Emre ÇEVİK

M.S. Thesis In Genetics and Bioengineering

January - 2012

AN AMPEROMETRIC BIOSENSOR BASED ON MODIFIED NANOPARTICLES WITH AN ELECTRON TRANSFER MEDIATOR FOR THE DETERMINATION OF PHENOL DERIVATIVES

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January 2012
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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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Prof. Dr. Halil Rıdvan ÖZ

Head of Department

This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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January 2012

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ABSTRACT

The main goal of this thesis is to enrich nanoparticles with electron transfer mediating groups and use them in an phenol biosensor application. To this end, different ligand systems featuring functional polymer and co-polymer segments and, also chain-end functionalities were developed. Ligand systems was functionalized by using mediator groups to facilitate electron transfer from the enzyme active site to the electrode. The enzyme was covalently immobilized onto the electrode by means of reactive groups whose hydrophilic moeities areused to create a suitable medium for the enzyme. These ligands will be utilized to functionalize nanoparticles with different cores, including metallic (Au), and magnetic (Fe,Pt and iron oxide) materials. Using these surface tailored nanoparticle scaffolds, surface binding of nanoparticles with enzyme horseradish peroxidase (HRP) was systematically studied to obtain optimum system for detection of phenol (or derivatives) levels. The system applied to real commercial phenolic samples used in the industry.

Keywords: Biosensors, Magnetic nanoparticles, HRP enzyme, Phenol Biosensrs.

PHENOL VE PHENOL TÜREVLERİNİN TAYİNİ İÇİN ELEKTRON TRANSFER MEDİYATÖR GRUPLARIYLA MODİFİYE EDİLMİŞ NANOPARÇACIK TABANLI BİYOSENSÖR GELİŞTİRİLMESİ

Emre ÇEVİK

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ÖZ

Bu tez çalışmasının amacı nanoparçacıkların electron transfer gruplarıyla modifiye edilmesi ve phenol biyosensoru uygulamalarında kullanılmasıdır. Bu amaçla, foksiyonel polimer ve ko-polimer içeren farklı ligand sistemleri geliştirilmiştir. Bu ligand sistemleri electron transfer aracı gruplarıyla ezimin aktif bölgesinden elektrot yüzeyine yapılan electron taşınmasını artırmak için daha fonksiyonel hale getirildi. Enzim kovalent olarak elektrot yüzeyine bağlandı ve reaktif grupların hidrofilik kısımları enzimler için uygun bir ortam oluşturdu. Kullanılan bu ligandlar (Au), manyetik (Fe, Pt ve iron oxide) nanoparçacıkları farklı katmanlar şeklinde modifiye ederek yüzey artırımıyla daha kullanılır hale getirdi. Yüzeyce geliştirilmiş bu nanoparçacıklara peroksidaz enzimi (horseradish peroxidase) (HRP) immobilize edildi ve sistematik olarak çalıştırılarak fenol ve fenol türevlarinin tayini gerçeklerştirildi. Bu system sanayide kullanılan ve fenol içeren gerçek örneklerde denendi.

Anahtar Kelimeler: Biyosensör,Manyetik nanoparçacık, HRP enzimi, Fenol biyosensorü

ACKNOWLEDGEMENT

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I express my thanks and appreciation to my family for their understanding, motivation and patience. Lastly, but in no sense the least, I am thankful to all colleagues and friends who made my stay at the university a memorable and valuable experience.

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

AIBN	2,2'-Azodi(isobutyronitrile)
Au	Gold electrode
Cys	Cysteamine hydrochloride
(-C_6H_5)	Phenyl group
DLS	Dynamic light scattering
E	Enzyme molecule
GMA	Glycidil metacrylate
HRP	Horseradish peroxidase
MPS	trimetox propyl trimethoxysilane
-OH	Hydroxyl group
P	Poly
Si	Silane containing group
Vfc	Vinyl ferrocene

CHAPTER 1

INTRODUCTION

The phenolic compounds play important roles in chemical industry for the production of a great variety of organic materials. They are present, for instance, in the production of plastics, insecticides, pharmaceuticals, paper and dyes, in oil refining, and in coal conversion [1-3]. Due to their toxicity and persistency, the determination of phenolic compounds has great importance in the environment [4–6]. In the earth soil and water surrounding plants are generally contaminated with toxic phenolic substances. The concentration of these compounds in natural water or soil may reach critical amount and this situation dangerours for environment, but on the whole they are present at the ppb level. There are many methods for the determination of phenolic compounds, Gas chromatography and spectrophotometry are two most popular ones among those methods [7-10]. In fact, these methods suffer from complicated sample pretreatment and unsuitable on-site monitoring. In order to overcome these problems; for the simple and effective determination of phenolic compounds many efforts have been made. Amperometric biosensors based on phenol determination with enzymes have been proven to be promising for this purpose [11].

1.1. PHENOLIC COMPOUNDS

Phenolic compounds are a class of chemicals consisting of a hydroxyl group (-OH) bonded to a phenyl group (-C_6H_5). The basic structure of phenol is (C_6H_5OH). Physical property of phenol is a solid crystal with the white color. Detection limit of the phenols is 40 ppb in air, and 1-8 ppb in water. It is quitely flammable and evaporates so slowly compared with water [12]. Phenol or Phenolic compounds can be both naturally forming chemicals and manufactured by man made. Naturally, it is found in waste of

human beings and animals, and decomposed organic materials. They are formed from aminoacids under UV of sunlight included in plants' hemicelluloses and tyrosine transformation in mammalian digestive tract [13].On the other hand, emissions play important role for releasing of phenol and phenolic compounds to the environment as a result of combustion process, from textile, medicine, paper and dye industries, from the structure of agricultural pesticides and disinfectants. Figure 1.1 shows some of the phenolic compounds used in this thesis [14].

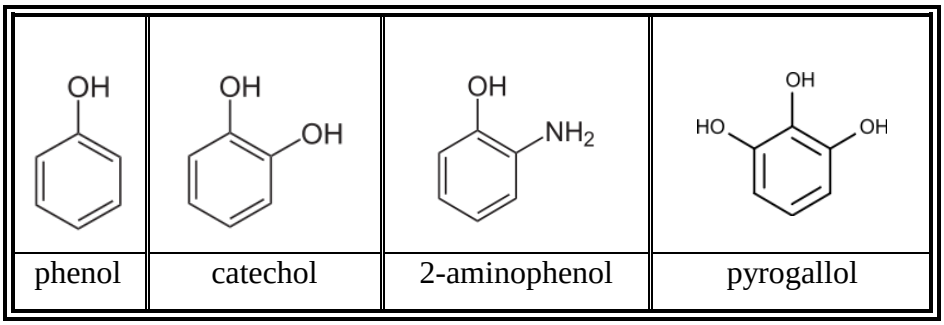


Figure 1.1. Phenols and some of the derivatives

1.1.1. Catechol

Catechol is an aromatic alcohol which is soluble both in water and organic solvents. It is mainly formed in the result of phenol and benzoic acid hydroxylation process. There are also some other processes to obtain catechol (Figure 1.2).

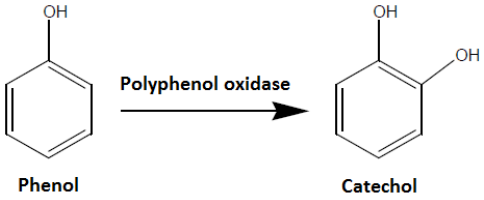


Figure 1.2. Enzymatic reaction phenol to catechol [13].

Catechol is employed in photography, rubber, synthetic material, cosmetic dye and insecticide production and drug synthesis. 4-tert-buthylcatechol is used to inhibit the polymerization process of synthetic materials [13].

1.1.2. Cresol

There are three main isomers of cresol: ortho, meta and para-cresol. Cresols are formed during coal and gasoline combustion. p-cresol are also formed during the decomposition of organic matter or synthesis of chlorinated phenols by fungi and plants

as a natural process. The occurrence of p-cresol is also related to the production of sewage by the petrochemical industry. The mixture of m-cresol and p-cresol is used in insecticide synthesis. The lisole which is a solution of cresols in potassium soap is used in medicine as it reveals strong disinfecting activity [14].

1.1.3. 2-aminophenol

2-Aminophenol is an amphoteric molecule and a reducing agent. In industry, it is synthesized via reducing the nitrophenol by hydrogen in the occurance of catalysts. 2-Aminophenol is used as a reagent for the synthesis of dyes and heterocyclic compounds [15].

1.1.4. Pyrogallol

Pyrogallol or benzene-1,2,3-triol is a white crystalline powder which is fisrtly synthesized by heating gallic acid by Schele in 1786. Pyrogallol is a strong reducing agent. Pyrogallol has been used as an autoxidize rapidly, especially in alkaline solution and the reaction has been employed for the removal of oxygen from gases [16]. It can be used in this way to calculate the amount of oxygen in air. Another usage area of pyrogallol is in hair dying.

1.2. TOXICITY OF PHENOLIC COMPOUNDS

Phenol is a toxic and hazardous substance even at low concentrations [17,18]. Phenol is toxic to microorganisms as well as higher freshwater organisms. A toxicity threshold of 64 mg phenol/liter was found for bacteria. Similar threshold values for protozoa and fungi have also been reported. The membrane of Escherichia coli cells grown in the presence of phenol were significantly impaired by the presence of phenol.

Possible pathways for exposure to phenol are drinking contaminated water, eating contaminated food, and contacting products that have phenol in them. Phenol can cause headaches, dizziness, high blood pressure, heart problems, shallow breathing, wheezing, coughing, vomiting, and stomach ulceration when it is taken into the body [17].

Serious health effects such as diarrhea, nausea, mouth sores, and dark urine have been reported for people who consumed water contaminated with phenol [19,20]. Short-term exposure to phenol in the air can also result in weight loss, respiratory irritation, headaches, dark urine and burning eyes, paralysis and severe injuries on the heart, kidneys, liver, and lungs, followed by death in some cases. Humans who had skin exposure to high amounts of phenol experienced skin burns, liver damage, dark urine, and irregular heart beat. Phenol can be used for medical purposes and is an effective antiseptic for skin and mouth washed in small amounts [21].

1.3. DETECTION METHODS OF PHENOLIC COMPOUNDS

There are many different analytical methods to detect phenolic compounds, such as chemical, electrochemical, chromatographic, flow injection, spectrometric analyses and so on.

1.3.1. Thin-layer Chromatography

The usage of TLC for the analysis of phenol was rare. Since the early 1960s, it has been a popular method in phenolic analysis and still plays a distinct role in the determination of phenolic acids in natural products [22]. Constantine et al. used double-development TLC to separate a mixture of flavonoids containing nine glucosides and seven aglycones. Even though quantification is not the main goal of TLC studies in general, densitometry is used in several studies to achieve this goal. The recoveries using a Standard Spiking Procedure were above 94%.

1.3.2. Gas Chromatography

This technique is used for analysis of many volatile compounds. It offers high sensitivity and selectivity especially when combined with mass spectrometry. The specific concern with the gas chromatography is the low volatility of phenolic compounds on the grounds that one chemical characteristic of the OH group in phenolic compounds is the hydrogen bonding capability increasing the melting point. Gas chromatography is a major chromatographic technique used for the analysis especially of phenolic acids in plants [22].

1.3.3. HPLC

HPLC has been used for the separation and characterisation of phenolic compounds for the last twenty years. One of the most imprtant advantages of using HPLC in the analysis of phenolic compounds is tha they enable the determination of low concentrations of analytes in the presence of many other interfering and coeluting components [22].

1.3.4. Spectrophotometric Methods

Phosphotungstate and phosphomolybdate spectrometries which was propesed by Folin et al. [23] is the earliest optical determination methods for phenolic compounds in water and the 4-AAP (4-aminoantipyrine) spectrometry is the most common method for detection of phenols.

Based on the variation of UV spectrum for phenolic compounds with alkanillity of medium, UV- spectrometry is used to determine phenols in gasoline and in sewage [24,25]. The phenolic compounds were deteremined in surface active agent and foods by Smullin and Englis [26,27]. Besides these, sensors which are based on voltammetric and amperometric methods are employed to detect phenolic compounds.

