i) The main classes of the enzyme: the first number of the code

(ii) The subclass of the enzyme: the second number of the code

(iii) The sub-subclass: the third number of the code

(iv) The serial number of the enzyme in its sub-subclass: the fourth number of the code

The main classes are:

Class 1 – Oxidoreductases catalyses the transfer of electrons and O and H atoms from one substrate to other.

Class 2 - Transferases catalyze transfer of a group, i.e. functional group from one molecule to another in the general form (Carr and Bowers 1980) :

$$A\text{-}B + C = A + C\text{-}B$$

Class 3 – Hydrolases catalyze cleaving C-O, C-N, and C-S bonds by addition of OH^- and H^+ to the atoms.

Class 4 - Lyases catalyze cleaving C-C, C-O, C-N, and C-S bonds by elimination or adding groups to double bonds and , leaving double bonds or rings (www.chem.qmul.ac.uk).

Class 5 - Isomerases create isomers of the original reactant of the reaction.

Class 6 – Ligases synthesize C-N, C-C, C-O, and C-S bonds and coupled to the cleavage of some nucleotide or high energy phosphate bonds in ATP (Carr and Bowers 1980).

As an example, EC number of ChOx is;

EC 1, shows the class of the enzyme (oxidoreducatases)

EC 1.1 shows the donor (CH-OH group)

EC 1.1.3 shows the acceptor (oxygen)

EC 1.1.3.6 shows the substrate (Cholesterol)

In enzymology, a ChOx (EC 1.1.3.6) catalyzes the chemical reaction;

Cholesterol + Oxygen $\rightleftharpoons$ Cholest-4-en-3-one + H_2O_2

1.1.4 Specifity of Enzyme Action

Enzymes are specific in action and some enzymes will act only on one particular substrate, when they are said to exhibit absolute specifity, some enzymes exhibit group specifity. So, enzyme-catalysed reactions are product-specific as well as being substrate-specific (Palmer and Bonner 2007).

Also, in addition to showing chemical specifity, enzymes exhibit stereochemical specifity.

1.1.4.1 The Fischer ‘’lock-and-key ‘’ Hypothesis

Enzyme specifity means a substrate fit into the enzyme as a key fits into its complementary site on a lock (Figure 1.7) and structures do not change during the binding process. This is suggested by Emil Fischer as early as 1890 (Palmer and Bonner 2007).

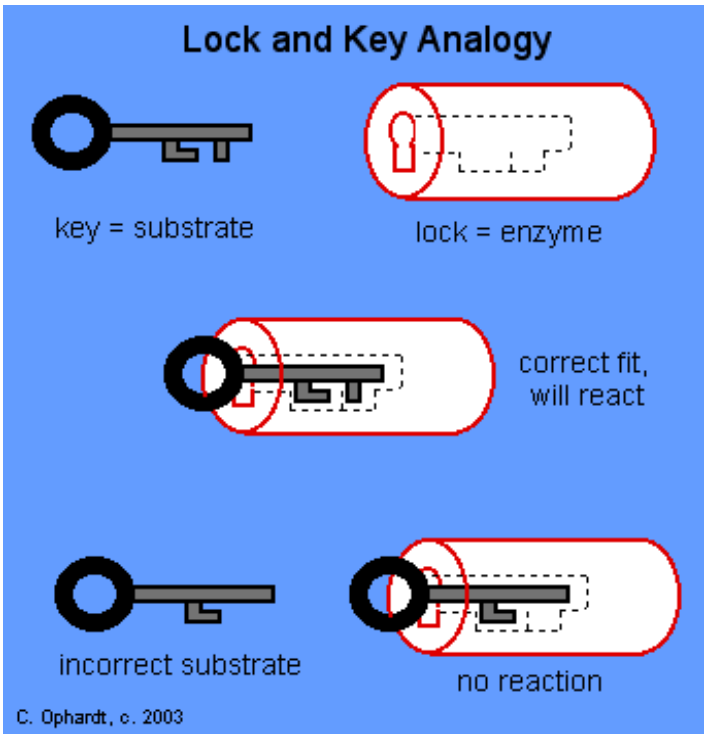


Figure 1.7 Diagramatic representation of the lock-and-key model

1.1.4.2 The Koshland ''Induced-Fit'' Hypothesis

Conformational change may bring about the binding a substrate to an enzyme (Palmer and Bonner 2007). However, the lock-and-key hypothesis does not give any information about the flexibility of proteins during the reaction.

In induced-fit hypothesis, Daniel Koshhland suggested that there is required conformational change in the enzyme to match substrate during the binding process (Figure 1.8).

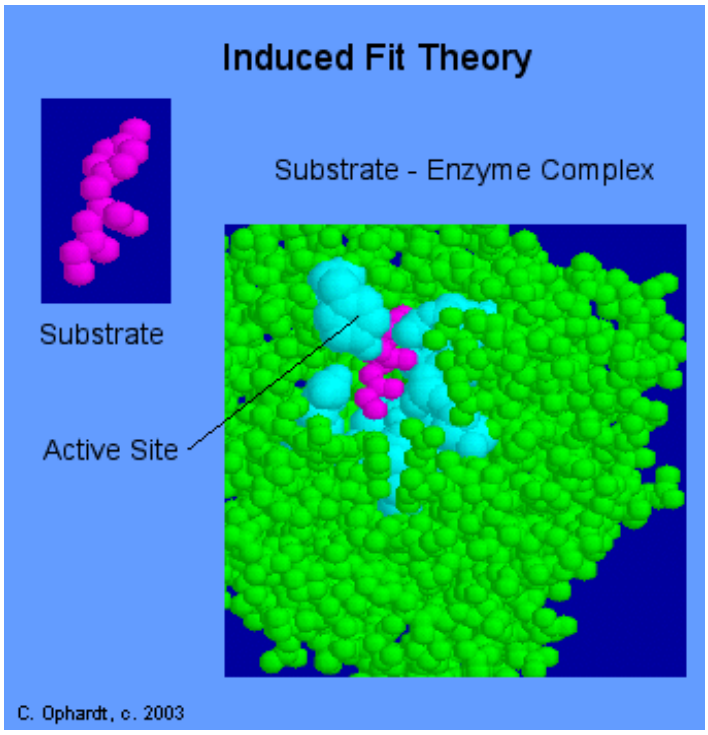


Figure 1.8 The representation of induced-fit hypothesis

1.1.5 Enzyme Activity

Catalytic effect of an enzyme is defined as enzyme activity. It is units (U) per miligram of enzyme or molecules of product formed per minute per molecule of enzyme (Medical Dictionary 2012). The overall rate of activity will be dependent on substance concentration (homepages.gac.edu). Because, enzymes and the substrate do not reacts. Actually enzyme brings its substrate into the proper configuration to react with another substance spontaneously.

The temperature effect on reaction rate is shown in (Figure 1.9). As the temperature rises, the movement of enzyme and substrate increases and results in increasing enzyme activity. However, the enzyme denaturation starts after increasing a certain point, and so no enzyme activity is observed (Mader 1996). The optimal temperature range for the commercial enzymes is between 40 ^{o}C and 60 ^{o}C (Mathewson 1998).

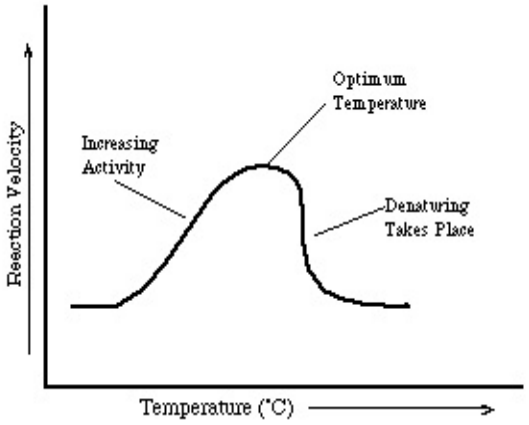


Figure 1.9 Effect of temperature on reaction rate (Worthington Biochemical Corporation. 2009)

pH effect on reaction rate is shown in Figure 1.10. As seen enzymes also have an optimal pH value for maintaining its configuration to exert its catalytic effect on a reaction (Mader 1996). Generally, commercial enzymes have an optimal pH range between 6.0 and 8.0. Otherwise, substrates do not bind and not react to produce a product.

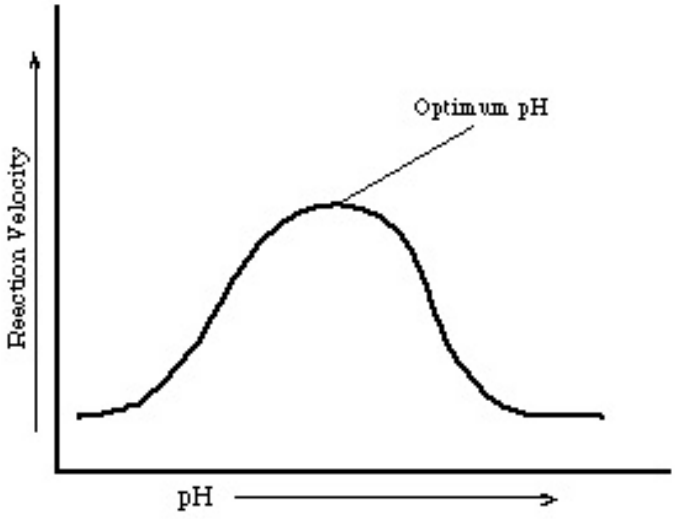


Figure 1.10 Effect of pH on reaction rate (Worthington Biochemical Corporation)

When the limiting factor of the reaction is enzyme, that is to say there is an enough substrate to saturate all enzymes, rate of reactions are directly dependent on the enzyme concentration. Then this reaction is independent of substrate concentration and becomes zero order reaction (Figure 1.11).

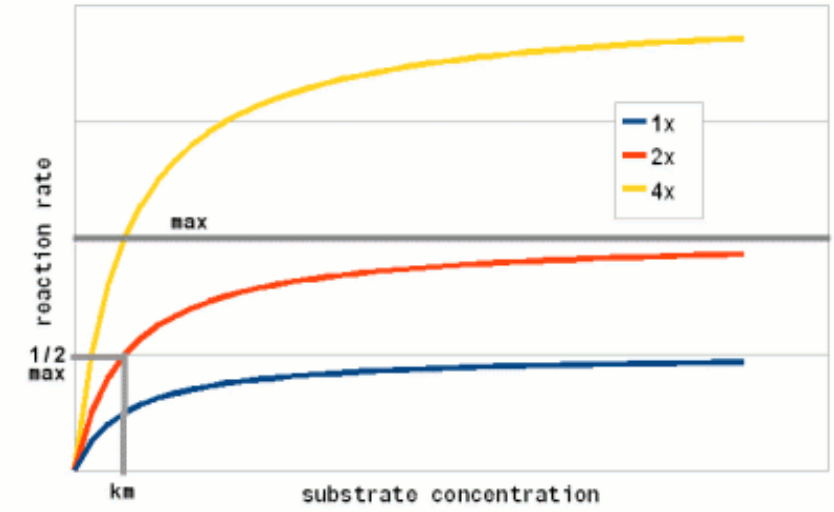


Figure 1.11 Dependence of rate of ‘’zero-order’’ reaction on enzyme concentration

Figure 1.11 shows the effect of three different enzyme concentrations (1x, 2x, and 4x) on reaction rate as a function of the substrate concentration. With an increasing substrate concentration reaction rate increases towards a maximum reaction rate and it has a direct dependence on the enzyme concentration. The reaction velocity will increase until it reaches a maximum point, when the amount of enzyme is constant. After maximum point, reaction velocity will not increase by increasing in substrate concentration. This is represented graphically in Figure 1.12.

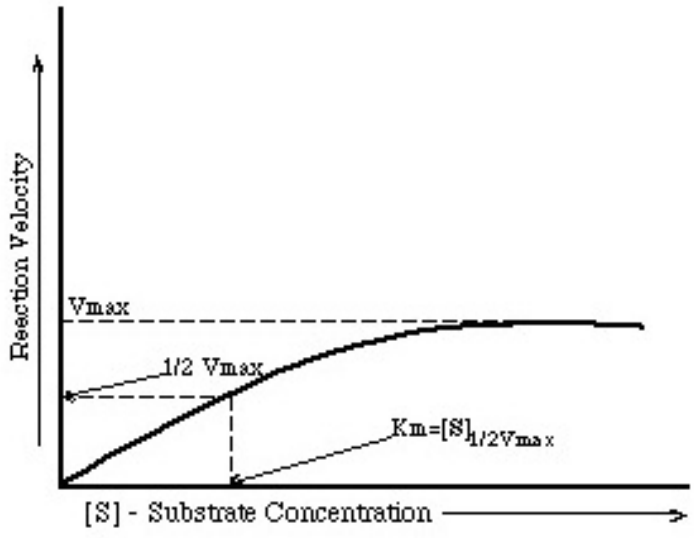


Figure 1.12 Effect of substrate concentration on rate of reaction

As a result, to see the effect of concentration of an enzyme on the reaction rate, temperature, pH, salt concentration and substrate concentration of reaction should be controlled. Each of them affects the rate of an enzyme reaction.

1.1.6 Enzyme Kinetics

Enzyme kinetics is the study of the working rate of the enzyme. Reaction rate of almost all enzyme catalysed reactions are first-order with respect to concentration of substrate at very low concentrations. However, rate will be zero-order (Figure 1.13) as the concentration of substrate increases gradually. In fact the rate will not affected by the substrate concentration.

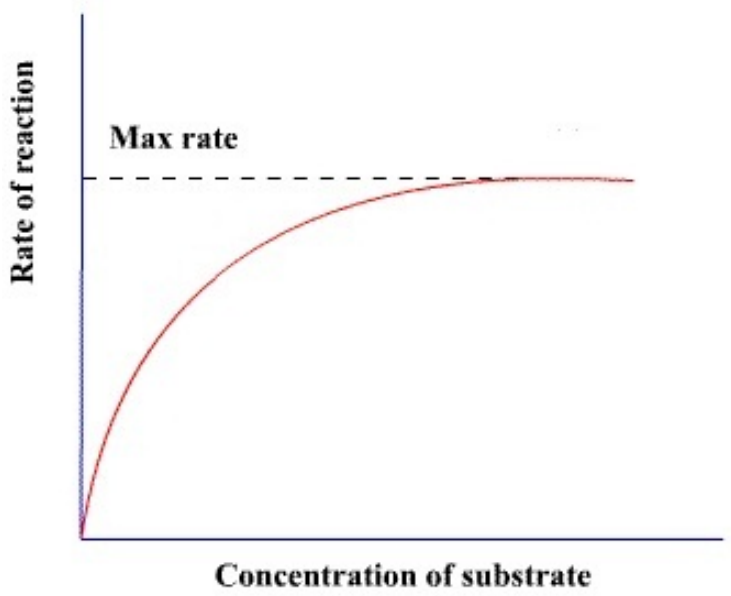


Figure 1.13 Rate behaviour according to substrate concentration for enzyme catalysed reaction

Michaelis and Menten explain this observation by being a mechanism supposes that the substrate (S) of the reaction binds to the enzyme (E) in the first step.