CHAPTER 2

BIOSENSORS

Sensor is a device which receives a physical, chemical or biological signal and replies to it in a distinctive manner. The sensors can be divided mainly into three groups:

a) Physical sensors which measure a physical quantity such as temperature, pressure, length etc.

b) Chemical sensors for measuring chemical components.

c) Biosensors which measure chemical substances by using a biological sensing element.

The difference between chemical sensors and biosensors is that recognition element in biosensors is biological. Biosensors have been developed to detect a variety of biomolecular complexes, including oligonucleotides, antibody-antigen interactions, hormone-receptor interactions, enzyme-substrate interactions, and lectin-glycoprotein interactions [28].

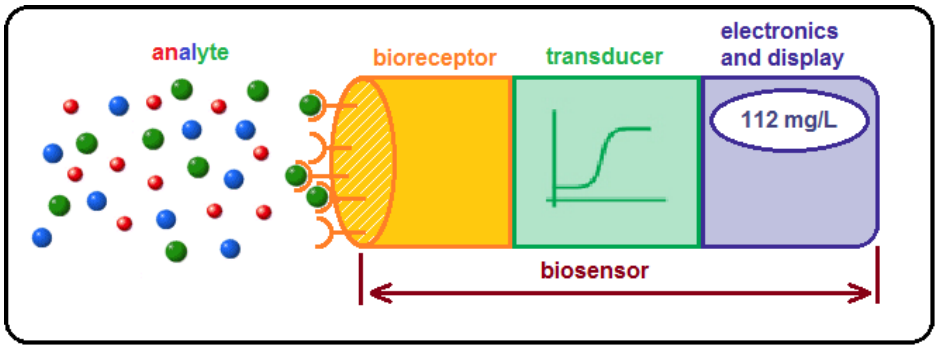


Figure 2.1. General Principle of a biosensor.

In general, biosensors consist of two components: a highly specific recognition element and a transducer that converts the molecular recognition event into a quantifiable signal (shown in Figure 2.1).

Biosensors can easily detect analytes in the micromolar to even femtomolar range. The main advantages of biosensors can be counted as speed of response and ease of use. On the other hand, instabilitity of biorecognition element (enzyme, tissue, etc.) is a limitation for biosensors. Another problem is the transducers' huge size [28].

2.1. BIOLOGICAL ELEMENTS

The major selectivity in biosensors is supplied by the biological elements. The most important point is that the biorecognition elements must attach themselves just to specific target elements but not the other ones. Biosensors have been developed to detect a variety of biomolecular complexes, including oligonucleotides, antibody-antigen interactions, hormone-receptor interactions, enzyme-substrate interactions, and lectin-glycoprotein interactions.

2.1.1. Enzymes

Enzymes are the biological catalists consisting of mainly proteins and usually a prosthetic group. The molecules which enzymes deal with are called substrates at the beginning of the reaction and after during reaction, they are converted into different molecules, called products.

The concept that enzymes form complexes with their substrate molecules was first featured in the late ninetenth century. During this period, Emil Fischer proposed the "lock and key" model for the stereochemical relationship between enzymes and their substrates (Figure 2.2) [29]. That makes the active site of every enzyme is unique to their own substrate. Enzymes can be used in a purified form or as in microganisms and tissues.

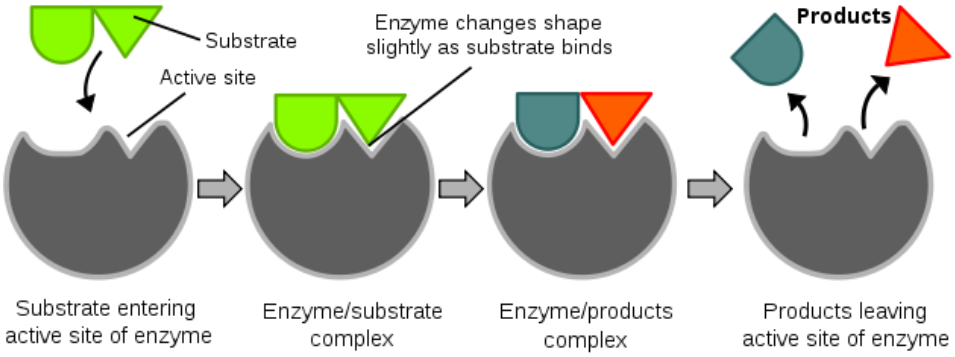


Figure 2.2. Schematic illustration of the lock and key model of enzyme – substrate interactions.

2.1.2. Tissue Materials

Both plant and animal tissues can be used as recognition agent. However, tissues may not be as selective as purified enzymes on the grounds that tissues include more than one enzyme. Furthermore, the response time might be longer because the substrate can diffuse slower in more tissue stuffs.

On the other hand, the sensors with tissue materials can have longer life time since enzymes are exposed to less degradation in their natural environment. Another advantage of the tissues is that tissues are much cheaper than enzymes [30].

2.1.3. Microorganisms

Microorganisms can assimilate organic compounds, resulting in a change in respiration activity, and can produce electro active metabolites. The usage of microorganisms in biosensors has similar advantages and disadvantages with the tissue materials when compared with enzymes [30].

2.1.4. Antibodies

Immunoassays and immunosensors are analytical systems that both use the remarkable specificity supplied by the molecular recognition of an antigen by antibodies. Antibodies are a family of glycoproteins known as immunoglobulins (Ig).

$$Ab+Ag \rightleftharpoons Ab\text{-}Ag$$

The binding affinity between an antibody (Ab) and an antigen (Ag) can usually be indicated by the equilibrium constant (K) as shown below:

$$K = \frac{[Ab - Ag]}{[Ab]\,[Ag]}$$

where Ab – Ag is the immunoassay complex formed by between antibody and its particular antigen. Those antibodies are very selective and sensitive so that they bind very strongly to their antigen which is the unique advantage of the biosensors with antibodies. On the other hand, the reaction between antibodies and antigen is not catalytic [30].

2.1.5. Nucleic Acids

Nowadays various types of natural and synthetic DNA and RNA molecules are available for electrochemical biosensors, as biorecognition element [31]. Both DNA and RNA binding species called aptamers are selected to detect from small molecules and proteins, to individual cell types and cellular organisms. Aptamers are extremely specific, and have affinities which rival antibodies.

2.2. IMMOBILIZATION TECHNIQUES OF BIOLOGICAL COMPONENTS

Immobilization is a technique to fix the recognition elements on the electrode surface.

The advantages of immobilization as follows:

- in many cases the enzyme is stabilized;
- the enzyme-carrier complex may be easily separated from the sample, i.e. the latter is not contaminated by the enzyme preparation;
- the stable and largely constant enzyme activity renders the enzyme an integral part of the analytical instrument [32].

There are several strategies to immobilize enzymes shown in figure 2.3;

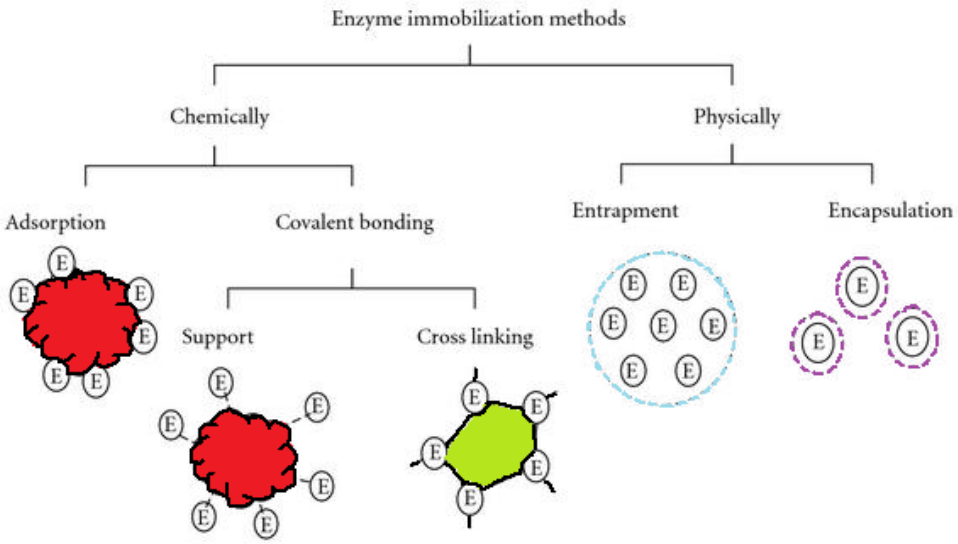


Figure 2.3. Schematic representation of immobilization methods used for biosensor construction. **E:**enzyme molecule.

2.2.1. Adsorption

Adsorption is the adhesion of atoms, ions, biomolecules or molecules of gas, liquid, or dissolved solids to a surface [33]. Adsorption can be divided into two parts: a)Physisorption (or physical adsorption) is adsoprtion in which the forces of attraction existing between adsorbate and adsorbent are Vander Waal's forces. b) Chemisorption (or chemical adsorption) Chemical adsorption is adsorption in which the forces of attraction existing between adsorbate particles and adsorbent are almost of the same strength as chemical bonds. Çevik et al. succesfully adsorbed urease enzyme on a metal chelated polymeric supporter [34].

2.2.2. Encapsulation

Encapsulation includes the trapping of molecules within capsule of different composition (particles, spheres, tubes, vesicles; made of hydro gels, Polymers, carbon, silica, lipids, etc.) and formed by different methods (by template molding, polymerization, self-assembly, emulsification, etc.). Encapsulation, as entrapment, has been reported to protect proteins from unfolding and degradation, ensuring larger activity times. On the other hand, microencapsulation needs relatively high

biocomponent concentrations and generates longer response times compared to the care when biocomponent is free in solution [35].

Encapsules can be divided into three main groups, according to their preparation methods:

a) layer-by-layer (LBL) self-assembly technique using polyelectrolyte

b) ionic cross-linking of alginate or alginate/chitosan mixtures

c) interfacial condensation or polymerization [36].

2.2.3. Entrapment

In this method, biomaterial is immobilized in a solution of organic or inorganic polymer during the matrix polymerization. However, there is loss of enzyme activity through the pores in the gel. Şenel et al. has been immobilized urease enzyme in a polymer matrix by using entrapment (Figure 2.4) [37].

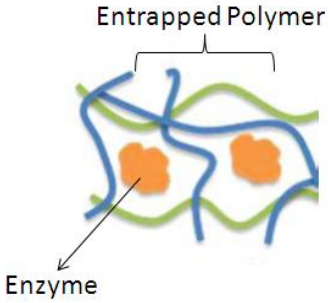


Figure 2.4. Entrapment of an enzyme molecule.

2.2.4. Cross-linking

The general meaning of cross-linking is that bonds of one polymer chain to another. This approach uses bifunctional agents to bind the biomaterials to solid supports, and has proven to be useful method for stabilizing adsorbed enzymes [28]. Glutaraldehyde, which reacts with lysine amino acid residues in the enzyme, is one of the popular materials used for cross-linking reactions. Glutaraldehyde also binds enzymes together to make a cross-linked enzyme aggregate and which prevent the leakages enzyme from gelly matrix.

2.2.5.Covalent bonding

Some functional groups which is tasked in the active sitye of enzyme can be covalently bonded to the support matrix (transducer or membrane) [29]. In this method, in order to the biological materials can bind, the sensor surface is treated as a reactive group.

2.3. TRANSDUCERS

Transducer is a material which converts the variable signals produced by biorecognition element into electrical signal.

2.3.1. Electrochemical Transducers

2.3.1.1. Potentiometric

Potentiometric sensor is based on measurement of potential at zero current. The analytical information is obtained by converting the recognition process into the potential, which is proportional (in a logarithmic fashion) to the concentration of the reaction product [38].

2.3.1.2. Amperometric

Amperometric methods rely on increasing or decreasing the applied potential to the cell until oxidation (or reduction) of the substance could be analysed. This method is one of the most common method used in biosensors. In the amperometric method voltammetry play important role. Voltammetry can be defined as the measurement of current as a function of applied potential. This method can be used for both qualitative and quantitative analysis of various molecular and ionic materials.

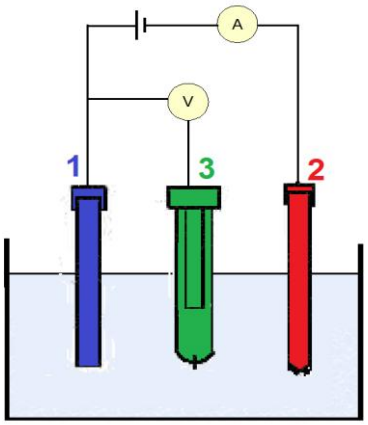


Figure 2.5. Three-electrode setup: (1) working electrode; (2) auxiliary electrode; (3) reference electrode

There are three different electrodes (Working, Auxiliary and Reference electrode) in amperometric measurements (Figure 2.5.). Electrochemical reactions happen at the surface of the working electrode. It can be defined as anode or cathode depending on which redox reaction occurs on the electrode. Working electrodes are generally manufactured from the inert metals such as gold, silver or platinum, inert carbon such as glassy carbon and mercury drop electrode can be also used as working electrode.

Auxiliary or counter electrode is a second electrode acting as the other half of the cell. Current flow occur between working and auxiliary electrode. Inert materials such as gold, platinum, or carbon can be used as auxiliary electrode.

Since reference electrode has a stable and well-known electrode potential, it is used to determine the potential of the other half cell. The most common reference electrodes are standard hydrogen electrode, saturated calomel electrode and silver chloride electrode [39].

2.3.1.3. FET-based Sensors

A field-effect transistor (FET) is a kind of transistor which is usually used to amplify the weak signal. FET can be used in potentiometric and voltammetric sensors (Figure 2.6).

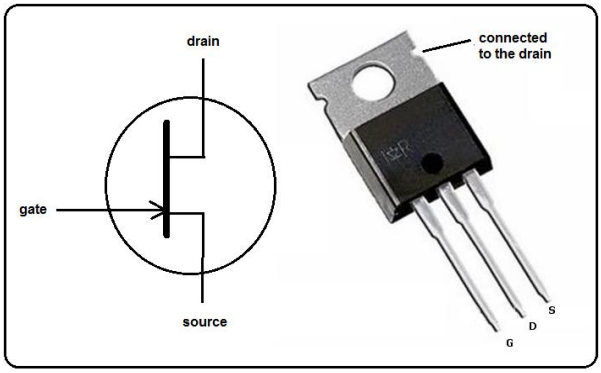


Figure 2.6. A type of field effective transistor

2.3.1.4. Optical Transducers

Optical transducers include the methods using light emission such as, fluorescence, luminescence, internal reflection spectroscopy, surface plasmon resonance and light scattering [39].

2.3.1.5. Piezoelectric Devices

When mechanical pressure is applied on certain solid materials (especials crystals, certain ceramics), a charge called “piezoelectricity” accumulates in those materials. The value of electrical field changes with respect to mass of material absorbed on its surface. The piezoelectric effect is a reversible process, it means when electric field effects to those kind of materials, they will generate mechanical force [39]. So piezoelectrical biosensors can measure slight changes of biorecognition element. For example antibody-antigen reaction can be sensed by this technique.

CHAPTER 3

NANOPARTICLES

In recent years, nanosized particles have been focus of sustained research and industrial interest, which is expected to continue for years to come. This is due to various potential applications in fields like electronics and magnetism, biochemistry, biomedicine, biosensors, enviromental protection.

Recently, magnetic nanoparticles have attracted increased interest due to its good biocompatibility, strong superparamagnetic, low toxicity, and easy preparation process. The successful applications of magnetic nanoparticles in the immobilization of bimolecular have also been reported [40].

3.1. GENERAL PROPERTIES OF NANOPARTICLES

Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. A bulk material should have constant physical properties regardless of its size, but at the nano-scale size-dependent properties are often observed. Thus, the properties of materials change as their size approaches the nanoscale and as the percentage of atoms at the surface of a material becomes significant. For bulk materials larger than one micrometer (or micron), the percentage of atoms at the surface is insignificant in relation to the number of atoms in the bulk of the material. The interesting and sometimes unexpected properties of nanoparticles are therefore largely due to the large surface area of the material, which dominates the contributions made by the small bulk of the material [40].

Nanoparticles often possess unexpected optical properties as they are small enough to confine their electrons and produce quantum effects. For example gold

nanoparticles appear deep red to black in solution. Nanoparticles of usually yellow gold and gray silicon are red in color. Gold nanoparticles melt at much lower temperatures (~300 °C for 2.5 nm size) than the gold slabs (1064 °C); [43] and absorption of solar radiation in photovoltaic cells is much higher in materials composed of nanoparticles than it is in thin films of continuous sheets of material. Other size-dependent property changes include quantum confinement in semiconductor particles, surface plasmon resonance in some metal particles and superparamagnetism in magnetic materials. Ironically, the changes in physical properties are not always desirable. Ferromagnetic materials smaller than 10 nm can switch their magnetisation direction using room temperature thermal energy, thus making them unsuitable for memory storage [41].

The high surface area to volume ratio of nanoparticles provides a tremendous driving force for diffusion, especially at elevated temperatures. Sintering can take place at lower temperatures, over shorter time scales than for larger particles. This theoretically does not affect the density of the final product, though flow difficulties and the tendency of nanoparticles to agglomerate complicates matters. Moreover, nanoparticles have been found to impart some extra properties to various day to day products. For example the presence of titanium dioxide nanoparticles imparts what we call the self-cleaning effect, and the size being nanorange, the particles cannot be observed. Zinc oxide particles have been found to have superior UV blocking properties compared to its bulk substitute. This is one of the reasons why it is often used in the preparation of sunscreen lotions.

Clay nanoparticles when incorporated into polymer matrices increase reinforcement, leading to stronger plastics, verifiable by a higher glass transition temperature and other mechanical property tests. These nanoparticles are hard, and impart their properties to the polymer (plastic). Nanoparticles have also been attached to textile fibers in order to create smart and functional clothing.

Metal, dielectric, and semiconductor nanoparticles have been formed, as well as hybrid structures (e.g., core-shell nanoparticles). Nanoparticles made of semiconducting material may also be labeled quantum dots if they are small enough (typically sub 10 nm) that quantization of electronic energy levels occurs. Such nanoscale particles are used in biomedical applications as drug carriers or imaging agents [42].

3.2. CHARACTERIZATION OF NANOPARTICLES

Nanoparticle characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. Characterization is done by using a variety of different techniques, mainly drawn from materials science. Common techniques are electron microscopy transmission electron microscopy(TEM) and scanning electron microscopy (SEM), atomic force microscopy (AFM), dynamic light scattering (DLS), x-ray photoelectron spectroscopy (XPS), powder X-ray diffraction (XRD), Fourier transform infrared spectroscopy (FTIR), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), ultraviolet-visible spectroscopy (UV), and nuclear magnetic resonance (NMR).

3.3. FUNCTIONALIZATION OF NANOPARTICLES

The surface coating of nanoparticles is crucial to determining their properties. In particular, the surface coating can regulate stability, solubility and targeting. A coating that is multivalent or polymeric confers high stability. For biological applications, the surface coating should be polar to give high aqueous solubility and prevent nanoparticle aggregation. In serum or on the cell surface, highly charged coatings promote non-specific binding, while polyethylene glycol linked to terminal hydroxyl or methoxy groups repel non-specific interactions. Nanoparticles can be linked to biological molecules which can act as address tags, to direct the nanoparticles to specific sites within the body, specific organelles within the cell, or to follow specifically the movement of individual protein or RNA molecules in living cells. Common address tags are monoclonal antibodies, aptamers, streptavidin or peptides. These targeting agents should ideally be covalently linked to the nanoparticle and should be present in a controlled number per nanoparticle. Multivalent nanoparticles, bearing multiple targeting groups, can cluster receptors, which can activate cellular signaling pathways, and give stronger anchoring. Monovalent nanoparticles, bearing a single binding site, avoid clustering and so are preferable for tracking the behavior of individual proteins [43].

3.4. NANOPARTICLES IN BIOSENSORS

Nanomaterials is playing an increasingly important role in the development of sensors. Biosensors represent an especially exciting opportunity for high-impact

applications benefiting from “nano” attributes. Sensitivity and detection range are the the vital parameters for a sensor. Many sensors operate through the variation of a surface parameter, like surface conductivity, with analyte concentration. Hence, the effective surface area of the device, the area directly interacting with the analyte, determines the sensitivity. In order to increase surface area, nanomaterials, especially nanoparticles, serves an easy answer. In recent years, many kind of nanoparticles with different properties have found and used broad application in biosensor technology. Due to their small size (normally in the range of 1-100 nm), nanoparticles exhibit unique chemical, physical, and electronic properties that are different from those of bulk materials, the high surface-to-volume ratio of nanoparticles has been exploited for improving the performance of biosensors. Reviewing the studies of recent years, we can find that many kinds of nanparticles, including metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles, and even composite nanoparticles, have been widely used in biosensors. Furthermore, different kinds of nanoparticles, and sometimes the same kind of nanoparticles, can play different roles in different biosensor systems. For instance, gold nanoparticles show potential to facilitate molecular bonding to detect glucose in the micromolar concentration range. Aided by silver nanoparticles, amperometric biosensors show improved biocompatibility useful in pesticide detection. Palladium nanoparticles have been tried to fabricate a sensitivity-enhanced electrochemical DNA biosensor. Functional nanoparticles (electronic, optical, and magnetic) bound to biological molecules (e.g. peptides, proteins, nucleic acids) have been developed for use in biosensors to detect and amplify various signals. Nowadays, nanoparticles relating to biosensors show significant maturation. Researchers tend to combine nanoparticles into the materials used for biosensors in order to improve the sensitivity of the system in potential sensing applications. Most recent studies show that biosensors composed with nanoparticles do take on rapid, simple, and accurate measurements, which offers exciting new opportunities for the development of biosensor capabilities [44].

3.5. THE FUNCTIONS OF NANOPARTICLES

Nanoparticles which include metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles, and composite nanoparticles, have been widely used in electrochemical sensors and biosensors. They can be used different roles in different electrochemical sensing systems based on their unique properties. However the main

aim of nanoparticles can be classified as: 1) immobilization of biomolecules; 2) catalysis of electrochemical reactions; 3) enhancement of electron transfer; 4) labeling biomolecule (Table 3.1).

3.5.1. Immobilization of Biomolecules

Nanoparticles can adsorb biocomponents strongly due to their large surface area and high surface free energy and became very important for immobilization of biomolecules in biosensor fabrication. Generally, biomolecules lose their bioactivity when the adsorption of them directly onto naked surfaces of bulk materials. This may result denaturation and loss of bioactivity. However, the adsorption of such biomolecules onto the surfaces of nanoparticles can retain their bioactivity because of the biocompatibility of nanoparticles. Since most of the nanoparticles carry charges, they can electrostatically adsorb biomolecules with different charges. Besides the common electrostatic interaction, some nanoparticles can also immobilize biomolecules by other interactions. Such as, gold nanoparticles can immobilize proteins through the covalent bonds formed between the gold atoms and the amine groups and cysteine residues of proteins [45]

3.5.2. Catalysis of Electrochemical Reactions

Catalytic properties of nanoparticles, especially metal nanoparticles, have excellent catalytic properties. They easly decrease the overpotentials of many analytically important electrochemical reactions, and even realize the reversibility of some redox reactions, which are irreversible at common unmodified electrodes, due to their high catalytic features into electrochemical sensors and biosensors. Because of the selective structure of nanoparticles, selective electrochemical analysis could be achieved. Raj et al [45] developed an electrochemical sensor for selective detection of dopamine in the presence of ascorbic acid, which was based on the catalytic effect of gold nanoparticles on the ascorbic acid oxidation. This resulted in the decrease of the oxidation overpotential of ascorbic acid and the effective separation of the oxidation potentials of ascorbic acid and dopamine, thus allowing the selective electrochemical detection.

3.5.3. Enhancement of Electron Transfer

Electrical contacting of redox-enzymes with electrodes is a key process in the construction of third-generation enzyme electrodes. While enzymes usually lack direct electrical communication with electrodes due to the fact that the active centers of enzymes are surrounded by considerably thick insulating protein shells, and the electron transfer between electrodes and the active centers are blocked, the conductivity properties of nanoparticles, mostly metal nanoparticles at nanoscale dimensions made them suitable for enhancing the electron transfer between the active centers of enzymes and electrodes acting as electron transfer "mediators" or "electrical wires"[46].