Then, reaction goes to form the product (P) and also the enzyme :

$$E+S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E+P \qquad (1)$$

Formation rate of product equals to the rate of formation of enzyme plus product from ES, which equals k_2 times [ES]. ES is not known, so this equation is not helpful. So, to solve ES two reasonable assumptions were used:

1- First assumption is that the [ES] is constant during the enzyme kinetic work (the formation rate of ES = the dissociation rate of ES).

2- Second assumption is that because of working at beginning of the reaction where the [P] is very low , formation of ES from enzyme + product is negligible.

Rate of ES formation = Rate of ES dissolution

$$k_1 \cdot [S] \cdot [E_{free}] = k_{-1} \cdot [ES] + k_2 \cdot [ES] \quad (2)$$

Total concentration of enzyme is known;

$[E]_{total} = [ES] + [E]$ and (3)

$[S]_{total} = [S] + [P]$ (4)

These two equations, 3 and 4, are for the mechanisms;

$$\frac{d[ES]}{dt} = k_1 [E][S] + (k_{-1} + k_2) [ES] \quad (5)$$

$$\frac{d[P]}{dt} = k_2 [ES] \quad (6)$$

[E], [S], [X] and [P] can not derived from these two equations as a function of time.

So, steady-state approximation is used;

$d[ES] / dt = 0$

So the equation 2 can be rewritten.

$$k_1 \cdot [S] \cdot ([E_{total}] - [ES]) = k_{-1} \cdot [ES] + k_2 \cdot [ES] \quad (7)$$

Solving for ES:

$$[ES] = \frac{k_1 \cdot [E_{total}] \cdot [S]}{k_1 \cdot [S] + k_2 + k_{-1}} = \frac{[E_{total}] \cdot [S]}{[S] + \frac{k_2 + k_{-1}}{k_1}} \quad (8)$$

By substituting this expression in equation 6, we can obtain

$$\frac{d[P]}{dt} = \frac{k_2[E_{total}]}{1 + (k_{-1} + k_2)/k_1[S]} \tag{9}$$

Therefore, enzymatic reaction has the velocity equation:

$$Velocity = k_2 \cdot [ES] = \frac{k_2 \cdot [E_{total}][S]}{[S] + \frac{k_2 + k_{-1}}{k_1}} \tag{10}$$

Finally,

$V_{max} = k_2 \times E_{total}$, and

$K_m = (k_2 + k_{-1}) / k_1$.

Substituting:

$$Velocity = V = \frac{V_{max}[S]}{[S] + K_m} \tag{11}$$

When we plot the graph of this steady-state kinetic data as a rate against the substrate concentration (Figure 1.14), we see that determination of kinetic parameter is difficult because of the curved line.

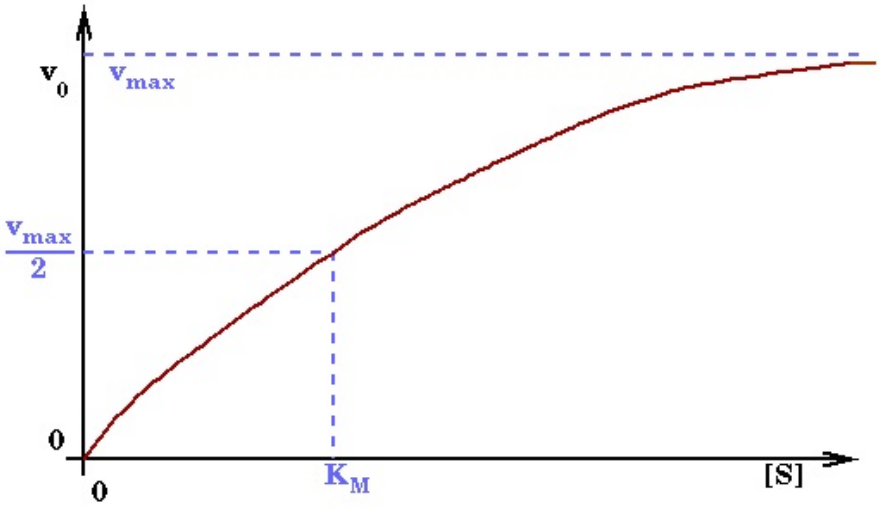


Figure 1.14 Michaelis-Menten plot (rate versus substrate concentration)

Hans Lineweaver and Dean Burk (1934) overcame this problem without making any assumptions. They simply look the Michaelis-Menten equation.

The Lineweaver-Burk Equation, is the equation of a straight line graph (y = mx + c) of plot of y against x has a slope of m and intercept on the y-axis (Figure 1.15) and obtained by taking reciprocals of V_{max} equation;

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]}$$

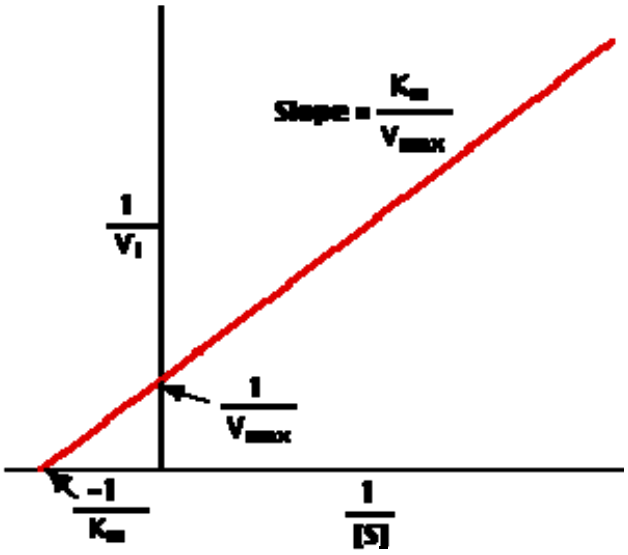


Figure 1.15 Lineweaver-Burk plot relating the 1/V to 1/substrate concentration

Because the reaction rate increases asymptotically with increasing substrate concentration approaching the maximum rate V_{max} , to characterize the enzyme concentrationi it is useful to choose the [S] at which the rate of reaction is at half of its maximum value $V_{max}/2$. This concentration is also equal to the Michaelis constant, K_m.

K_m is an inverse measure of the affinity of binding between the enzyme and its substrate so, the lower the K_m, the greater the affinity.

Assumptions of enzyme kinetic analyses:

1- The product production has linear relation with time.

2- The concentration of substrate is constant during the assay and exceeds the concentration of enzyme.

3- The product is formed by a single enzyme.

4- The spontaneous creation of product without enzyme is negligible.

5- There is no cooperativity between enzyme and the substrate.

1.1.7 Enzyme Immobilization

Immobilization of an enzyme is the attachment of an enzyme to the inert, insoluble support. ımmobilization can provide increased resistance to changes in conditions such as pH or temperature. And also allows enzymes to be stable in place during the reaction and separated from the products easily.

The advantages of immobilizing enzymes to a solid support are (Boyukbayram, et al. 2006):

- Repetitive usage of a single batch of enzymes
- Rapid termination of the reaction (continuous operational mode)
- Enhanced stability
- Enzyme is isolated from the product (isolation is important in the pharmaceutical and food industries)
- Separation of enzyme from product easily
- Low operation cost

Other than stability, retention of the activity of the immobilized enzyme should be considered. There is a loss of enzyme activity in the immobilization process because of the interaction with the matrix. Hence, immobilization method should be chosen carefully.

1.1.7.1 Immobilization Methods

Immobilization methods are showed schematically in Figure 1.16 (Kim, Korea University).

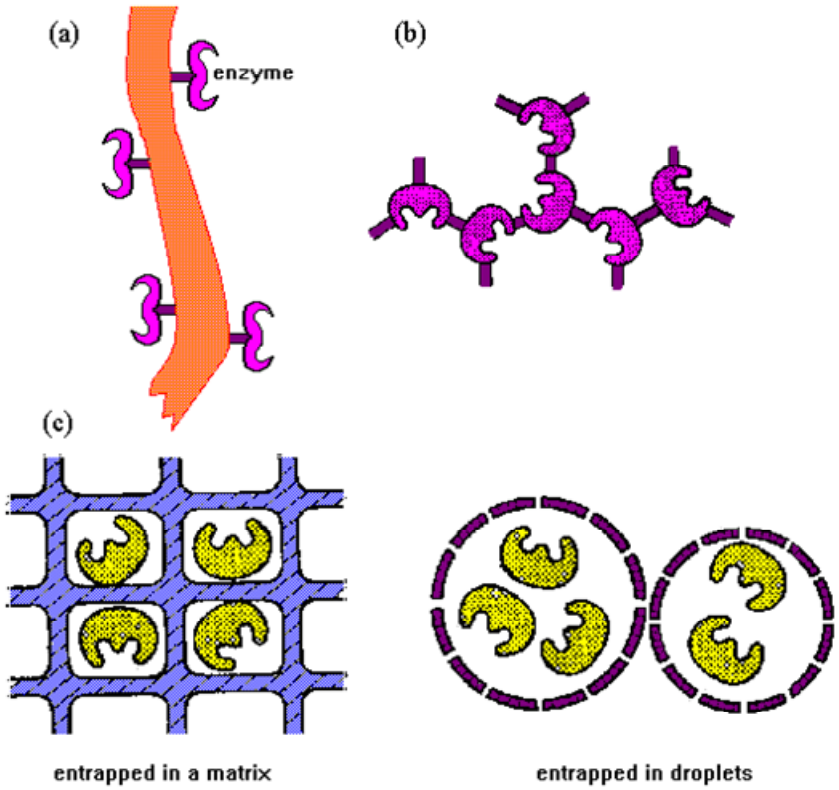


Figure 1.16 Enzyme immobilization methods

1.1.7.1.1 Carrier Binding Method

It is the method of binding of enzymes to water-insoluble carriers. In this method, binding techniques and the selection of the carriers is very important. According to the binding mode, this method can be classified into three as adsorption, ionic binding and covalent binding.

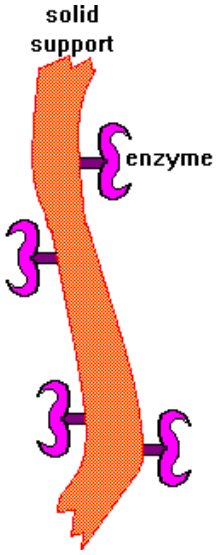


Figure 1.17 Carrier-binding method

1- physical adsorption: in this method enzyme is adsorbed on the surface of water-insoluble carriers physically.

2- ionic binding: in this method enzyme binds to water-insoluble carriers ionically.

3- covalent binding: in this method enzyme binds to the water-insoluble carriers covalently.

Table 1.1 Advantages-Disadvantages of carrier-binding methods

Method	Advantages	Disadvantages
Physical adsorption	♦ Little or no conformational change of the enzyme active center ♦ Simple and cheap	♦ Non-specific
Ionic binding	♦Little changes in the conformation and the active site of the enzyme ♦ High activities in most cases	♦Leakage of enzymes from the carrier may occur in substrate upon variation of pH
Covalent binding	♦ The wide range of choices is possible by selecting carrier materials and binding method. This allows a great deal of flexibility in designing an immobilized enzyme	♦ Relatively expensive ♦ Active site may be modified through the chemical reactions used to create covalent bonding.

1.1.7.1.2 Cross-Linking Method

In this method, the immobilization of enzymes is performed by the formation of intermolecular cross-linkages between the enzyme molecules, so based on the formation of chemical bonds.

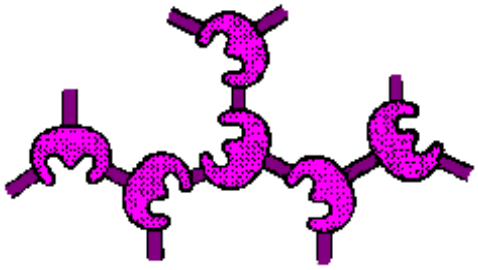


Figure 1.18 Cross-linking method

Advantages: Enzyme strongly bound, so desorption occurs very little.

Disadvantages: May cause significant changes in the active site of enzymes and also severe diffusion limitation and preparation steps may lead to significant loss of enzyme activity.

1.1.7.1.3 Entrapping Method

This method has wide applicability. Because other than covalent binding and cross-linking method, enzyme does not bind to the matrix or membrane in this method. This method is based on confining enzymes in the lattice of a polymer matrix or enclosing enzymes in semipermeable membranes.

This method can be classified into the lattice and micro-capsule types.

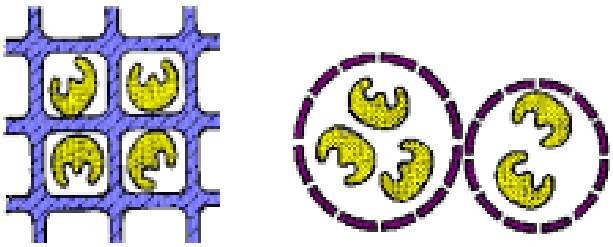


Figure 1.19 Entrapping method

Advantages: Only physical confinement of enzyme.

Disadvantages: High diffusion barrier.

Immobilization of an enzyme has many advantages over using of free enzyme, such as easier fabrication, better operational stability and longer-term stability. Good operational stability is premise of accurate measurement for repetitive uses and long-term life-time, beneficial to biosensor transport and storage (Kıralp, et al. 2003).

Although immobilization of an enzyme has some important advantages, they also has specific disadvantages. Therefore, it is important to find a suitable method and conditions for the immobilization of a particular enzyme for the intended application.

1.2 Biosensors

Biosensors are chemical sensing device that used for conversion of a biochemical signal into quantifiable electric signal as a part of the sensor (Besombes, et al. 1997). The working principle of this device based on the physico-chemical transducer by integrating the specifity of biomolecule with micro-electronics (Figure 1.20).

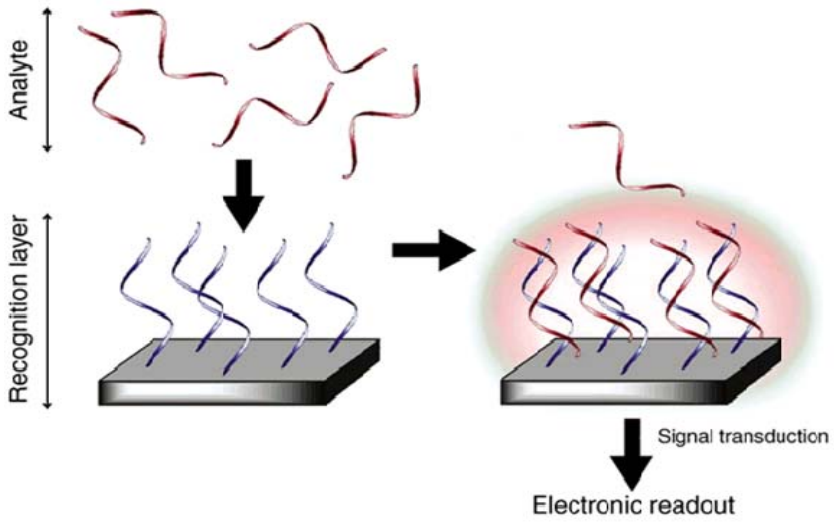


Figure 1.20 Parts of a biosensor

The word ‘’biosensor’’ is first used by Clark and Lyons in 1962. Biosensors are used for many applications in clinical diagnosis, biomedical and environmental monitoring, food technology and industrial, (Sharma and Rogers, 1994).

The most important and the key factor of fabrication a biosensor is the selection of biomolecule that forms a complex between an immobilized (Zhang, et al. 2003), biologically active compound and a desired analyte. Second aspect is monitoring/quantitating the ineractions between them.

The principal parts of a biosensor are (Figure 1.21)

i) bioreceptors; as enzymes, antibodies, nucleic acids or whole cells, usually adsorbed on solid substrates

ii) transducer; of the signal that is generated when the sensitive element specifically reacts with the analyte to be determined

iii) electronic circuit; for signal elaboration.

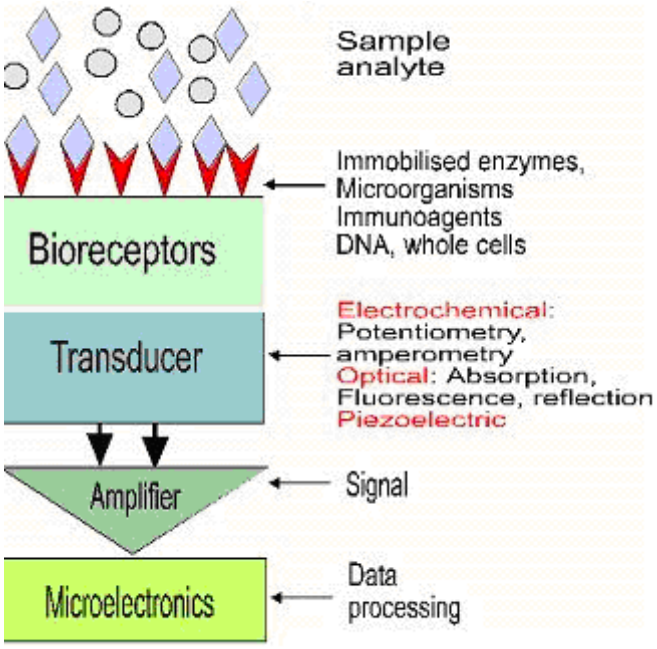


Figure 1.21 The principle components of biosensor

Functioning of enzyme system in the biosensor has some problems such as loss of enzyme, maintanence of stability, shelf-life of the biosensor, need to reduce the time of enzymatic response and offer a disposable devices (Amine, et al. 2006). To overcome these problems several immobilization procedures have been developed (Ahuja, et al. 2007).

It is obvious that biosensor development is limited by the immobilization protocol. In the fabrication of biosensors the immobilization of the biological molecule is important procedure. As the immobilization procedure must reproducibly keep the bio-recognition molecule close to the transducer surface while retaining its biological activity. A suitable method for immobilization is required for the development of biosensor (Taylor, 1991, Lev et al., 1995 and Lin and Brown, 1997). For optimum biostability and reaction efficiencies, the host matrix should be the one that isolates the biomolecule, protecting it from self-agregation and microbial attack, while providing the same local aquaeuos micro-environment as in biological media.

1.2.1 Cholesterol Biosensor

Many studies showed that increased concentrations of cholesterol are related to cardiovascular diseases (Bittman 1997, Bowman, et al. 2003, Rifai, et al. 2004). So, the monitoring of blood cholesterol concentrations is important for prevention and therapy. Cholesterol (Figure 1.22) determination is also important for the investigation of relation between cholesterol blood level. For all these purposes determination of cholesterol in blood is very important, since high levels in blood signalise danger of either formation or occurance of hypertension, hyperthyroidism, anaemia and coronary artery diseases. For this reason it is necessary to determine cholesterol concentration using rapid and accurate analytical technique (Biosens Bioelectron. 2007). The total content of blood cholesterol is sum of free cholesterol and its esters of fatty acids. To determine this total content by cholesterol biosensor then can be modified with enzyme cholesterol esterase (Nakaminami, et al. 1999) which hydrolyses cholesterol esters to free cholesterol and fatty acids. Then cholesterol biosensor can determine the naturally cholesetrol in blood as a sum of total cholesterol.

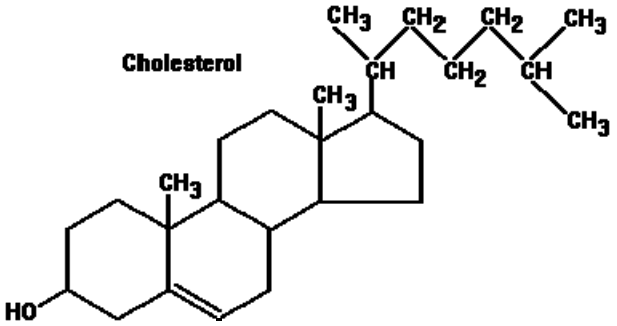
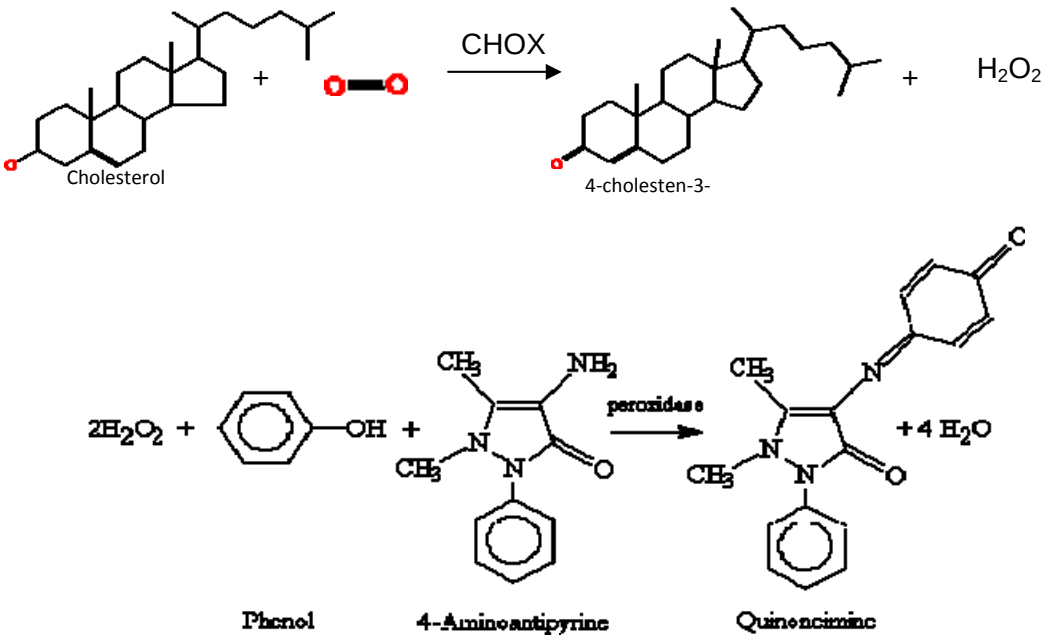


Figure 1.22 Structure of cholesterol

The normal level of cholesterol in blood/serum is 150-250 mg/dl. Among the various methods available for determination of total cholesterol (Pasin, et al. 1998, Richmond 1992) enzymatic method, employing ChOx and HRP, is simple, sensitive and specific therefore suitable for routine analysis (Allain, et al. 1974, Ragland, et al. 2000). It is based on the following chemical reactions:



1.3 Proton Conducting Polymers

There are variety of proton conductive materials that can carry electrical current by their protons or introduced protons at various temperature from room temperature to ~ 800 °C (Vaivars, et al.). These are such as inorganic salts with hydroxide groups, organic polymers and ion-conducting polymers (Lee, et al. 2005). Although protons do not have any electron shell, they takes on some H(1s) character by interacting strongly with the electrons of their environment. Although hydrogen (proton) transfer is long-range transport (i.e.diffusion) in metals, proton mobility requires also structural reorganizations in non-metallic environments.

Proton conductors are solid electrolytes in which protons or protonated molecular species are mobile in the host lattice. Fischer, Hofacker, and Rathner recognized that the dynamics of the proton environment is involved in proton conductivity in the late 1960s (Herz, et al., 2003). Then different classes of materials have attracted increasing attention as proton conductors including polymers, ceramics, and intercalation compounds (Schuster, et al., 2003). The polymer/ acid systems have advantages in terms of reduced leakage and corrosion problems, and they represent less costly alternatives to perfluorinated polymers. The main advantage is relatively inexpensive when compared with conventional proton-conducting polymers (Schuster, et al., 2003). In the last few years, researches have been driven by the goal of developing suitable proton-conducting materials for application in sensors, fuel cells, smart windows, batteries and most importantly (Pomes and Rous, 1996).

Proton conducting polymers can be divided into three types according to the range of temperature:

1- High temperatures, perovskite type oxides

2- Low temperature, hydrated polymer membranes

3- Intermediate temperatures, relying on proton transfer between nitrogen acting as proton donor and proton acceptor (Karadedeli, et al., 2005).

1.3.1 Proton Conduction Mechanisms

1.3.1.1 Grotthuss Mechanism

In this mechanism, protons move along extended hydrogen bonded by two steps:

1- First step is, intermolecular, proton transfers from a proton donor to a proton acceptor.

2- Second step is, intramolecular, reorientation of proton acceptor, making it available to take up another proton (Bozkurt, et al., 2003).

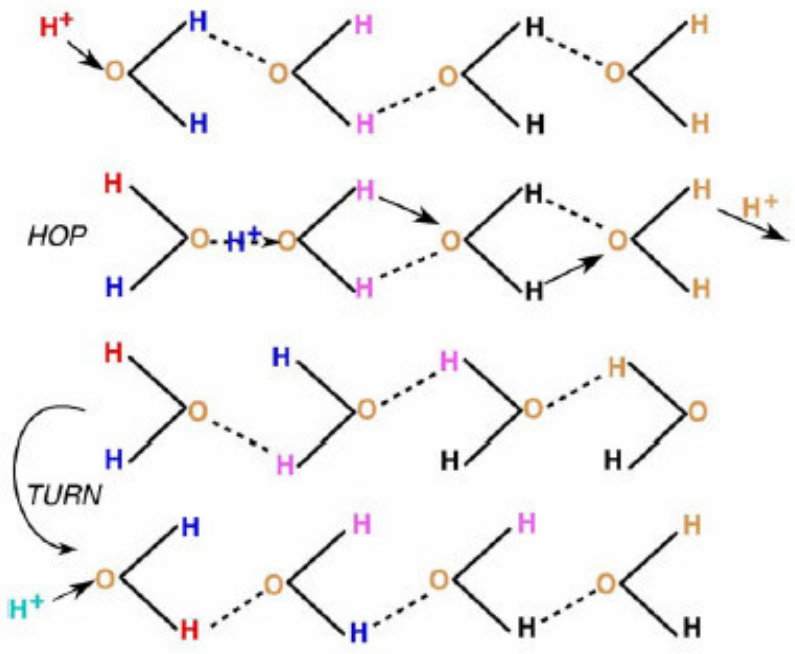


Figure 1.23 Basic ideas of the Grothuss's mechanism

In Figure 1.23, in the first row, there is covalent OH bond between approaching H^+ and the O of the first water molecule. This covalently bonded proton is shared between the next water molecule and form a protonated water dimer $(H_5O_2)^+$. This step

described as a ‘’hopping step’’ by Grothuss himself. This hopping propagates along the water molecules in the next row. After the H^+ leaves the last water molecule in the wire of second row, the total dipole moment of the water molecule wire is reversed in the third row. If another H^+ is to be transferred in the same direction as before, the four water molecules need to rotate back (turn step) to their configurations as in the fourth row. So, a ‘red’ H^+ enters and a ‘blue’ H^+ leaves the water molecule wire. (Sevil and Bozkurt, 2004).

1.3.1.2 Vehicle Mechanism

Another model is “Vehicle Mechanism”. Proton transport takes place with the aid of a vehicle such as H_2O or NH_3, so that the mobile species is actually the protonated complex ion, e.g. H_3O^+ or NH_4^+ (Figure 1.24). The transport properties are largely depending on water content of those membranes. This model further requires that the non-protonated vehicle move in opposite direction of the protonated vehicle during the conduction process (Bozkurt, et al. 2003).

Unlike the Grotthuss mechanism, this model does not require the existence of extended hydrogen-bonded network for proton transport. Proton conductivity by this mechanism should depend on the ease by which the vehicle can translate though the lattice. In contrast, the conductivity for the Grotthuss Mechanism will depend on

the ease by which the proton hops (tunnels) and acceptor reorients (Bozkurt, et al. 2003).

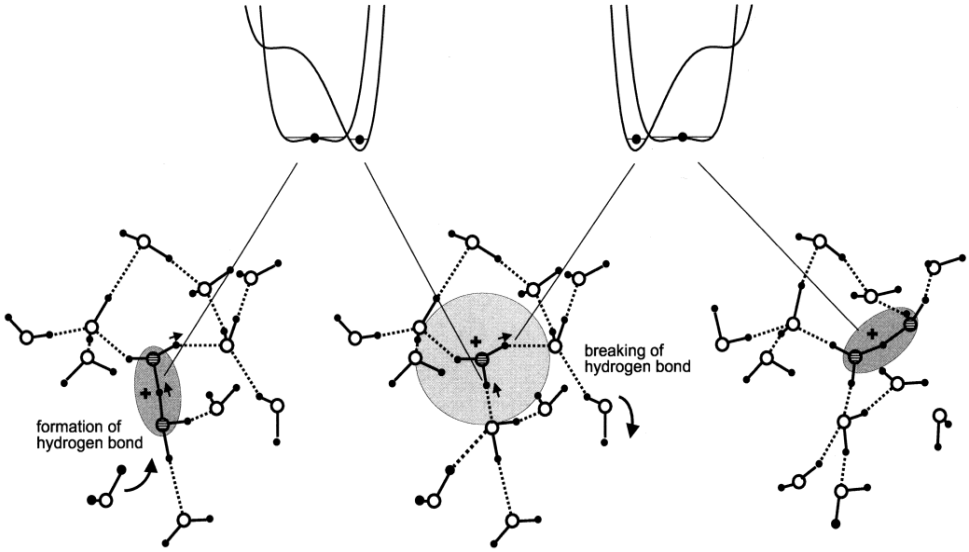


Figure 1.24 Vehicle Mechanism (Benhabbour, et al., 2005)

There is an increasing interest in anhydrous proton conducting polymer electrolytes due to their use as membranes in fuel cells at intermediate temperatures (T>100 °C) (Bozkurt, et al., 1999).