Table 3.1. Different nanoparticles in electrochemical systems

Functions	Properties	Nanoparticles	Sensor advantages	Examples
Biomolecule immobilization	Biocompatibility To large surface area	Metal nanoparticles Au,Ag	Improved stability	Antibody immobilization onto nanoparticles remains stable for 100 days
Catalysis of reactions	High surface energy	Metal nanoparticles	H_2O_2 sensor	Improved sensitivity and selectivity
Enhancement of electron transfer	Conductivity; Small dimensions	Au,Pt Metal nanoparticles Au,Ag	Improved sensitivity	Electron transfer rate of 5000 /s for GOx enhanced by gold nanoparticles
Labeling biomolecules	Small size; modification	Semiconductor nanoparticles; CdS, PbS Metal nanoparticles Au,Ag	Improved sensitivity; Indirect detection	DNA sensor labeled with Ag nanoparticles accomplish detection limit of 0.5 pM

3.5.4. Labeling biomolecules

The labeling of biomolecules, such as antigen, antibody and DNA with nanoparticles plays an increasingly important role in developing sensitive electrochemical biosensors. Biomolecules labeled with nanoparticles can retain their bioactivity and interact with their counterparts, and based on the electrochemical detection of those nanoparticles the amount or concentration of analytes can be

determined. Dissolution of the nanoparticle labels – mostly metal and semiconductor nanoparticles[47,48] and measuring the dissolved ions with stripping voltammetry represents a general electroanalytical procedure, as stripping voltammetry is a very powerful electrochemical analytical technique for trace metal measurements Metal nanoparticle labels can be used in both immunosensors and DNA sensors, and gold nanoparticles are the most frequently used among all the metal nanoparticle labels available [49].

3.6. REDOX ACTIVE POLYMERS

Polymers are being used as conductors with a different of novel applications. Nowadays scientists try to combine their expertise to produce enhanced and remarkable redox polymer. Some of the organic compounds have been used as electron transport mediator. They are classified as charge transfer complexes, ionic radical salts and organometallic and conjugated organic polymers. Such polymers have interestingly electrical and optical features but these properties are found previously in inorganic systems. Such polymers which are electrically conductive species different from the inorganic crystalline semiconductors e.g.[50,51].

3.6.1. Redox Active polymers in Biosensors

Conducting polymers have been used and serves as a suitable matrix for enzymes and biological components. Conducting polymers are thus finding ever increasing use in diagnostic medical reagents [52]. In order to enhance speed, sensitivity and fertility of a biosensor in the measurement of vital analytes conducting polymers play important role [53-55]. Immobilization of enzymes into electrode surface conducting polymers permit the localization of active biological components on electrode of any size and shape and it is appropriate for the fabrication of amperometric biosensors. Conducting polymers have attracted to increase flexibility in the suitable chemical, which can be functionalized or modified if needed. It is possible to synthesize or modified by chemical methods in required electronically and mechanical properties. Furthermore polymers can be modified by itself in order to bind biological molecules [56]. Besides, conducting polymers allows direct aggregation of the polymer on the surface of electrode, while immobilizing the biological components. Thus, the film thickness became more important in order to control distribution of the immobilized

enzymes. Interaction between the polymer and biologically active components will improve the development of the conducting polymers and any kind of technology in this field. It has been reported that conducting polymers transfer electrons from enzyme active site to surface of the electrode in the biosensor applications. They are also providing three dimensional structures for this purpose [57].

Electrode surface can be easily modified with conducting polymers and they used for both immobilizing agent of biologically active components and electron transfer. Electric charge produced by the biochemical reaction and transferred to electronic circuit by conducting polymers [58]. This property of conducting polymers along with the possibility to entrap enzymes during electrochemical polymerization has been exploited for the fabrication of amperometric biosensors [59]. In addition to that electrically conducting polymers shows alteration and size exclusion properties owing to which their highly sensitive and specific interactions between substrate [60]. Literature survey indicates that numerous published articles found about conducting polymers which they are serve as good matrices for the immobilization of enzymes. Furthermore they provide fast response, good medium for the enzyme catalyzed reaction.

3.6.2. Vfc and GMA based Biosensors

The charge transfer from redox enzyme to the electrode surface is difficult. However using some metallic complexes or ferrocene containing groups obtained from coimmobilaztion, is proposed to obtain attractive reagentless biosensors. polyvinylferrocene (PVfc) has been investigated due to its organometallic properties for various enzyme electrodes for the amperometric determination of glucose, sucrose, urea, cholesterol, phenols, and hydrogen peroxide. The homopolymer PVFc is a widely used and well known material with very high potential for used in amperometric biosensors. Due to having metal part in the center it shows excellent electron transfer property. The regeneration is also important for the co-enzymes and redox reactions mediators should be involved, the speed of enzymatic reaction can be controlled by regeneration components in the electron relay chain from enzyme to the electrode. The introduction of a mediator in an enzymatic system leads to an acceleration of the electron transfer, a decrease in the applied working potential (the reduction of the mediator is detected at the electrode) and an increase in sensitivity [61]. Glycidyl methacrylate (GMA) is a monomer which is able to bind enzyme covalently via its

epoxy groups. In a previous study, horseradish peroxidase (HRP) was successfully bonded to the P(GMA-*co*-Vfc) film electrode via epoxy groups. Vinylferrocene (VFc), due to its organometallic properties for various enzyme electrodes, was chosen as a redox mediator in the design of GMA based polymers. Şenel et al (2010) has been successfully made peroxide biosensor by using GMA and Vfc containing polymer. They reported that this conducting polymer increased electron transfer rate and made the biosensor more sensitive [62].

3.7. PHENOL BIOSENSORS

Phenolic compounds are generally categorized by chromatographic methods before detection. However, these separations are difficult and need so much time and chromatography equipments are not only expensive but also not generally portable. Instead of chromatographic techniques, scientists have been used a device, which detect phenol or phenolic contents in aqueous medium even at low micro molar concentration [63]. Due to its high selectivity electrochemical biosensors became important alternative tools for the detection of hazardous compounds for environmental pollutants control. These biosensors are useful for the detection of phenolic compounds owing to being cheap and easy alternative and they are portable, fast test devices. Many biosensors have been developed in the past using the catalytic activity of the redox enzymes for phenol determination. enzymes such as tyrosinase, peroxidase, laccase are mostly used in the phenol biosensors [64].

In addition, they have the potential for becoming limits of detection are not always as low as with chromatographic techniques, but for screening and field monitoring purpose electrochemical and, particularly, amperometric techniques are gaining more and more importance.

3.7.1. HRP based Phenol Biosensors

In the literature there are several amperometric biosensors, which made up different transducer based on HRP enzyme. These biosensors have been and used to determine mostly phenolic compounds and hydrogen peroxide contents [64]. Generally, they are used for detectin of contaminated water and food products.

Horseradish peroxidase (HRP) catalyses the oxidative coupling reaction of phenolic compounds. During the reaction, H_2O_2 needed as co-substrate which means it is used for the complete recovery of the enzyme [65].

An amperometric phenol biosensor based on the immobilization of HRP onto a novel copolymer electrode made up with random copolymer of electroactive 3-methylthienyl methacrylate (MTM) and side chain epoxy group containing glycidyl methacrylate (GMA) monomers was prepared via free-radical polymerization. One of the phenol oxidases, HRP was immobilized using different immobilization methods including chemical bonding or entrapment or chemical bonding/entrapment to poly(GMA-co-MTM)-based electrodes for phenol detection [64].

3.7.2. Phenol Biosensors Based on Nanoparticles

A phenolic biosensor based on ZnO(zinc oxide) nanoparticles has improved by Lee et al[66]. In this biosensor, ZnO nanoparticles dispersed chitosan nanocomposite providing an advantageous microenvironment in terms of its favorable isoelectric point for tyrosinase loading and the immobilization of tyrosinase enzyme.

Zhang et al., (2003) [65] have used hybrid sol-gel titania matrix for the construction of sensitive mediator free tyrosinase biosensor. Under optimum pH titania retained the tyrosinase activity and stability attached on to the surface of a glassy carbon electrode.

Another aproach was acetylcholinesterase biosensor based on nanoparticles ZrO_2/chitosan by Yang et al [67]. They proposed this biosensor to determine pesticides in vegetable samples. Acetylcholinesterase was covalently immobilized onto ZrO_2/chitosan nanocomposite matrix. The experimental conditions were optimized showed that pesticides inhibit the activity of enzyme with an effect of decreasing of oxidation current.

Magnetic core–shell (Fe_3O_4–SiO_2) nanoparticles modified film onto the surface of carbon past electrode for covalently immobilization of Laccase enzyme, done by chemical crosslinking with glutaraldehyde and free aldehyde groups to determined the

hydroquinone concentration. Magnetic core–shell nanoparticles supply microenvironment for retaining the bioactivity of laccase [68].

In this thesis we aimed to detect toxic phenolic compounds by using HRP enzyme immobilized to nanoparticles with different polymer matrices for a new biosensor system. Phenolic compound mainly used in industry and these compounds are very dangerous for human and environment therefore detection is important. There are several methods for the detection of them but they are not cost effective and not so fast. In order to detect these chemicals instantly and more sensitive we developed a new biosensor system. This system includes contucting polymers and enhanced with super paramagnetic nanopartiles. The response time of the system around 3 second and real sample measurements show us this biosensor useful for the detection of phenolic compounds.

CHAPTER 4

EXPERIMENTAL

4.1. MATERIALS

HRP (EC 1.11.1.7, RZ > 3.0, 250 U/mg), $FeCl_2.4H_2O$ (EG Nr.: 13478-10-9) NH_3 (EG Nr.: 7664-41-7) and benzene (EG NR.: 71-43-2) were obtained from Sigma. Phenol (EG Nr.:203-632-7), catechol (EG Nr.:204-427-5), pyrogallol (EG Nr.:201-762-9), Cresol 2-aminophenol were obtained from Alfa Aesar. Glycidyl methacrylate (GMA) and 2,2'-azobis (isobutyronitrile) (AIBN), purchased from Fluka and Across Chemical Co., were used without further purification. (3-mercaptopropyl)trimethoxysilane (MPS) (EC 2245885) was obtained from Aldrich.

4.2. INSTRUMENTATION

The FT-IR absorption spectra (4000–400 cm^{-1}) were recorded with a Bruker FTIR-ATR spectrophotometer. Native structure of the free and immobilized HRP were determined by using UV–Visible spectrophotometer (Shimadzu UV-1700). NMR spectra were recorded in $CDCl_3$ using a Bruker 400 MHz spectrometer. X-ray powder diffraction (XRD) analysis was conducted on a Rigaku Smart Lab operated at 40 kV and 35 mA using Cu Kα radiation (λ = 1.54059Å). The thermal stability was determined by thermogravimetric analysis (TGA, Perkin Elmer Instruments model, STA 6000). The TGA thermograms were recorded for 5mg of powder sample at a heating rate of 10 ^oC/min in the temperature range of 30–800 ^oC under nitrogen atmosphere. Transmission

electron microscopy (TEM) analysis was performed using a FEI Tecnai G2 Sphera microscope. A drop of diluted sample in alcohol was dripped on a TEM grid.

Electrochemical measurements were performed using a CHI Model 842B electrochemical analyzer. A gold plate working electrode (1 cm^2), a platinum plate counter electrode (1 cm^2), an Ag/AgCl-saturated KCl reference electrode and a conventional three-electrode electrochemical cell were purchased from CH Instruments. All amperometric measurements were carried out at room temperature. They were performed in stirred solutions by applying the desired potential and allowing the steady state current to be reached. Once prepared, the HRP electrode were immersed in 10 ml of a pH 7.5 10 mM PBS solution and the amperometric responses to the addition of known amount of phenolic compounds solution were recorded, respectively.