1.3.1.3 Proton Conducting Perovskite Type Oxides

Perovskite oxides, which are usually referred to as high temperature proton conductors (HTPCs), are important class of protonic conductors. When oxygen-deficient materials, have vacancies leads to significant oxygen ion conductivities at elevated temperatures in a moisture-free oxygen gas atmosphere, are heated in a water-containing atmosphere and water can dissolve into the perovskite to fill up these oxygen vacancies, which is that introducing protons into the structure. So all of the perovskite type oxides require the incorporation of H2O into the structure for them to become proton conductors. The transport of protons occurs through hopping between two neighboring oxygen ions (Grotthuss mechanism) and not by the movement of hydroxyl ions (Vaivars, et al.).

1.3.1.4 Low Temperature Proton Conducting Polymers

Nafion (Figure 1.25), perfluorinated sulfonic acid membranes, lead to development of solid protonic polymers [27]. They have high ability of proton conductivity in hydrated state with long term electrochemical stability (Ahmad, et al., 2006).

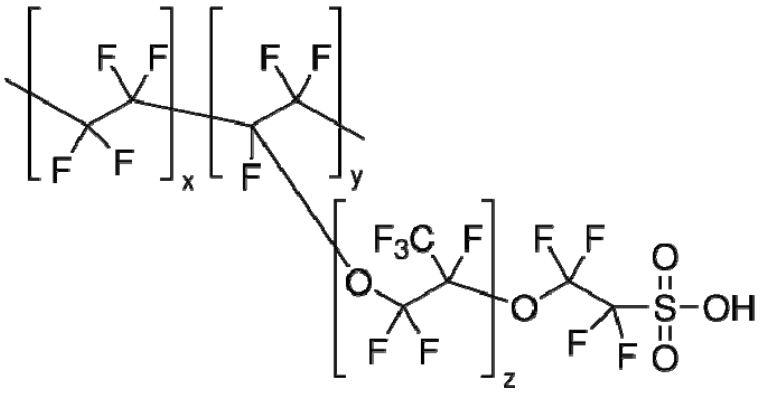


Figure 1.25 Chemical Structure of Nafion

Cluster-network model (Figure 1.26), proposes inverted micelle structures, ionic clusters of perfluroalkylsulfonates, interconnected by narrow channels, was suggested to understand conduction mechanism of Nafion (Gancarz, et al., 2006).

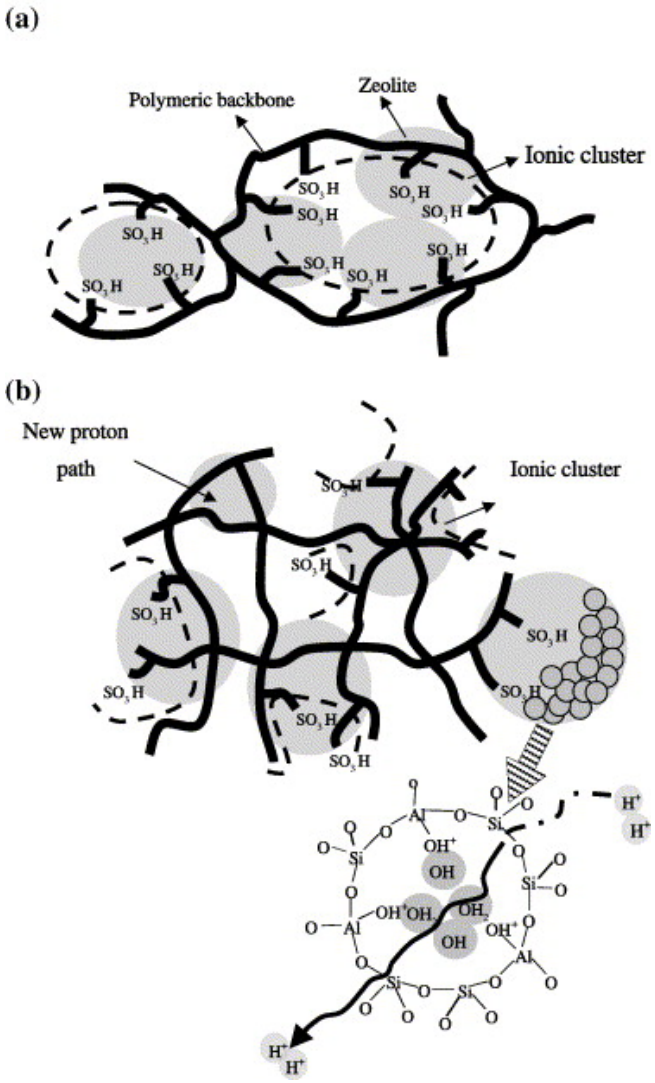


Figure 1.26 (a), (b) The cluster- network model

When hydrated, the high mobility of protons in the SO_3H groups at the end of side chains leads to highly conductive membranes. The water of hydration then leads to a further phase separation. A stationary micro-structure is formed and then absorbs and desorbs water at moderate temperatures. Hydrophobic part of this micro-structure provides good mechanical stability even in the presence of water. On the other hand, hydrated hydrophilic domains provide very high proton conductivity (Karadedeli, et al., 2005) (Figure 1.27).

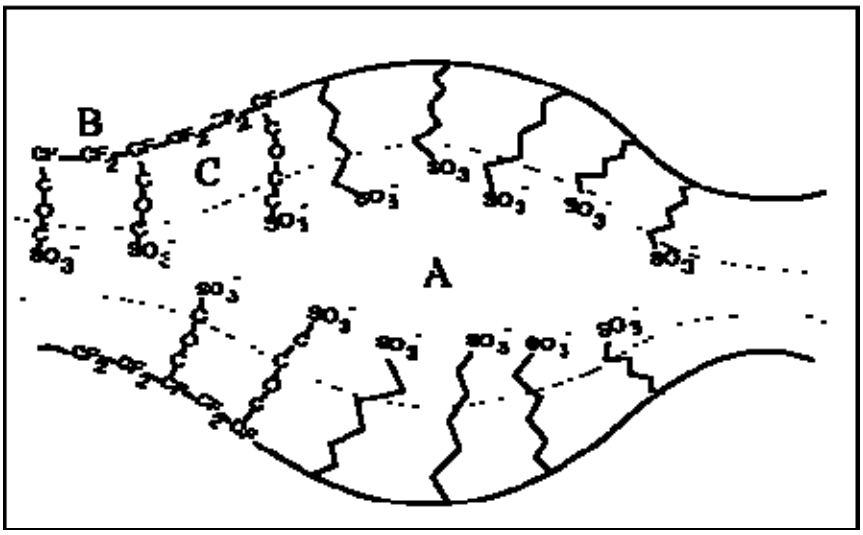


Figure 1.27 Channelles in Nafion (Schematic structure of Nafion)

In Figure 1.27 the nafion polymer contains three different phases. The sulphonic acid groups are clustered in hydrophilic inclusions (A) where most of the counter ions and water are located. These clusters are approximately 30 Å in diameter and are interconnected by aqueous channels with a diameter of approximately 10 Å. The Teflon-like polymer backbone (B) is essentially hydrophobic while the interfacial domain (C) is intermediate in hydrophilicity.

The water content plays a vital role in proton conduction in these sulfonated polymers because they follow the vehicular mechanism. Mobility of the water molecules in the Nafion membrane lead to transportation of proton (Emregül, et al., 2006) and protons on the SO_3H (sulfonic acid) groups "hop" from one to another acid site.

The disadvantages of low temperature proton conducting polymers are:

1- poor ionic conductivities at elevated temperatures or at low humidity conditions.

2- high cost of perfluorinated ionomer membrane is also a major limiting reason for its widespread application (Gancarz, et al., 2006).

3- evolution of toxic intermediates and corrosive gases liberated at temperatures above 150 ^{o}C

1.3.1.5 Intermediate Temperature Proton Conducting Polymer

The formation of protonic defect and provide strong fluctuation proton donor to acceptor are necessary to see proton conductivity in intermediate temperature proton conducting polymer. There are only few materials have been developed to show high proton conductivity at intermediate temperatures (100-400 ^{0}C) (Karadedeli, et al., 2005).

Polymers basic sites interact with the acidic polymers or acids via hydrogen bonds. Then, proton conduction occurs through structure diffusion in these systems. Non-vehicular mechanism is the proton transport under anhydrous or low humidity conditions. Only protons are mobile from site to site without an assistance of diffusible vehicle molecules (Işık, et al., 2003).

1.4 Chitin

Chitin, polysaccharide, occurs widely in lower animals, fungi, etc. and very little is known about the metabolism of chitin in animals (Muzzarelli 1973). This natural polymer that can be called poly-N-acetyl-D-glucosamine, can be formally considered a derivative of cellulose where the C-2 hydroxyl groups have been completely replaced by acetamido groups (Figure 1.28). It is constituted of β-(1-4)2-acetamido-2-deoxy-D-glucose units, some of them being deacetylated (Muzzarelli 1973). However it is clear that it acts as a carbohydrate and nitrogen reserve and associated with proteins to form glycoproteins. The supporting or protecting function of chitin seems to be of minor importance.

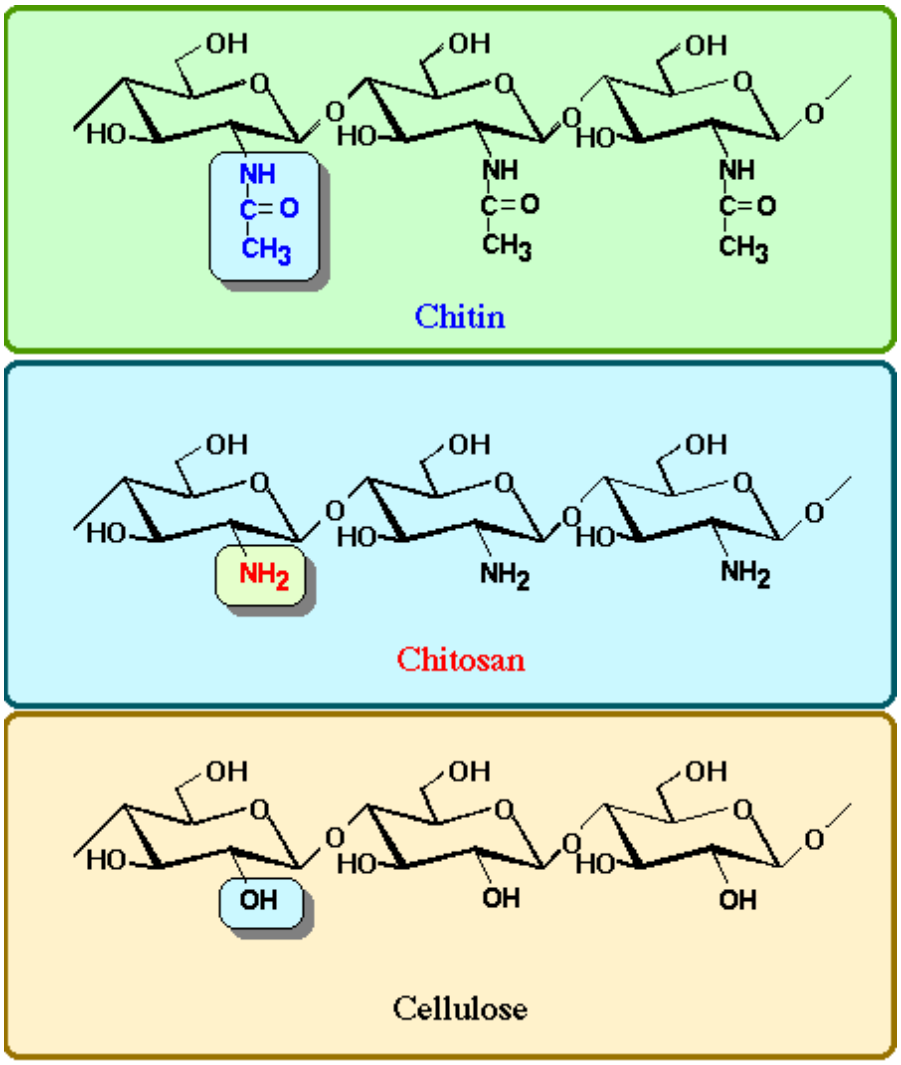


Figure 1.28 Structure of Chitin, Chitosan and Cellulose

Harsh treatments are required to free chitin from the accompanying materials (BeMiller 1965). Chitin is usually prepared from crustacean shells by treatment with strong acids. It is a white solid insoluble in water, organic solvents, dilute acids and cold alkalis of any concentration.

1.5 Chitosan

Chitosan is obtained, first time, by Hoppe-Seyler as a product in 1894 by fusing potassium hydroxide at 180^{o}C with chitin (Hoppe-Seyler 1894).

The principle repeating units are given in (Figure 1.29)**:**

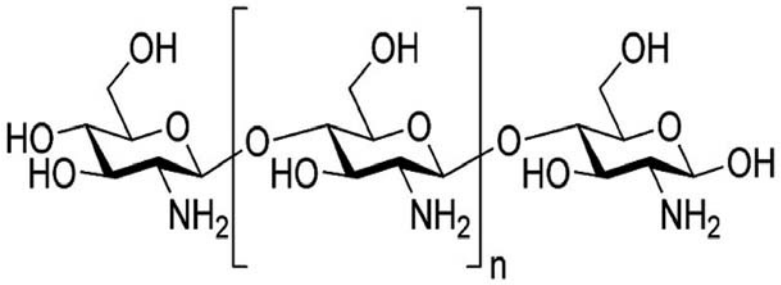


Figure 1.29 The repeating units of chitosan

Chitosan was also studied by Araki, (Araki 1895) and von Furth and Russo concluded that three out of four acetyl groups can be removed from chitin (Furth and Russo 1906).

Chitosan is a high molecular weight polysaccharide, currently manufactured at industrial scale in USA and Japan, from shrimp and crab wastes. However, as pointed out by Muzzarelli et al. (Muzzarelli 1973), other sources mainly of fungal origin, are quite attractive for their production because of the good qualities exhibited by the fungal product. The methods for isolating chitin from crustacea involve usually decalcification in a dilute hydrochloric acid followed by deproteinization in a hot aqueous sodium hydroxide solutions. After that, the chitin is deactylated to chitosan (Bough, et al. 1978). In the case of the fungal wastes, they are simply treated with boiling concentrated sodium hydroxide solutions to obtain the chitosan-glucan complex (Muzzarelli, et al. 1980). Chitin and chitosan products with distinc chemical characteristics can be obtained that may then be applied in specific field, by employing different conditions during the

manufacturing process (Bough, et al. 1978, Brine and Austin 1981, Muzzarelli, et al. 1981).

In fact, chitosan is a preferred form of chitin as it can be dissolved very easily in different solvents to form solution. Chitosan is the product after the N-deacetylation of the naturally occuring polymer chitin (Muzzarelli 1973). Chitin is a mucopolysaccharide widely distributed on the earth as a supporting material of crustacera. As its crystal structure is very hard because of acetamide group at the position C-2, they are few solvents dissolving chitin.

Chitosan is insoluble in water and inorganic solvents ; it will, however, dissolve in dilute solutions of acetic acid. In solution the positive hydrogen ion from the acid associates with the amino group in chitosan producing a net positive change on the polymer giving it potential precipitation and flocculating properties. A 2.0% w/w chitosan (in 5% v/v glacial acetic acid) solution was employed.

At pH>6.5 the negatively charged molecules will interact with the protonated amino groups of chitosan producing destabilisation. Below pH 6 where both materials have the same charge, very poor recovery is obtained. In the alkaline region recovery is still high (85% at pH 9,5) although the number of protonated amino groups in chitosan decreases (Muzzarelli 1973).

Chitin and chitosan had proved to be valuable products for using in different applications such as adhesives, textile and paper additives, etc. (Bough 1976, Muzzarelli 1977, Berkeley 1979). And also because of similarity of chitosan to mucopolysaccharides of gastric mucin, the efficacy of purified polysaccharides in preventing and treating peptic ulceration has been evaluated (Hillyard, et al. 1964). The existance of normal mucus-alkaline secretion in the stomach, which participates in the maintanence of the function and integrity of gastric mucosal barrier, seems to be one of the most important factors of protection to the gastric mucosa (Johansson and Kollberg 1978). A chitin-derivative such as chitosan becomes adhesive, viscous at low pH and binds acids. It has been suggested that high acid binding capacity of chitosan may account for antiulcer property of this substance in prevention of Shay ulcers (Konturek, et al. 1981).

Theoretically, it is possible to form a membrane, surroundinng a liquid core, by electrostatic interactions between two oppositely charged polymers (Rha, et al. 1988,

Hwang, et al. 1985). We have developed a new method of encapsulation which is simple and fast. This is practically possible with chitosan being available since many anionic polysaccharides have been already available for some time. The direct reaction between chitosan and AA, or PTSA, infact creates hydrogel. In this study, we demonstrate, also, the bio-compatiblity of entarraped enzyme in these hydrogel networks.

1.6 Alginic Acid

AA (Figure 1.30) is a polyuronide found in brown seaweeds (Muzzarelli 1973), whose chemical formula is reported below:

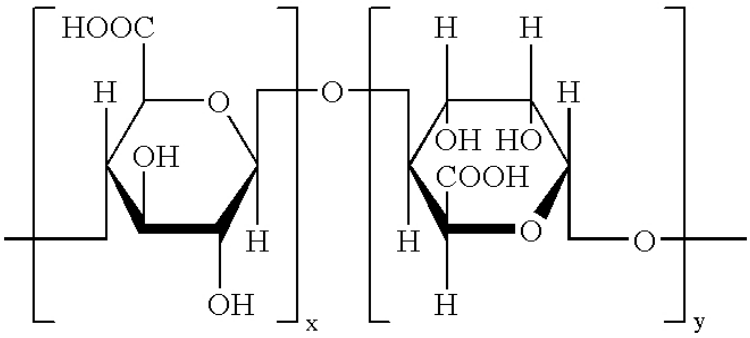


Figure 1.30 Structure of Alginic Acid

Mannuronic and gluronic acids are the two constituents of AA : this information was reached after Nelson and cretcher establish that the uronic acid content of AA is 100% based on a decarboxylation reaction, and that mannuronic acid was a constituent of the polymer.

Appearantly, AA is a heteropolymer including both the uronic acid. However, there is no evidence against the hypothesis of two different polymers, is made up solely of mannuronic acid, and the other of guluronic acid (Muzzarelli 1973).

Isolation of AA presents the obvious problem of avoiding degradation. The seaweed is reduced to powder, and using base exchange reactions, the AA is alternatively made soluble and insoluble, so as to seperate it from other components (Miklestad 1967). The preliminary treatment involves hot water and lime water giving a solution of mannitol, laminarin, fucoidan and salts. Then, a dilute acid treatment eliminates acid soluble substances, while converting the alginate to free AA.

Occasionally, an infrared spectrum was reported (Cozzi, et al. 1968) for AA and cobalt alginate where the strong band at 1735 cm^{-1} was assigned to the free carboxyl group of AA, while the band at 1600 cm^{-1} was assigned to the salified carboxyl group. In any case, this instrumental evidence of salification does not add new light to the existing knowledge about interaction of metal ions with the polymer, as the salification reactions are well known from precipitation studies, and the chelating ability of the polymer is not evident from the hydroxyl group region of the infrared spectrum.

1.7 p(Toluenesulfonic) Acid

Sulfonic acid is a derivative of sulfuric acid ($HOSO_2OH$), with general formula RSO_2OH, where R is an aliphatic or aromatic hydrocarbon. OH in sulfuric acid ($HOSO_2OH$) has been replaced by a carbon group or a compound where a hydrogen atom has been replaced by treatment with sulfuric acid.

PTSA or tosylic acid (TsOH), also known as 4-methylbenzenesulphonic acid, is water, alcohol and other polar organic solvent soluble white solid with the formula $CH_3C_6H_4SO_3H$ (Figure 1.31) (http://en.wikipedia.org/wiki/p-toluenesulfonic_acid). PTSA is strong acid when compare to sulphuric acid. In watery environments it is almost completely ionized even at low pH.

The extra methyl group para- to the sulphonic acid group in PTSA has a weakly activating effect on the benzene ring, which makes it slightly more prone to electrophilic aromatic substitution. For PTSA (Figure 1.32)**,** the closely related substance benzene sulphonic acid (Figure 1.33) can be used as a surrogate in view of the chemical similarity between the two compounds (Figure 1.31)**.**

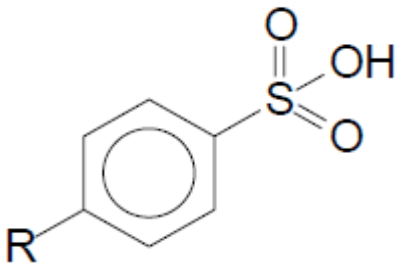


Figure 1.31 Structure of benzenesulphonic acid and PTSA

Acidity of the sulphonic acid group is influenced by two factors:

1. The methyl group exerts an electron donating effect, which makes the negative charge on the resulting sulphonate ion after deprotonation slightly less stable.

2. The resonance effect still stabilises the negative charge on the sulphonate ion by dividing the charge on the oxygen atoms.

As a result, the acidity of the sulphonic acid group is not expected to change significantly compared to benzene sulphonic acid. Calculation of the pKa confirms this expectation: –2.58 for PTSA and –2.8 for benzene sulphonic acid. Thus the reactivity of PTSA (Figure 1.32) and benzenesulphonic acid (Figure 1.33) is very similar and aquatic toxicity data from benzenesulphonic acid can be used for PTSA.

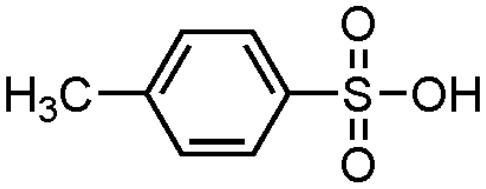


Figure 1.32 Structure of PTSA

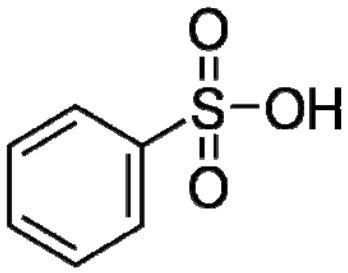


Figure 1.33 Structure of benzenesulphonic acid

Because of H atom sulfonic acid is acidic and stronger than a carboxylic acid. They are widely used in the detergent industry. Sulfonate cleaners do not form an insoluble precipitates in hard water (www.chemicalland21.com).

Alkylbenzene sulfonic acid is the largest-volume synthetic surfactant because of its relatively low cost, good performance. It is the fact that alkylbenzene sulfonic acid can be dried to a stable powder and the biodegradable environmental friendliness.

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Toluene sulfonic acids are used in (www.chemicalland21.com);

- the production of toluenesulfonamide as the parent material for the production of saccharin
- paints, hot-melt adhesives, nitrocellulose, coating materials, thermosetting resins and phenolic resins as a raw material of flow-promoting agents
- electroplating solutions as a basic material
- preparing hydrazine based blowing agents
- the synthesis of isocyanate compounds as catalysts
- the production of thermosetting resins as catalysts
- number of biologically active compounds, pharmaceuticals, dyes and pigments candidates as synthetic intermediates

PTSA is used in (www.chemicalland21.com);

- the manufacturing of plasticizers as a non-oxidizing catalyst
- the epoxy-phenolic resins as a curing agent
- the formation of acetal as a catalyst. In that process, water must be removed azeotropically by distilation from the reaction mixture to escape reversible reaction

1.8 Aim of the Study

1) To perform the synthesis of chitosan/AA polymer network
2) To determine the physically most stable polymer network of chitosan/AA by mixing chitosan and AA at different stoichiometric ratios.
3) To check the possibility of immobilization of ChOx in chitosan/AA network
4) To characterize the enzyme entrapped polymer network of chitosan/AA
5) To determine the maximum reaction rate (Vmax) and Michaelis-Menten constant (Km) for the immobilized cholesterol oxidaase in chitosan/AA network
6) To perform the synthesis of chitosan/PTSA polymer network
7) To determine the physically most stable polymer network of chitosan/PTSA by mixing chitosan and PTSA at different stoichiometric ratios.
8) To check the possibility of immobilization of ChOx in chitosan/PTSA network
9) To characterize the enzyme entrapped polymer network of chitosan/PTSA
10) To determine Vmax and Km for the immobilized ChOx in chitosan/PTSA network
11) To see the proton conductivities of both polymer networks by proton exchange mechanism between chitosan and AA and chitosan and PTSA.

CHAPTER II

EXPERIMENTAL

2.1 Chemicals

Chitosan (low molecular weight, 80% deacetylated) was purchased from Fluka. The percent degree of deacetylation will decrease the proton conductivities of both polymer network by decreasing the number of proton, used in proton conductivity, by deacetylated part of the chitosan structure. ChOx [E.C. 1.1.3.6] with a specific activity of 26.4 U mg^{-1} solid and HRP [E.C. 1.11.1.7] with a specific activity of 181 U mg^{-1} solid were purchased from Sigma-Aldrich. Cholesterol, AA, PTSA, isopropanol (99%), triton X-100 (t-octylphenoxypolyethoxyethanol), 4-aminoantipyrine, and glycial acetic acid (GAA) were purchased from Sigma-Aldrich. Phenol was purchased from Carlo Erba. Phosphate buffer (pH.7.0) was prepared using sodium monobasic and sodium dibasic in distilled water.

2.2 Instrumentation

The infrared spectra for chitosan, AA, PTSA, and chitosan/AA and chitosan/PTSA conducting networks were carried out on a Scimitar Series Varian 1000 FTIR. UV-Visible spectra were recorded to determine the activities of immobilized enzyme using a Shimadzu UV-1601 UV-Visible spectrophotometer. Setaram Labsys TGA/DTA were used for thermogravimetry (TGA) and differential thermal analysis (DTA) in a temperature range of 25-300^{0}C. Elemental analyses (EA) of the conducting polymer networks were performed using LECO, CHNS-932. The proton conductivity studies of the polymer networks were performed using a Novocontrol dielectric impedance analyzer in the frequency range 0.1 Hz - 3 MHz at 10 ^{0}C intervals. The networks samples prepared as films sandwiched between platinum blocking electrodes.

2.3 Experimental Procedures

2.3.1 Preparation of Cholesterol Solution

Cholesterol is soluble in alcohol and also in water in the presence of surfactants (Kumar, et al. 2000, Trettnak and Wolfbeis, 1990, Charpentier and Murr 1995, Masoom and Townshnd 1985). Cholesterol solutions were prepared in situ by dissolving cholesterol in isopropanol, Triton X-100, and the phosphate buffer (pH.7.0) at ratio of 10:4:86 by volume, respectively.

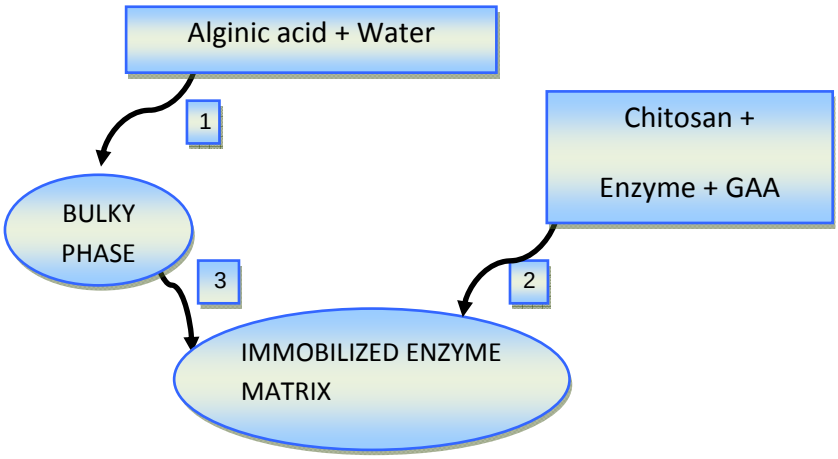
2.3.2 Preparation of 4-Aminoantipyrine Solution

4-aminoantipyrine (158 mg/mL), phenol (146 mg/mL) and HRP (10 mg/dl) were mixed in (pH 7.0) phosphate buffer. It is used for the formation of colored solution of products of ChOx catalysed reaction. The spectrophotometric determination of cholesterol is based on the color of quinoneimine dye produced by coupling of peroxide with 4-aminoantipyrine and phenol in the presence of HRP (Kumar, et al. 2000, Deeg and Ziegenhorn 1983, Braco, et al. 1992).