4.3. METHODS

4.3.1. Preparation of Fe_3O_4 nanoparticles

A 0.2 M aqueous solution of ferrous chloride ($FeCl_2.4H_2O$) was prepared in deionized water. A 2M aqueous solution of NH_4OH is added dropwise to precipitate metals as hydroxide gel. The hydrated iron gel is thoroughly washed and transferred to a flask fitted with a water condenser. It is to be noted that the presence of anion contaminants, such as Cl^-, NO_3^-, might impede the reaction by forming soluble salts with Fe^{2+}. The gel was stirred under reflux for 4h at 100 °C under N_2 flow to ensure inert atmosphere. The continuous influx of the solvent during the reflux process breaks the gel network into more energetically favorable small crystalline iron-oxide regions [69]. The solid product after refluxing was filtered and oven-dried.

4.3.2. Preparation of poly(glycidylmethacrylate) (SiPGMA)

Telomerization of poly(glycidylmethacrylate) was accomplished by dissolving glycidylmethacrylate (50 mmol) and (3-mercaptopropyl)trimethoxysilane (2 mmol) in benzene (10 mL), followed by the addition of 2 wt % AIBN. The mixture was degassed by using Argon gas and sealed under vacuum. After degassing, the tubes were placed in constant temperature baths controlled to 80 ^{o}C. After two days, White precipitates were formed upon the addition of diethyl ether to the residue solution at −20 °C. Precipitated polymer was washed with diethyl ether and reprecipitated in this manner two more times. The product was then dried under vacuum. The average degree of polymerization (*n=19*) was determined by ^{1}H NMR spectroscopy. In $CDCl_3$, the integral values of peaks around δ = 3.25ppm (one protons of epoxy group) and δ = 0.75 ppm (three protons of $SiCH_3$) were used to calculate the degree of polymerization.

4.3.3. Preparation of Vinyl ferrocene co poly(glycidylmethacrylate) SiP(GMA-co-Vfc)

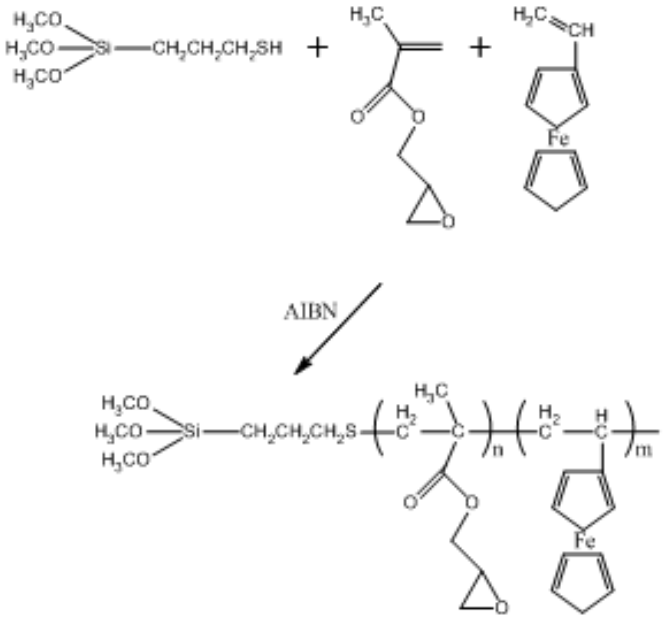


Figure 4.1. Shematic illusturation of SiP(GMA-co-Vfc) telomerization.

Telomerization of SiP(GMA-co-Vfc) was accomplished by dissolving glycidylmethacrylate (50 mmol), MPS (2 mmol) and Vfc(5mmol) in benzene (10 mL), followed by the addition of 2 wt % AIBN (Figure 4.1). The mixture was degassed by using Argon gas and sealed under vacuum. After degassing, the tubes were placed in constant temperature baths controlled to 80 ^{o}C. After two days, White precipitates were formed upon the addition of diethyl ether to the residue solution at −20 °C. Precipitated copolymer was washed with diethyl ether and reprecipitated in this manner two more times. Precipitated product was then dried under vacuum.

4.3.4. Silanization of Fe_3O_4 nanoparticles

The synthesized SiP(GMA-co-Vfc) was silanized onto nanosize magnetic particles in toluene/methanol (80:20 volume ratio) solution at the reflux temperature for 2 days. The silanized particles were washed repeatedly with organic solvents and collected by centrifugation. After washing, the particles were dried in vacuum (Figure 4.2).

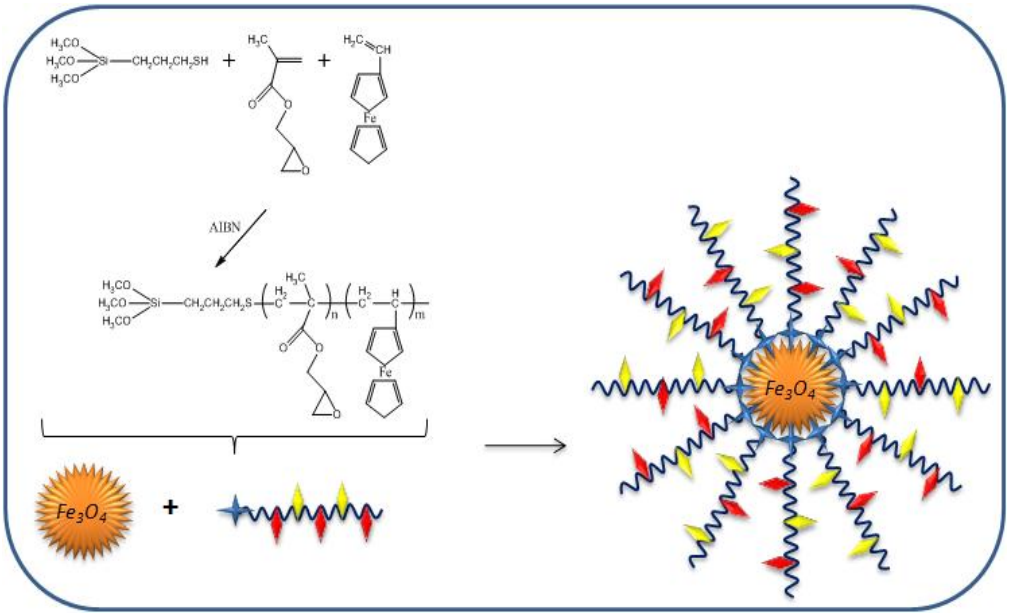


Figure 4.2. Modification Fe_3O_4 nanoparticles with SiP(GMA-co-Vfc).

4.3.5. Fabrication of enzyme electrode

The surface of the gold electrode was first polished with 0.05 μm α-Al_2O_3 powder successively, and washed ultrasonically in deionized water for 10 s. After that, the gold electrode was dipped into a 3:1 H_2SO_4–H_2O_2 mixture [70], rinsed with distilled water, and dried. The cleaned gold electrode was immediately immersed in a 0.1 M cysteamine solution for 24 h at room temperature (Figure 4.3). The resulting electrode was thoroughly washed with water to remove physically absorbed cysteamine. Cysteamine decorated Au electrode was immersed in Fe_3O_4- SiP(GMA-co-Vfc) containing phosphate buffer solution (0.1 M, pH 7.5) for 12 h, then washed with water. The enzyme, HRP, was covalently attached to the modified Au electrode surface by using the free epoxy groups on the polymer arms of Fe_3O_4. The electrode was immersed in a phosphate buffer solution (0.1 M, pH 7.5) containing HRP (10 mg/mL) enzyme for 12h. The resulting enzyme electrode was rinsed with phosphate buffer solution (pH 7.5) in order to remove the excess amount of the enzyme [71].

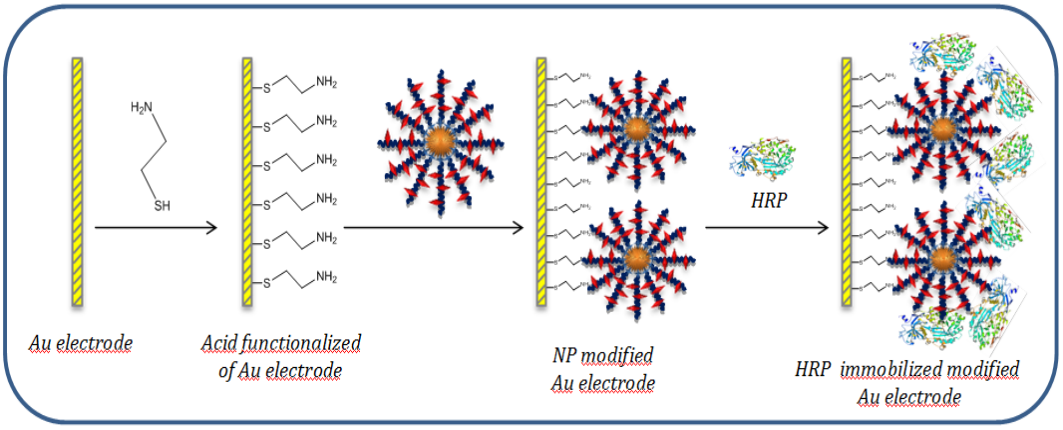


Figure 4.3. Preparation of enzyme electrodes.

4.4. OPTIMIZATION OF ENZYME ELECTRODES

4.4.1. Optimum Potential

Amperometric response of the Au/Cys/ Fe_3O_4-Si(Vfc-GMA) /HRP biosensor measured in the potential range between 0 and -80 mV to investigate the optimum potential on the different applied potential range for catechol determination.

4.4.2. Optimum pH

The pH stabilities of HRP immobilized electrode were employed by measuring the current response to catechol in the acetate and phosphate buffers (0.1M) between pH 5.0 and 9.0.

4.4.3. Optimum Temperature

The effect of temperature on the activity of the enzyme electrode was studied between 20 and 55^{o}C using catechol solution under standard assay conditions by amperometric measurements.

4.4.4. Stability

4.4.4.1. Storage and Reuse

The electrodes immobilized with HRP was stored in phosphate buffer (0.1 M, pH 7.5) at 4 and 25^{o}C for 30 days. Its activity was measured at frequent intervals. After each activity assay the samples were washed with the buffer and left stored until the next assay in buffer solution.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. CHARACTERIZATION OF SIP(GMA-CO-VFC) AND MODIFIED MAGNETIC NANOPARTICLES

5.1.1. FT-IR Analysis

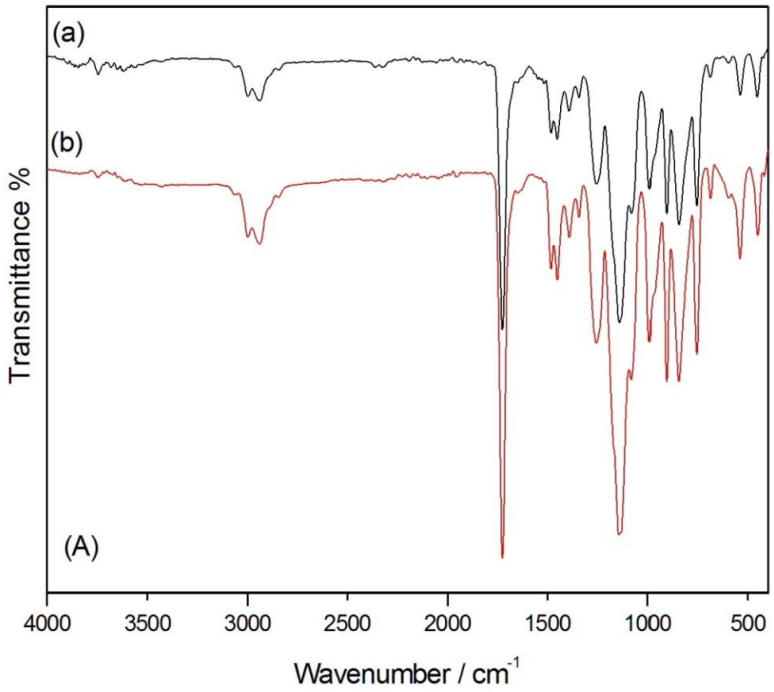


Figure 5.1. FTIR Spectra of SiP(GMA-co-Vfc) and Fe_3O_4- SiP(GMA-co-Vfc).