2.3.3 Preparation of Chitosan/AA Network and Entrapment of Cholesterol Oxidase

First the physically most stable chitosan/AA ratio was determined by mixing 0.10 grams of chitosan with different amounts of AA. Chitosan was mixed with water containing ChOx, and AA was mixed with 1% GAA (Scheme 2.1). Then the two phases were put together to get enzyme entrapped polymer network (EEPN). We expected that the most stable matrix will be with the ratio of 2, where protonation of chitosan and the %content of chitosan in the network is maximum. AA is a dibasic acid hence, we used x=2 (x is the number of moles of chitosan per moles of –COOH units in AA). However,

the EA and the FTIR results showed that the most suitable matrix was with x=1. Therefore, throughout the study, chitosan/AA matrices were prepared with x=1. During complexation, 1.5 mg of ChOx enzyme was entrapped in 1mL of AA (0.1g/mL) and 1mL of chitosan (0.1g/mL) mixture, and the maximum water absorbing capacity was found to be 1.0 mL in 2.0 mL of AA (0.1g/mL) and chitosan (0.1g/mL). This EEPN was used for activity determinations.



Scheme 2.1 Preparation of chitosan/AA network

2.3.3.1 Activity Assay

2.5 mL of cholesterol solutions of different concentration was prepared. Immobilized ChOx enzyme was waited 2,4 and 6 minute. This time range was determined by doing several experiments and these time range is the time for maximum ChOx enzyme activity in the prepared cholesterol solutions. After waited the immobilized enzyme in this solutions, 2.5 mL of 4-aminoantipyrine solutions were added to complete the formation of quinoneimine dye. And then it was measured at 500 nm with a double beam spectrometer.

2.3.3.2 Determination of Cholesterol Oxidase Activity

The activity of ChOx was determined spectrophotometrically using Kumar method (Kumar, et al. 2000). One unit converts 1.0 μmol of cholesterol to 4-cholesten-3-one per min at pH 7.0 at 25 ^{0}C. Different concentrations of cholesterol solutions were prepared, then a 2.5 mL of cholesterol solution was contacted with the ChOx immobilized chitosan/AA. Different incubation times (2, 4 and 6 min) were applied. After these incubation times, 2.5 mL of a solution containing 4-aminoantipyrine, phenol and HRP were added and waited for 10 min to complete quinoneimine dye formation. The spectrophotometric determination of cholesterol is based on the color of quinoneimine dye produced by coupling of peroxide with 4-aminoantipyrine and phenol in the presence of HRP (Kumar, et al. 2000, Deeg and Ziegenhorn 1983, Braco, et al. 1992). The product was determined spectrophotometrically at 500 nm.

2.3.3.3 Determination of Kinetic Parameters

To determine the maximum reaction rate and Michaelis-Menten constant (V_{max} and K_m), different concentrations (0.1, 0.01, 0.03, 0.05, 0.005 mM) of cholesterol solutions were prepared in phosphate buffer (pH 7.0) and activity assay was applied. V_{max} and K_m were calculated from Lineweaver-Burk plots (Palmer 1995).

2.3.3.4 Determination of Optimum Temperature and pH for Immobilized Cholesterol Oxidase

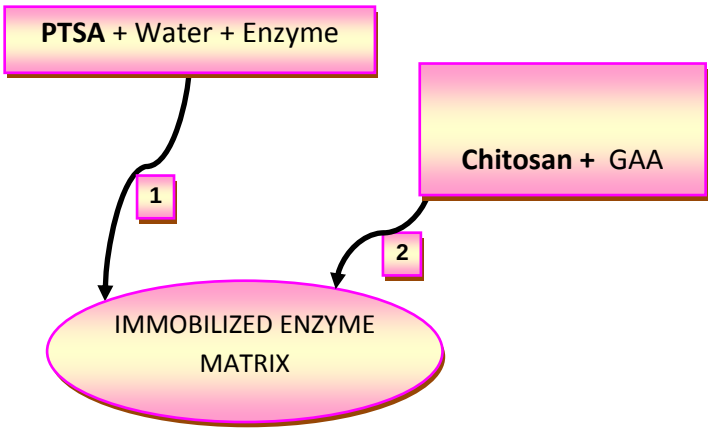
Optimum temperature and pH were determined by keeping the substrate concentration at 10 K_m. To find optimum temperature, activity assay was applied by changing the incubation temperature between 10 ^{0}C and 50 ^{0}C. The chitosan/AA network was synthesized with a stoichiometric ratio of x=1. To determine optimum pH, an assay was applied by changing the pH between 5 and 8 at 25 ^{0}C.

2.3.3.5 Determination of Operational Stability and Shelf Life

The enzyme bioreactor, at optimum activity conditions, was used in 44 activity assays in one day to determine the operational stability of chitosan/AA network and 26 activity assays in one day to determine the operational stability of chitosan/PTSA network. The shelf life of the enzyme bioreactor was determined by performing activity assays within 15 days for chitosan/AA network.

2.3.4 Preparation of Chitosan/PTSA Network and Entrapment of Cholesterol Oxidase

To prepare enzyme entrapped chitosan/PTSA polymer network, different ratios of chitosan/PTSA were prepared. We expected that the physically most stable matrix will be with the ratio of x=1 (x is the number of moles of chitosan per moles of SO_3H units in PTSA), where the protonation of chitosan and %content of chitosan is maximum in the network. However, the FTIR results and elemental analyses results showed that the most stable ratio was 1:3. To get the EEPN, 0.10 grams of chitosan mixed with GAA (1%) and then enzyme and PTSA were added to them as shown in Scheme 2.2.



Scheme 2.2 Preparation of chitosan/PTSA network

2.3.4.1 Determination of Cholesterol Oxidase Activity

The activity of ChOx was determined spectrophotometrically using Kumar method (Kumar, et al. 2000). One unit converts 1.0 μmol of cholesterol to 4-cholesten-3-one per min at pH 7.0 at 25 ^{0}C. Different concentrations of cholesterol solutions were prepared, then a 2.5 mL of cholesterol solution was contacted with the ChOx immobilized chitosan/PTSA network. Different incubation times (2, 4 and 6 min) were applied. After these incubation times, 2.5 mL of a solution containing 4-aminoantipyrine, phenol, HRP were added and waited for 10 min to complete quinoneimine dye formation. The spectrophotometric determination of cholesterol is based on the color of quinoneimine dye produced by coupling of peroxide with 4-aminoantipyrine and phenol in the presence of HRP (Kumar, et al. 2000, Deeg and Ziegenhorn 1983, Braco, et al. 1992). The product was determined spectrophotometrically at 500 nm.

2.3.4.2 Determination of Kinetic Parameters

Kinetic parameters (V_{max} and K_m) were calculated from Lineweaver-Burk plots (Palmer 1995). To do this, different concentrations of cholesterol solutions were prepared and activity assay was applied.

2.3.4.3 Determination of Optimum Temperature and pH for Immobilized ChOx

To find optimum temperature, activity assay was applied by changing the incubation temperature between 10 ^{0}C and 50 ^{0}C and keeping substrate concentration at 10 K_m. To determine optimum pH, activity assay was applied by changing the pH between 5 and 8 and keeping substrate concentration at 10 K_m , at 25 ^{0}C.

2.3.4.4 Determination of Operational Stabilities and Shelf Life

EEPN was used in 26 activity assay in one day at optimum activity conditions to determine the operational stability. The shelf life of the EEPN was determined by performing activity assays within 25 days.

2.3.5 Determination of Proton Conductivity

Frequency dependent proton conductivity of the samples was measured by impedance method as a function of temperature alternating current (AC) conductivity, σ_{ac} versus frequency curves are plotted and the direct current (DC) conductivity, σ_{dc} of the samples was derived for the σ_{ac} data, as described in previously (MUKOMA, et al., 2004) DC conductivities at several temperatures of the chitosan/AA and chitosan/PTSA networks.

CHAPTER III

RESULTS AND DISCUSSION

3.1 Immobilization of Cholesterol Oxidase in Chitosan/AA and Chitosan/PTSA Networks

ChOx was immobilized in chitosan/AA and chitosan/PTSA networks by following the procedures as defined in the experimental part.

3.1.1. Kinetic parameters

First ChOx was immobilized in chitosan/AA and chitosan/PTSA networks. For the most suitable matrix preparation, 1.5 mg (39.6 U) of ChOx was immobilized in these polymer networks.

Then, these EEPN were used for the activity assay while varying the substrate concentration at constant temperature and pH (Palmer 1995). Kinetic parameters were calculated using Lineweaver-Burk plots. K_m is the Michaelis-Menten constant and shows the affinity of enzyme to its substrate. V_{max} is the maximum rate for enzymatic reaction. The calculated values were given in Table 3.1.

Table 3.1 Kinetic parameters for free and immobilized ChOx in chitosan/AA and chitosan/PTSA networks

	K_m (M)	V_{max}
Free ChOx	$2.6x10^{-1}$	$3.0x10^{-1}$ (μmol/min.ml)
Immobilized ChOx (chitosan/AA)	$2.05x10^{-5}$	$5.3x10^{-6}$ (μmol/min.mg complex)
Immobilized ChOx (chitosan/PTSA)	$2.6x10^{-4}$	$2.7x10^{-5}$ (μmol/min.mg complex)

Thus, as given in the Table 3.1, K_m of the immobilized ChOx decreased compared to that of the free enzyme. The low K_m value indicates that the immobilized ChOx enzyme interacts with its substrate for a longer time (compared to free enzyme) and thus, the immobilized enzyme catalysed reaction gives lower amount of product than that of free enzyme catalysed reaction. As a result, V_{max} of this long time interaction between immobilized enzyme and its substrate reveals smaller V_{max} (Table 3.1).

3.1.2 Temperature Influence on Enzyme Entrapped Polymer Network

The temperature optimization process is very important in enzyme immobilization and biosensor construction because temperature is important parameter for the enzyme activity of the enzyme network. Thermal stability of the EEPN was studied by measuring the absorbance of colored products of enzyme catalyzed reaction at different temperatures (10 ^{0}C – 50 ^{0}C). The effect of temperature on the immobilized enzyme activity is shown in Figure 3.1 and Figure 3.2. Although free enzyme is optimally active at a temperature of 37 ^{0}C and lost its activity after that point (MacLachlan, et al. 2000), immobilized enzyme shows appreciable activity between 10

^{0}C and 50 ^{0}C with a maximum activity at 40 ^{0}C in chitosan/AA network. The reason for not loosing activity with increasing temperature may be due to the minimized protein denaturation through the protection by chitosan/AA matrix. However, after 40 ^{0}C, there is a sudden decrease in the activity due to the denaturation of enzyme. EEPN provides a suitable immobilization medium for ChOx and can be used as a biosensor for a wide range of temperature. However, ChOx shows maximum activity at 30 ^{0}C when immobilized in chitosan/PTSA. Although, this matrix can not increase the temperature of maximum activity, it prevent enzyme in large temperature range around to 50 ^{0}C.

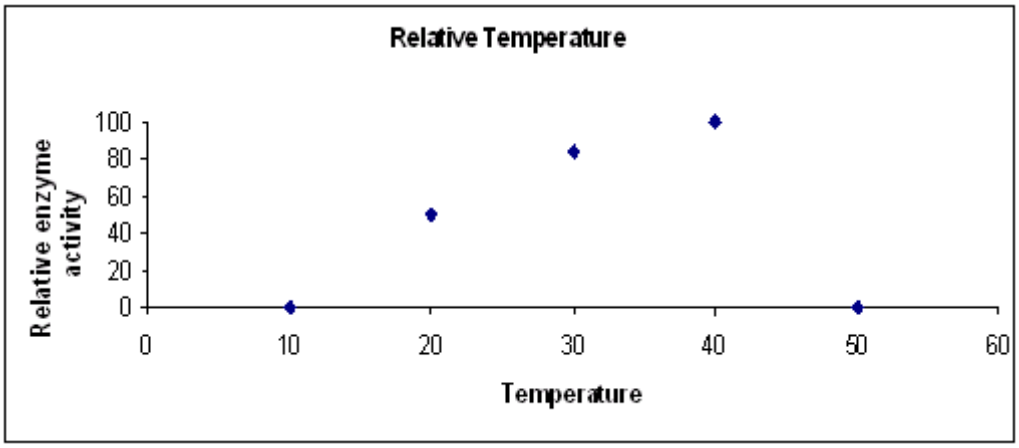


Figure 3.1 Optimum temperatures of chitosan/AA network

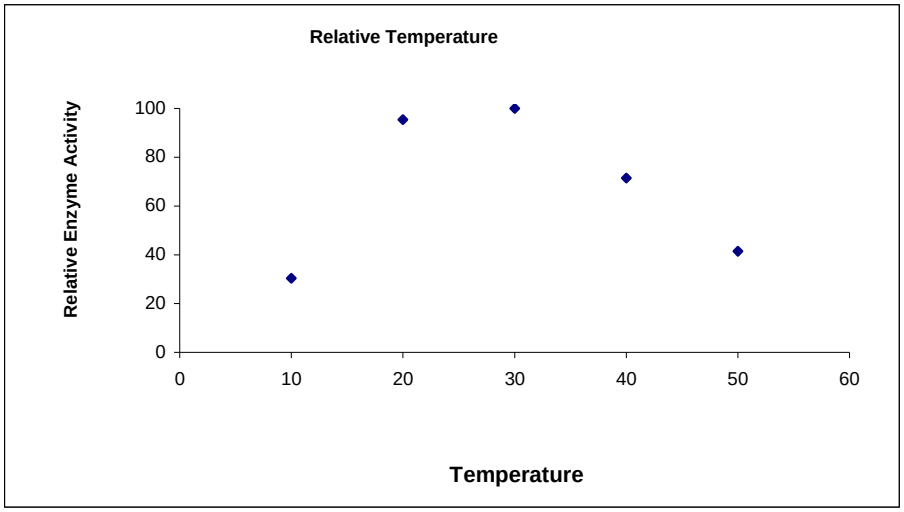


Figure 3.2 Optimum temperatures of chitosan/PTSA enzyme biosensor

3.1.3 pH Influence on Enzyme Entrapped Polymer Network

Enzyme can be denatured especially in high and low pH media. Hence, pH optimization is important in enzymatic assays. Figure 3.3 and Figure 3.4 shows the response of the EEPN as a function of pH. The effect of pH on immobilized ChOx activity was investigated between a pH range between 5 and 8. Although, free ChOx shows maximum activity at pH 7.0, immobilized ChOx enzyme showed maximum activity at pH 7.5 in chitosan/AA complexand at pH 7.0 in chitosan/PTSA complex. Chitosan/PTSA complex revealed a reasonable activity between pH 6 and 8. The protonation of medium does not affect the enzyme in chitosan/AA network, thus, pH of enzyme in EEPN is different than that of the bulk. Decreases in activity at pH 7.0 and 6.5 (Figure 3.3 and Figure 3.4, respectively) are due to the isoelectric point of the enzyme. At this point, the pH of microenvironment of enzyme is lower than 7.0 and 6.5 and this is the isoelectric point at which molecule carries no net electrical charge, hence the activity decreased rapidly due to the difficulty in substrate binding. At extreme pH values the enzyme was irreversibly denaturated. As a result, chitosan/AA and chitosan/PTSA matrix provides enzyme broader ranges of pH.