The FT-IR spectra of Fe_3O_4- SiP(GMA-co-Vfc) nanocomposite and SiP(GMA-co-Vfc) were presented in Figure 5.1. The methylene vibration between 2910 and 2940 cm^{-1} and the methyl vibration at 2960 cm^{-1} among the characteristic vibrations of the telomere. The vibration at 1740 cm^{-1} represents the ester configuration, whereas the epoxide group is unchanged both for Fe_3O_4-SiP(GMA-co-Vfc) and SiP(GMA-co-Vfc) nanocomposite characterized by a peak at 910 cm^{-1} [72-74]. The inorganic lattice vibration appears in the range 400–700 cm^{-1}. As prepared powder presents characteristic peaks that are exhibited by the commercial magnetite powder: metal-oxygen band, v_1, observed at 590 cm^{-1} corresponds to intrinsic stretching vibrations of the metal at tetrahedral site ($Fe_{tetra}\leftrightarrow O$), whereas metal-oxygen band observed at 445 cm^{-1}, v_2, is assigned to octahedral-metal stretching ($Fe_{octa}\leftrightarrow O$) [75-81].

5.1.2 NMR Spectra

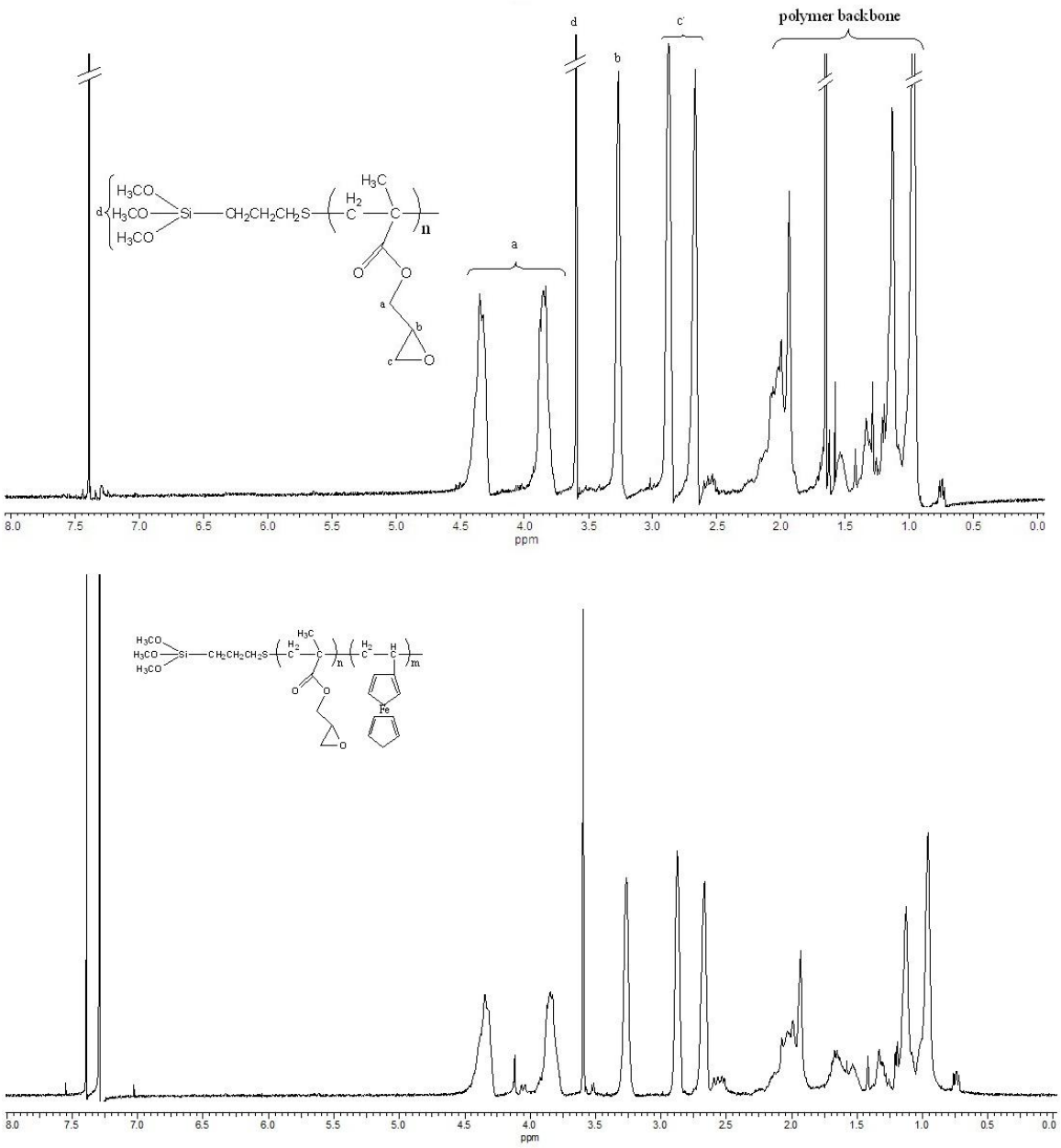


Figure 5.2. NMR spectra of polymer SiPGMA and SiP(GMA-co-Vfc).

The structures of SiP(GMA-co-Vfc) was characterized by ^{1}H NMR spectroscopy. The chemical shift assignments of the telomere were based on those obtained for poly(GMA) [85]. The ^{1}H NMR spectra of polymer and telomere (Figure 5.2), in $CDCl_3$, shows resonance signals between 0.7 and 2.4 ppm which belong to aliphatic methylene and methyl protons in the polymer backbone. In the spectrum of telomere the peak at 3.64 ppm is due to the presence of silane connected methyl groups protons. Also, the peaks at 2.62, 2.82 and 3.22 ppm are specific resonance signals of epoxy ring protons,

respectively. The 1H NMR spectrum of GMA-co-Vfc displays resonance signals between 0.7 and 2.4 ppm originating from the aliphatic methylene and methyl protons in the polymer backbone. The peaks of the epoxy protons appear between 2.5 and 3.5 ppm. The resonances between 3.8 and 4.3 ppm stem from the methylene protons adjacent to the epoxy moiety and the protons of the ferrocenyl group [82].

5.1.3. TG Analysis

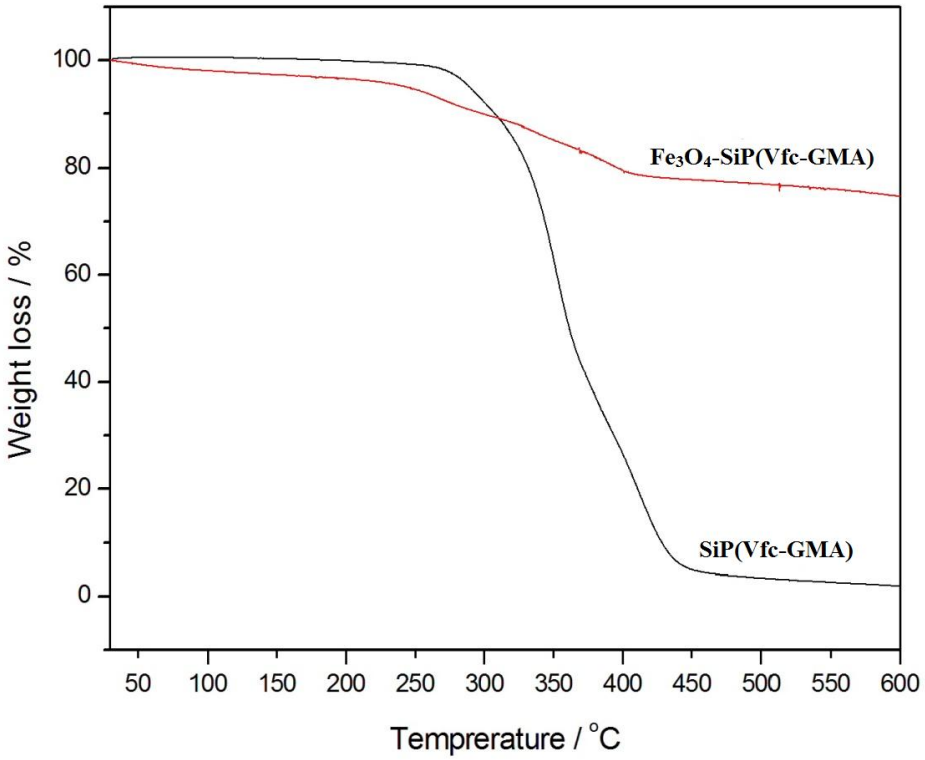


Figure 5.3. Thermal Gravimetric Analysis of Polymers.

The thermal analysis is carried out to evaluate the mechanism for the formation of nanocomposite and to observe the effect of heating on structural changes of both SiP(GMA-co-Vfc) and nanocomposite. Fe_3O_4- SiP(GMA-co-Vfc) nanocomposite shows a slight weight loss, while SiPGMA exhibits a considerable thermal stability up to 300 ^oC. SiP(Vfc-GMA) exhibits one stage decomposition (shown in Figure 5.3) of which it starts at around 300 ^oC and observed up to ~ 450 ^oC is due to the

decomposition of organic group which corresponds to ca. 95% wt loss and 5 % weight still exists due to the presence Si in the polymer. Fe_3O_4 nanocomposite (Figure 5.3) shows one-step weight loss which started at 250 ^oC and was observed upto 430 ^oC which is due to the removal of adsorbed water and breakdown of the polymer backbone in the nanocomposite. Degradation of SiP(GMA-co-Vfc) over the iron oxide begins at a much lower temperature. This behavior could be originated from the fact that iron oxide particles behave as catalysts thus reducing the degradation temperature of SiP(GMA-co-Vfc) [83,84]. Remaining 80% weight is due to the Fe_3O_4 content in the nanocomposite, which reveals the ratio between organic and inorganic phase as 20%:80% This analysis confirms the strong interaction between Fe_3O_4 nanoparticles and SiP(GMA-co-Vfc) forming a stable nanocomposite.

5.1.4. XRD Analysis

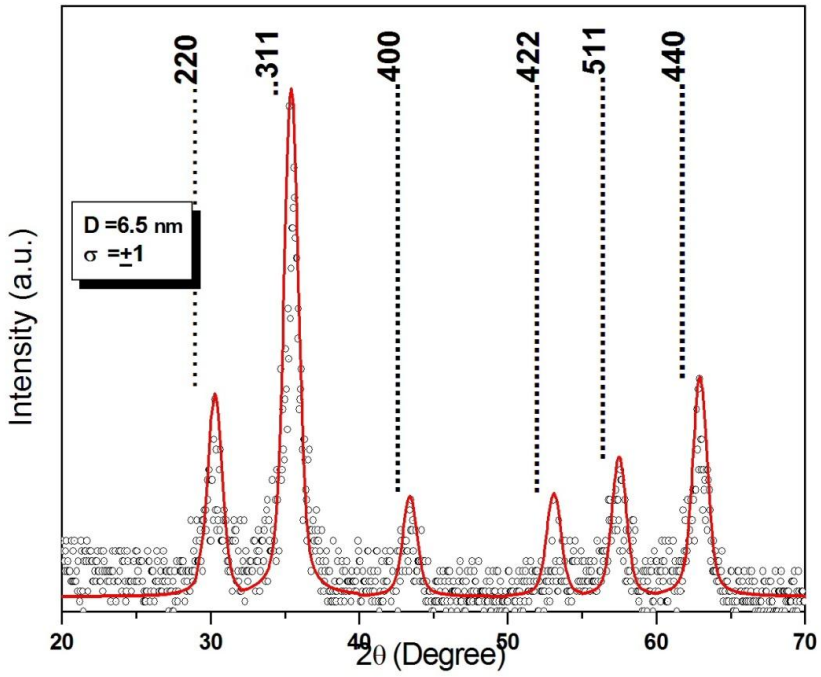
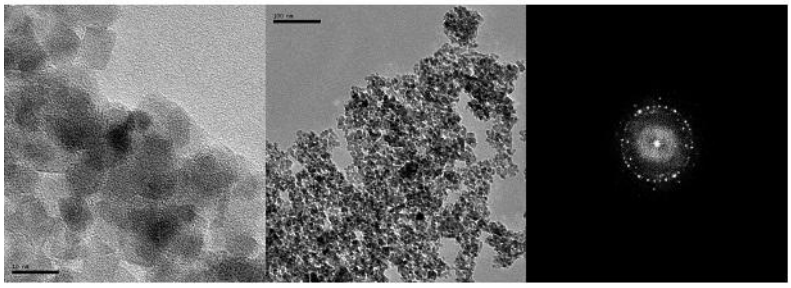


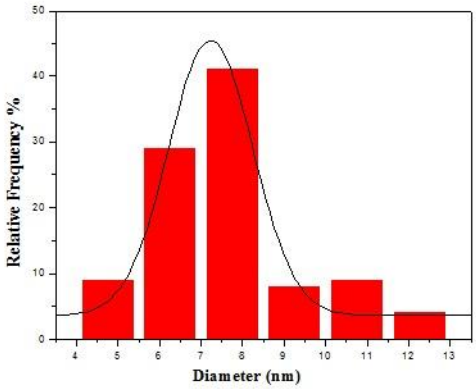
Figure 5.4. XRD Results of SiP(GMA-co-Vfc) grafted Fe_3O_4 nanoparticles.

Phase investigation of the product was performed by XRD, and XRD pattern of SiP(GMA-co-Vfc) grafted Fe_3O_4 was shown in Figure 5.4. The XRD pattern indicates that the inorganic core is iron oxide, Fe_3O_4, and the diffraction peaks are broadened owing to its small crystallite size. All the observed diffraction peaks could be indexed by the cubic structure of Fe_3O_4 (JCPDS no. 19-629) indicating a high phase purity of iron oxide. The mean size of the crystallites was estimated from the diffraction pattern by line profile fitting method using the equation (1) given in References 85 and 86. The line profile was fitted for observed 6 peaks with the following miller indices: (220), (311), (400), (422), (511), (440). The average crystallite size, D and σ, was obtained as 6.5±1.0 nm as a result of this line profile fitting.

5.1.5. TEM Analysis



a) b) c)



d)

Figure. 5.5. TEM Analysiz of SiP(GMA-co-Vfc) grafted F_3O_4 nanoparticles.

TEM analysis was performed (Figure 16) to investigate the morphology and size of the Fe_3O_4- SiP(GMA-co-Vfc) nanocomposite, and a micrograph is presented in Figure 5.5a. The particle size distribution was obtained from several micrographs, counting a minimum of 75 nanoparticles, and is presented in Figure 5.5b The average particle size, DTEM, for the samples was calculated as 7.2. ± 0.2 nm for the Fe_3O_4-SiP(GMA-co-Vfc) nanocomposite using a log-normal fitting to the histograms obtained from several TEM micrographs. Fe_3O_4 particles exhibit near spherical morphology. SAED (Selected Area Electron Diffraction) pattern of Fe_3O_4- SiP(GMA-co-Vfc) nanocomposite is given in the inset of Figure 5.5c which is typical of polycrystalline

nanoparticles. The particle size obtained from the TEM analysis for the Fe_3O_4-SiP(GMA-co-Vfc) nanocomposite is much larger than the crystallite size obtained from X-ray line profile fitting, revealing the polycrystalline nature of the synthesized nanocomposite.

5.2. ELECTROCHEMICAL AND UV/VIS SPECTRAL CHARACTERIZATION OF ENZYME ELECTRODE

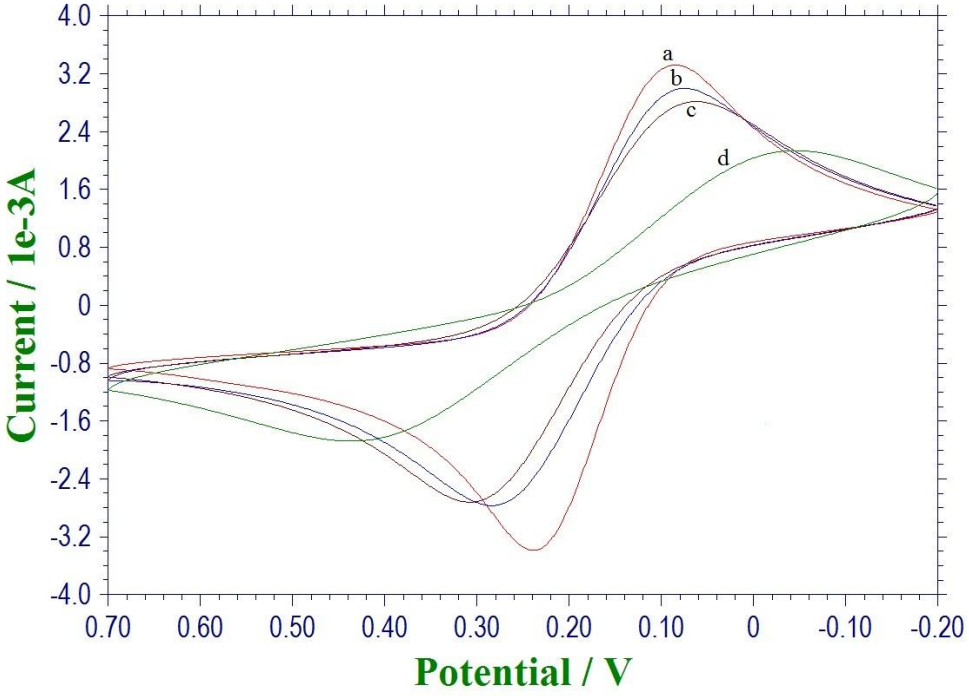


Figure 5.6. Cyclic Volammetry results of modified electrodes. ; **(a)** bare Au electrode, **(b)** Au/Cys, **(c)** Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc), and **(d)** Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc)/HRP, respectively.

Cyclic voltammetry of ferrocyanide, as redox marker, is a valuable and useful technique to follow the barrier of the modified electrode. Cyclic voltammograms of 5 mM $[Fe(CN)_6]^{3-/4-}$ between -0.2 and 0.7 V shown in Figure 5.6 were obtained at modified electrodes; (a) bare Au electrode, (b) Au/Cys, (c) Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc), and (d) Au/Cys/ Fe_3O_4-SiP(GMA-co-Vfc)/HRP, respectively. Well-defined

cyclic voltammogram, characteristic of a diffusion-controlled redox process, is observed at the bare Au electrode. After the modification of the Au electrode by dipping into Cys solution, and the covalent attachment of the Fe_3O_4- SiP(GMA-co-Vfc) dendrimers and HRP, the well-defined peaks of the bare electrode was greatly diminished, confirming that the surface of the Au electrode was successfully modified.

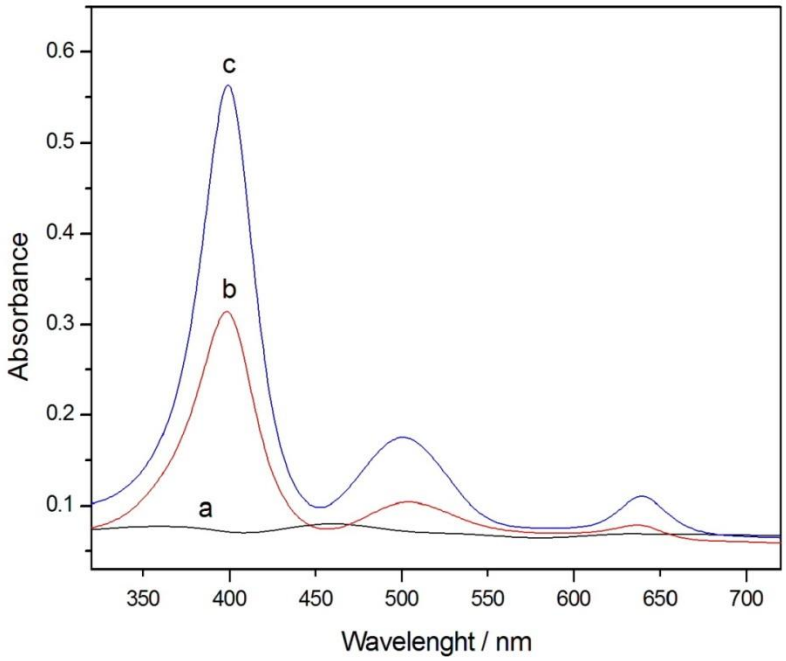


Figure 5.7. UV/vis Spectra of Fe_3O_4- SiP(GMA-co-Vfc) (a), NP- Fe3O4-SiP(GMA-co-Vfc)/HRP (b), and HRP (c).

The efficiency of the entrapment of HRP on nanoparticles modified Au electrode was verified by UV/vis spectroscopy according to litareture [87]. A shifting or disappearing of the Soret band in the UV/vis spectra of HRP give an information about the environment surrounding HRP. Figure 5.7 shows the UV/vis spectra of Fe_3O_4-SiP(GMA-co-Vfc) (a), NP- Fe_3O_4- SiP(GMA-co-Vfc)/HRP (b), and HRP (c). Fe_3O_4-SiP(GMA-co-Vfc) (curve a) had no adsorption band in a range of 320 – 720 nm. The

free HRP (curve c) showed three peaks at about 400, 500, and 640 nm [88]. The position of adsorption bands (400 nm) for Fe_3O_4- SiP(GMA-co-Vfc) /HRP (curve b) was almost the same as those for free HRP, suggesting that the HRP immobilized on Fe_3O_4- SiP(GMA-co-Vfc) indeed maintained its native structure.

5.3. AMPEROMETRIC DETECTION OF PHENOLIC COMPOUNDS

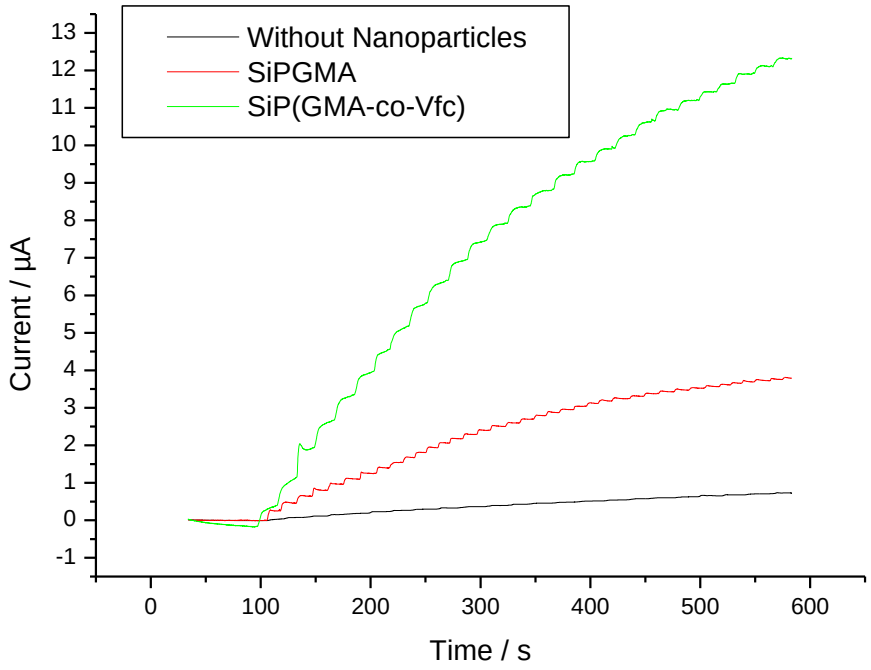


Figure 5.8. Amperometric responses of different enzyme electrodes (same amount of substrate was aded for each in PBS).

Figure 5.8 shows a typical current-time response plot of phenol biosensor after the addition of successive aliquots of 0.5 mM *p*-cresol to the PBS. The enzyme electrode exhibited a rapid response to the chance of substrate concentration and reached steady-state current with-in ~4 s. The effect of the nanoparticles on the biosensor performance for p-cresol was compared in Figure 5.8 and the Au/Cys/ Fe_3O_4-SiP(GMA-co-Vfc)/HRP electrode shows ten times higher response than the Au/Cys/

Fe_3O_4-SiPGMA/HRP and Au/Cys/ Fe_3O_4-SiPGMA/HRP electrode shows eight times greater response than Au/Cys/HRP electrode. Additionally, the effect of the nanoparticles on the biosensing of the phenol, catechol, 2-aminophenol and pyrogallol at the Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc)/HRP electrodes are seven, eight, nine and four times higher than the Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc)/HRP electrode, respectively. Both electrodes showed showed higher response in the measurements of other phenolic compounds. This was showed that the incorporation of VFc and Fe_3O_4 nanoparticles increased the efficiency of the biosensor.