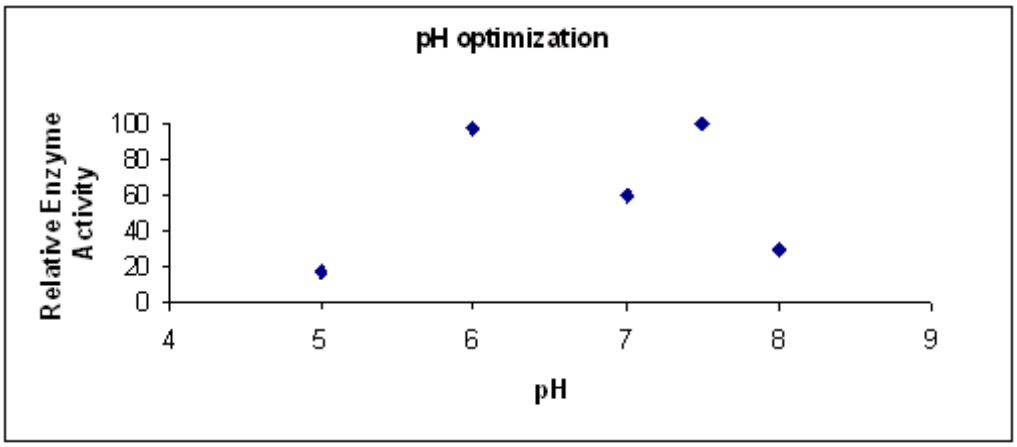


Figure 3.3 pH influence on chitosan/AA enzyme biosensor

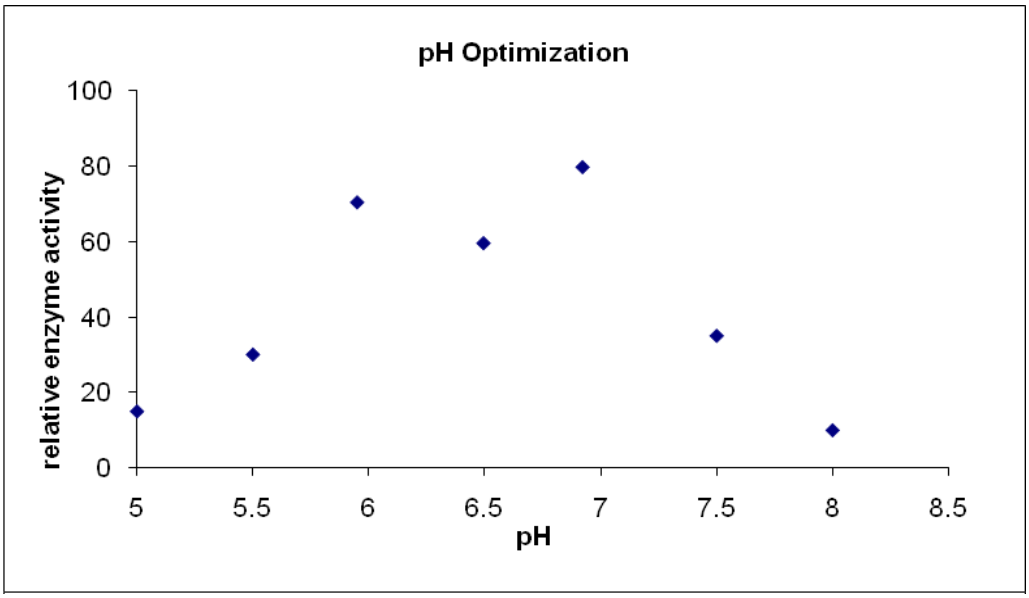


Figure 3.4 pH influence on chitosan/PTSA enzyme biosensor

3.1.4 Operational Stability and Shelf Life of the Enzyme Biosensor

The operational stability was studied by applying activity assay (under optimum conditions) for 44 times for chitosan/AA network and 26 times for chitosan/PTSA network in the same day at constant temperature, pH and substrate concentration.

There is not much activity loss for the first 15 usage. It lost 25% of its activity for the other 20 days. At the end of the 44 measurements, the bioreactor lost 37% of its initial activity when immobilized in chitosan/AA network (Figure 3.5). When enzyme immobilized in chitosan/PTSA network, it lost 10% of its activity in 5 usage and 25% of its activity other day (Figure 3.6). At the end of the 26 usage, enzyme lost 50% of its initial activity. This shows that immobilized enzyme has advantages of repeated usage over free enzyme and an improved operational stability.

The activity assay was also applied for every 5 days within 15 days to display the shelf life of immobilized enzyme (Figure 3.7 and 3.8). As given in Figure 3.7 and Figure 3.8, EEPN exhibits 20% activity after five days for chitosan/AA network and 65% activity after five days for chitosan/PTSA network. Because of decreasing enzymatic activity during the storage period, it is concluded that biosensor constructed with these enzyme and polymer matrix shows good stability for a short time.

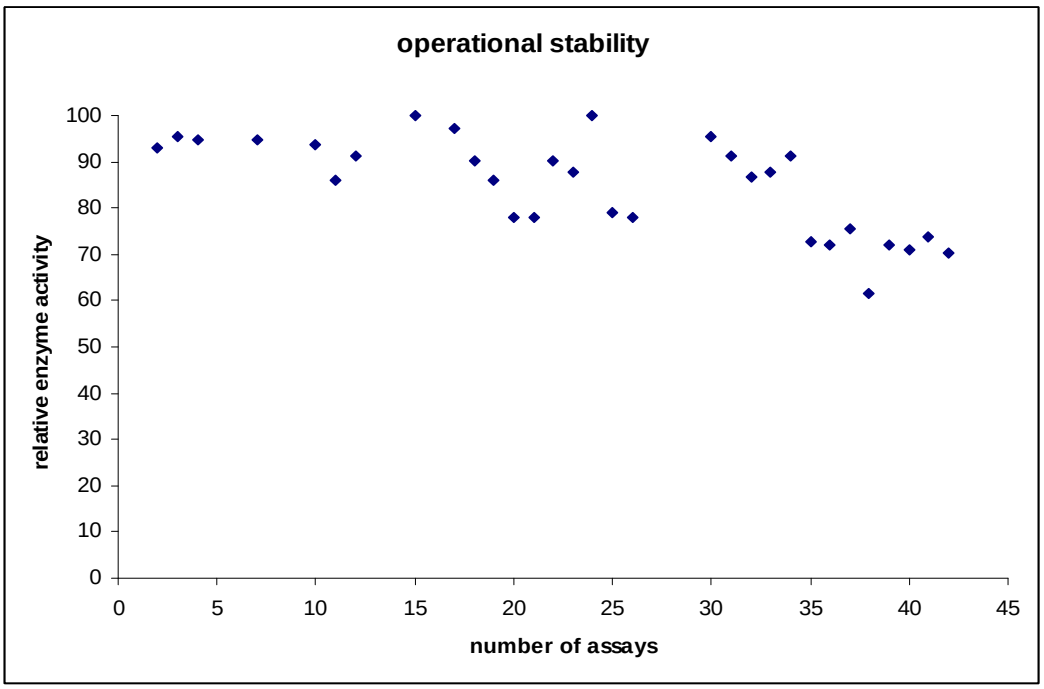


Figure 3.5 Operational stability of chitosan/AA enzyme biosensor

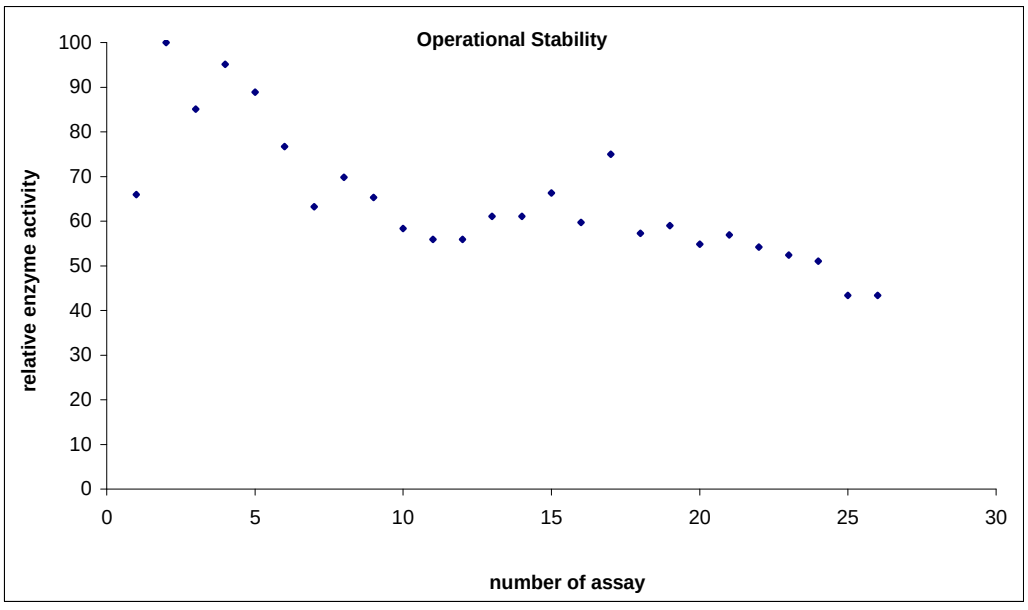


Figure 3.6 Operational stability of chitosan/PTSA enzyme biosensor

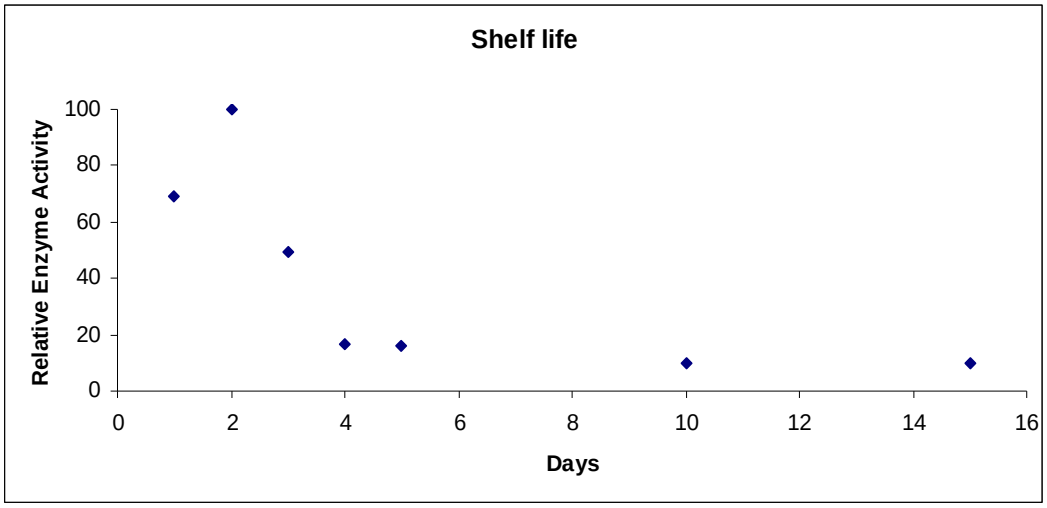


Figure 3.7 Shelf life of the biosensor (chitosan/AA)

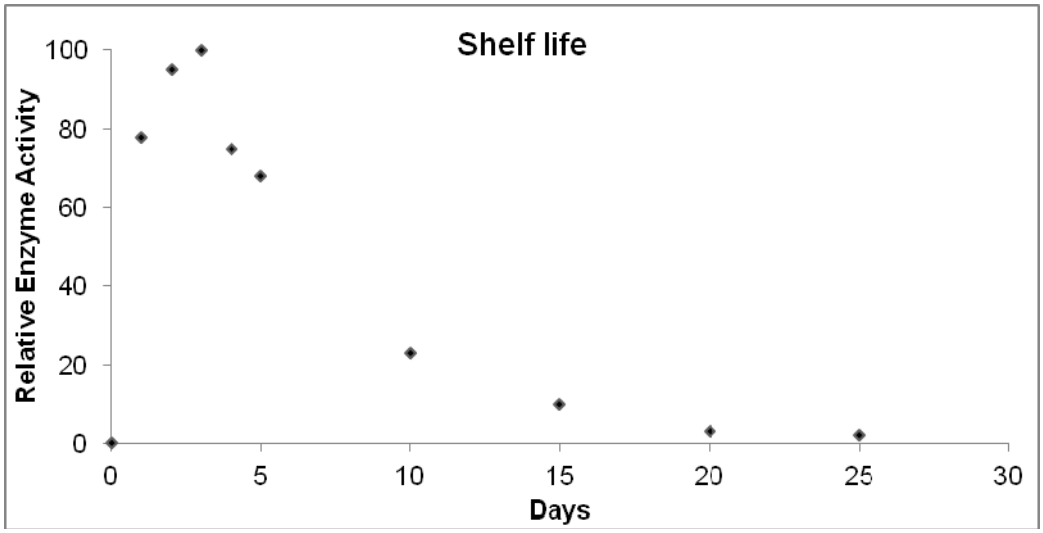


Figure 3.8 Shelf life of the biosensor (chitosan/PTSA)

3.2 FTIR Results

The FTIR spectra of the samples were obtained with a Varian 1000 FT-IR spectrophotometer. Figure 3.9 shows the FTIR spectra of chitosan/AA complexes. The band at 1597 cm^{-1} was attributed to the bending vibration of the amino group in chitosan (Silverstein and Webster 1998), which disappeared upon complexation with AA. The peaks at 1380, 1420, 2870 and 2920 cm^{-1} were due to the C-H. The peak at 3420 cm^{-1} is belong to the –OH stretching. The bands at 1595 and 1622 cm^{-1} (Silverstein and Webster 1998) represent the symmetric and asymmetric bending vibrations of $-NH_3^{+,}$ respectively. The disappearance of $–NH_2$ and appearance of $-NH_3^+$ indicated the protonation of the amino group in the chitosan with the addition of AA. Similarly, the intensity of strong C=O stretching of AA at 1731 cm^{-1} is decreased and new broad peak is assigned to asymmetric stretching of $-CO_2^-$ unit (Mukoma, et al. 2004) (see Figure 1). These results demonstrated the proton exchange reaction between chitosan and AA leading to polymer complexation. Also, the absoption band at 1641 cm^{-1} was hidden by the $-NH_3^+$ band, related to the C=O stretching peak of the chitin form, indicated that chitosan was not fully deacetylated. FTIR spectra of chitosan/PTSA networks is like in the Figure 3.9. As in the case of the chitosan/AA network, protonation of chitosan by PTSA is supported by the disappearance of amino group ($-NH_2$) in chitosan structure and the appearance of $–NH_3^+$. Although we expected that the best protonation was achieved when the ratio of chitosan:PTSA was, x=1, from the FTIR spectra, we can conclude that the best protonation was achieved when the weigth ratio of chitosan:PTSA was 1:3. Also, physical stability of this ratio network was the highest.

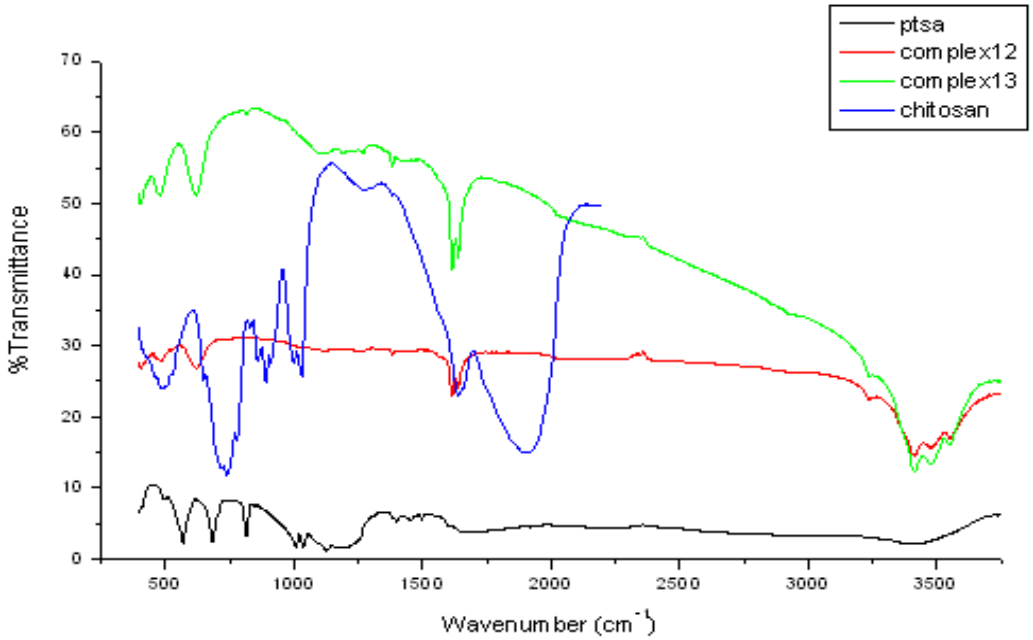


Figure 3.7 FTIR spectrum of pure chitosan, PTSA and chitosan/PTSA networks of different weight ratios

3.3 DSC and TGA Analysis

In the immobilization process, thermal stability of network is an important property. The DSC thermogram reveal the formation of two endothermic transition at around 60 ^{0}C and 180 ^{0}C (Figure 3.10.a), which resulted respectively from elimination of water and the formation of amide linkages in the chitosan/AA complex (Smitha, et al. 2005). The DSC result is in accordance with TGA data of the chitosan/AA complex and shows that with increasing temperature the weight of the sample decreases gradually up to 180 ^{0}C and faster at temperatures ranging from 180 ^{0}C to 300 ^{0}C (Figure 3.10.a and b). Figure 3.11 shows the thermal stability of chitosan/PTSA network. The decrease in gravity began at about 100 ^{0}C which indicated the elimination of water from network, and at about 250 ^{0}C indicated the decomposition of network, which is higher temperature than that of chitosan/AA. We conclude that the thermal stability of chitosan network with PTSA is higher than with AA.

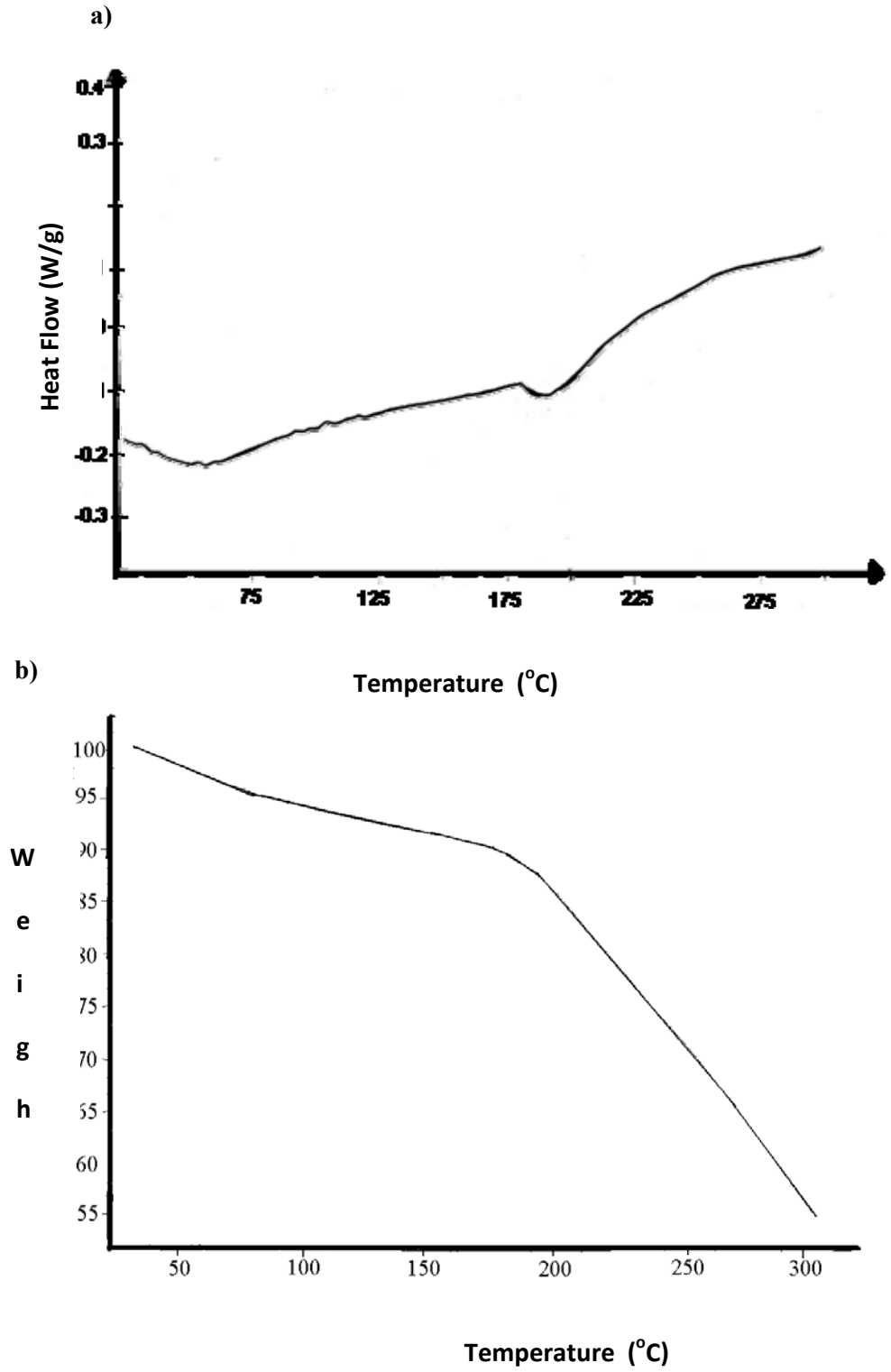


Figure 3.8 (a)DSC and (b)TGA Results of Chitosan/AA complex (x=1)

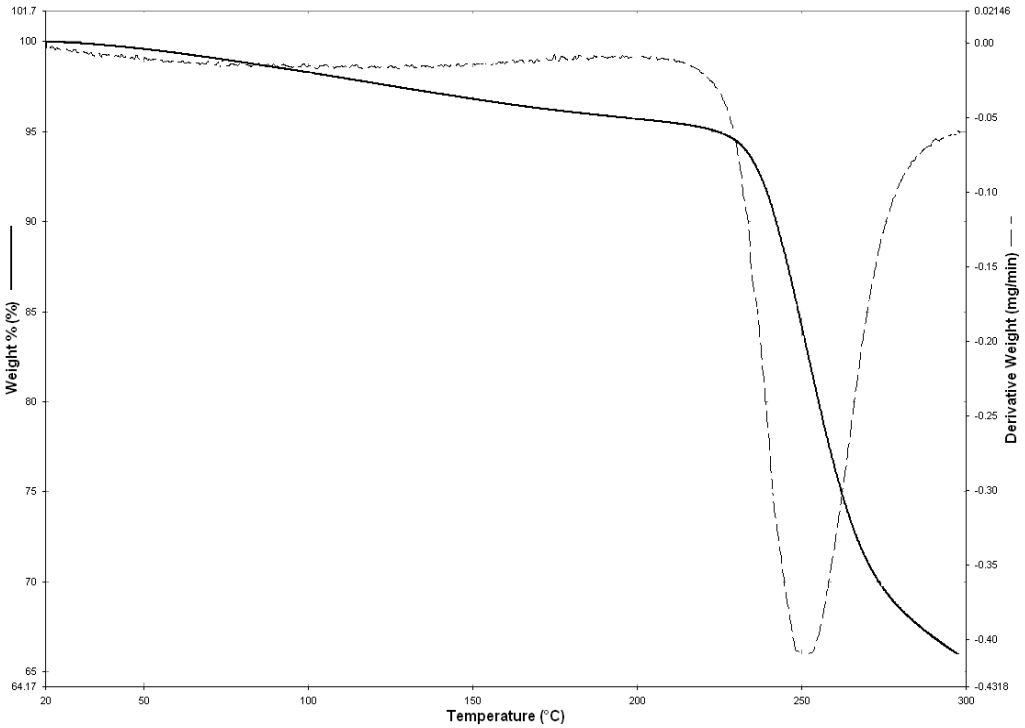


Figure 3.9 TGA Results of (a)Chitosan, (b)Chitosan/PTSA complex (x=1)

3.4 Elemental Analysis

Different mixing ratios of chitosan/AA and chitosan/PTSA complexes were prepared and dried for two days under vacuum for EA. Table 3.2 and Table 3.3 shows the feed and the complex content of the chitosan/AA and chitosan/PTSA complex polymer electrolytes.

In Table 3.2 and Table 3.3 results reveal respectively, that the chitosan content varies between 33% and 80% in the chitosan/AA and 35% and 69% in the chitosan/PTSA in the feed. Whereas, it varies between 44% and 64% in the chitosan/AA complex and 47% and 69% in the chitosan/PTSA complex. According to the results, the feed ratio is expressed x=1, since it refers to higher chitosan content in chitosan/AA complex and x= 0.33 for chitosan/PTSA complex. Chitosan enhances the stability of the complex, making it more stable than the products obtained with other mixing ratios.

Table 3.2 Results of elemental analysis (chitosan/AA)

Sample (Chitosan:AA)	mol % Chitosan in the feed	mol % Chitosan in the complex
1:2	33	44
1:1	50	64
2:1	67	62
4:1	80	62

Table 3.3 Results of elemental analysis (chitosan/PTSA)

Sample (Chitosan:PTSA)	mol % Chitosan in the feed	mol % Chitosan in the complex
1:1	35	47
1:2	53	67
1:3	69	69

3.5 Proton Conductivity

In Table 3.4, we can conclude that clearly the conductivity of the samples depends on the temperature. At higher temperatures the degrees in conductivity can be explained by the loss of humidity. Chitosan has very low electrical conductivity due to non ionizable unit in the structure (Mohamed, et al. 1995). However, it gains some mobilized ions, i.e., H_3O^+ after dissolving with AA under humidified conditions. Also, protonation of $–NH_2$ groups result in the formation of chitosan based polyelectrolyte and proton conductivity may also occur over the protonated and unprotonated unit in the complex matrix.

Table 3.4 Results of proton conductivity measurements

Temperature (K)	Proton Conductivity (S/cm)	
	Chitosan/AA	Chitosan/PTSA
353	1.4×10^{-3}	0.9×10^{-3}
333	1.9×10^{-3}	1.4×10^{-3}
313	2.5×10^{-3}	1.9×10^{-3}
293	2.7×10^{-3}	2.2×10^{-3}

CHAPTER IV

CONCLUSION

New polymer networks containing chitosan/AA and chitosan/PTSA were synthesized. These networks were obtained by mixing different ratios of chitosan and AA and chitosan and PTSA. Then, these were used for immobilizing ChOx.

These polymers' protonation reactions between chitosan/AA and chitosan/PTSA were studied by FT-IR spectroscopy. FT-IR results show the protonation of chitosan by AA and PTSA. Composition of the polymer networks were studied by EA and results show that chitosan content was changed from 44% and 64% in chitosan/AA network and 47% and 69% in chitosan/PTSA network and these results in accordance with the physical stability of the network which depends on the amount of chitosan in it. Also, TGA and DSC results demonstrated that although chitosan decompose at lower temperature than complexes of chitosan with AA and PTSA. This shows that protonation of chitosan by AA and PTSA or complexation of chitosan with AA and PTSA were achieved successfully. The proton conductivity of the chitosan/AA and chitosan/PTSA is nearly the same and 2.1 x 10^{-3} S/cm at 323 K.

Also, ChOx enzyme can be immobilized successfully in these polymeric networks. ChOx entrapped chitosan/AA and chitosan/PTSA polymer networks have high temperature resistance, operational stability and shelf-life and also reasonable values for pH optimization. For EEPN, the results are as follows:

- K_m value was found as 2.05×10^{-5} and 2.6×10^{-4} and V_{max} value was found as 5.3×10^{-6} and 2.7×10^{-5} μmol/min.mg complex for chitosan/AA/ChOx and chitosan/PTSA/ ChOx, respectively.

- Maximum temperature was found as 40 ^{0}C for chitosan/AA/ChOx and 30 ^{0}C for chitosan/PTSA/ ChOx.
- The maximum pH was found to be 7.5 for chitosan/AA/ChOx and 7 for chitosan/PTSA/ChOx.
- The proton conductivity of the chitosan/AA and chitosan/PTSA is nearly same and increase with decreasing temperature.

So, entrapped ChOx shows activity in broader range of temperature and pH than free ChOx.

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APPENDIX

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