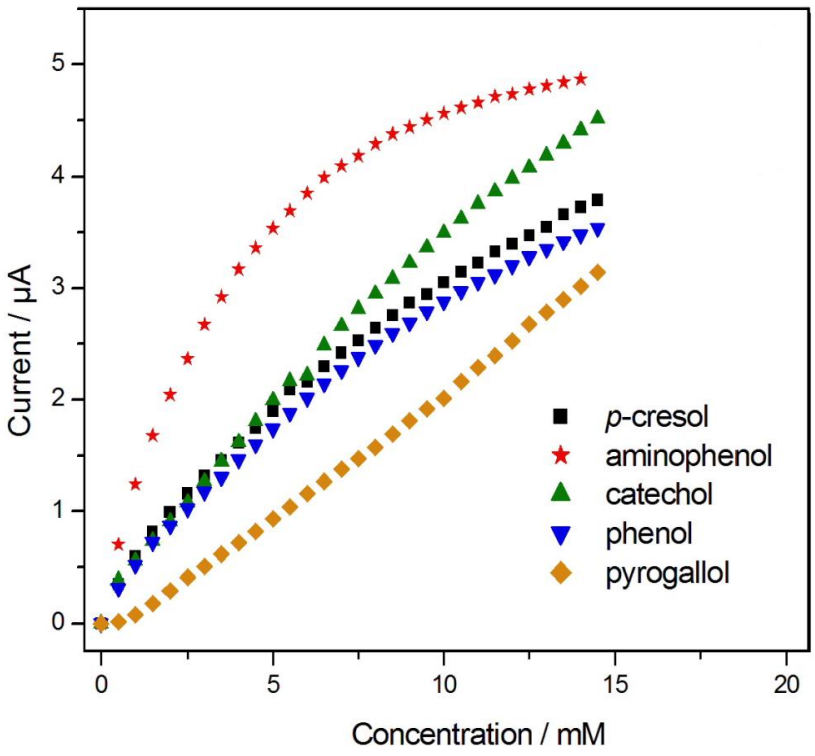


Figure 5.8.1. The calibration curve graphs of the prepared enzyme electrode with different phenolic compounds

The calibration curves of the enzyme electrode under optimized conditions were shown in Figure 5.8.1 The enzyme electrode responded rapidly to the concentration increments of the various substrates and the response deviates from a straight line and eventually reaches a plateau. The analytical performances of the different modified electrodes derived from Figure. 5.8.1 The linear range spanned the concentration of *p*-cresol from 0.5 to 7.0 mM, aminophenol from 0.5 to 3.5 mM, catechol from 0.5 to 11.0 mM, phenol from 0.5 to 8.5 mM and pyrogallol from 0.5 to 15.0 mM, respectively. The sensitivity of the biosensor to the substrates followed the order as: catechol>aminophenol>phenol> pyrogallol> *p*-cresol, which was similar to the MWCNT-PPy based biosensor [64].

As shown on the Figure 5.8.1, catechol gives the best response, so the measurements and analysis except real sample detection (it is applied to phenol) are applied to catechol. The linear range for SiP(GMA-co-Vfc) can be observed to be up to 22mM with correlation coefficient (R) of 0.99078 then a plateau is reached. SiP(GMA-co-Vfc) coated phenol biosensor has a good detection limit of 25 μM (signal-to-noise = 3), a high sensitivity of 1.75 μA/mM and a short response time (within ∼3 s).

5.4. OPTIMIZATION OF ENZYME ELECTRODES

5.4.1. Optimum Potential

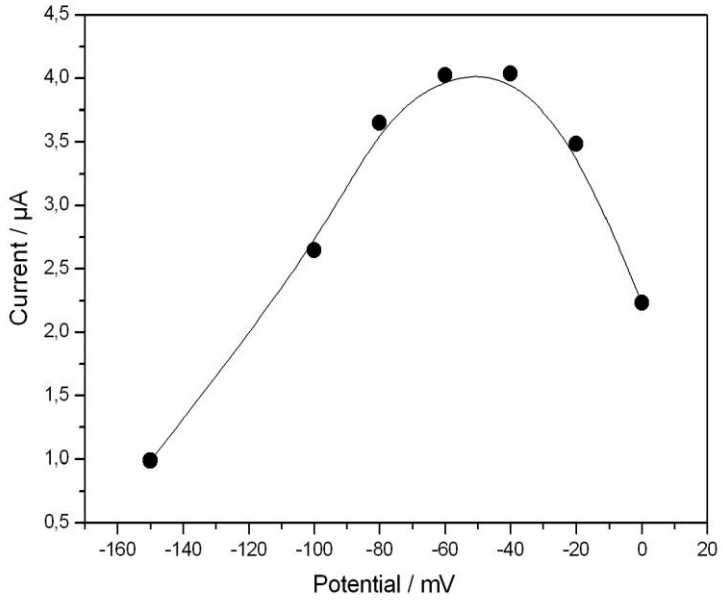


Figure 5.9. Amperometric response of the Au/Cys/ Fe_3O_4- SiP(GMA-co-Vfc) /HRP biosensor at different applied potentials. Catechol concentration 1 mmol in 10mM phosphate buffer at pH 7.5. [H_2O_2] 50 mmol/L.

In order to obtain a better response, the applied potential should be considered during biosensor applications. Due to this importance the optimum potential was investigated on the different applied potential range for catechol determination. As shown in Figure 5.9, the amperometric response increases between 0 and -40 mV and then remained practically constant until -80 mV. It should be emphasized that in the potential range between 0 and -80 mV, phenol species reduction occurs. After the -80mV if potential goes to more negative values one thing clearly seen that sensitivity starts decreasing. This sharp decreasing probably caused by irreversible HRP inactivation as describe in Csoregi et al. [89] Hence, both to obtain good sensitivity, the potential of -50 mV were set for the further experiments and to provide enzyme inactivation.

5.4.2. Determination of Optimum Peroxide Concentration.

Figure 21 represent that the peroxide concentration which used in the measurement. The peroxide is very important to get good sensitivity and avoid the formation of inactive enzyme [90].

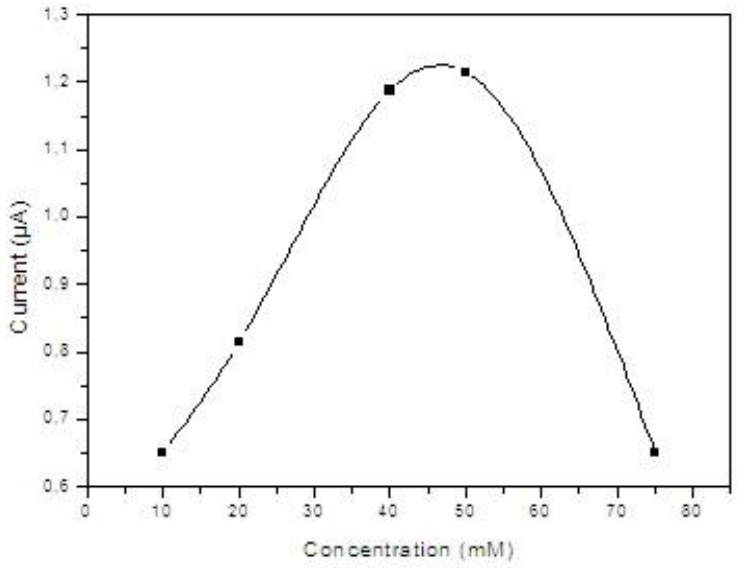


Figure 5.10. Effect of Hydrogen peroxide (H_2O_2) concentration.

Figure 5.10 is shown the dependence of hydrogen peroxide concentration of electrode responses where inthe fixed amount of catechol. The current response incrase with increasing H_2O_2 concentration when it reach 50mM. When it reaches to maximum response which is 50mM, the current starts to decrease. Due to this result the ratio was fixed as 50mM and that amount of peroxide is enough to get good results without any enzyme inactivation.

5.4.3. Optimum pH

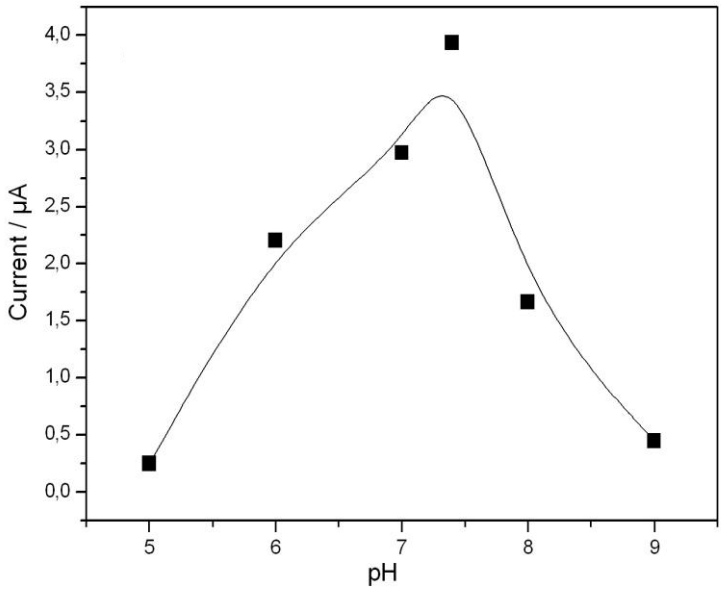


Figure 5.11. Effect of the pH on the current response of enzyme electrode to catechol solution at an applied of -0.50V vs. Ag/AgCl.

The pH effect on the amperometric response of HRP immobilized electrode was investigated by measuring the current response to catechol. The effect of the pH on the enzyme electrode was presented in Figure 5.11. The current increases repectively from pH 5.0 to 9.0 and after achieving the maximum current at pH 7.5, the current decreases, indicating that the optimum pH 7.5 can be used for the detection of catechol.

5.4.4. Optimum Temperature

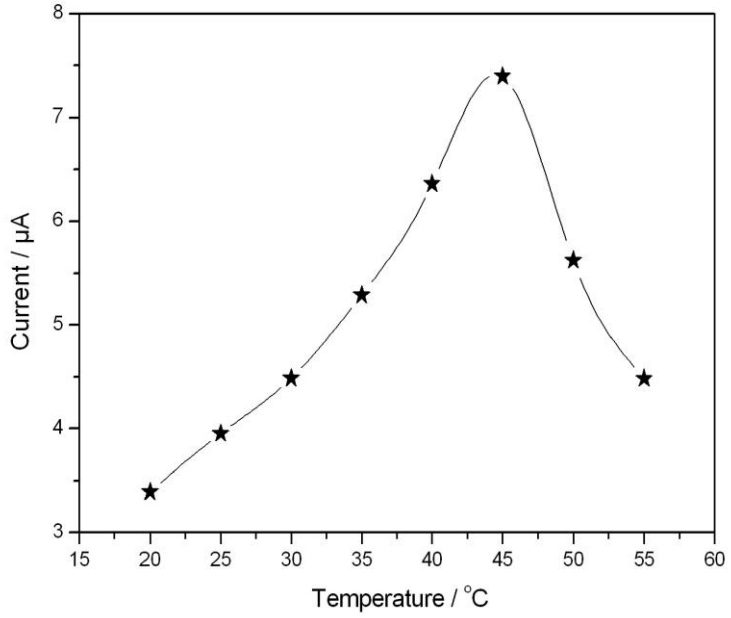


Figure 5.12. Effect of temperature on the amperometric response of enzyme electrode to catechol solution in 10mM PBS solution, pH 7.5 at an applied potential of -0.50V vs. Ag/AgCl.

Other important effect on the activity of the enzymatic reactions is temperature. The activity of the enzyme electrode has been investigated using catechol solution in 10mMPBS, pH 7.5 solution by amperometric measurements at temperature varying from 20 to 55 ◦C, shown in Figure 5.12. It was observed that the response increased with temperature increase, reaching a maximum at 45 ◦C, and then decreased. This could have been caused by denaturation of HRP at the higher temperatures.

5.4.5. Stability and Operational stability

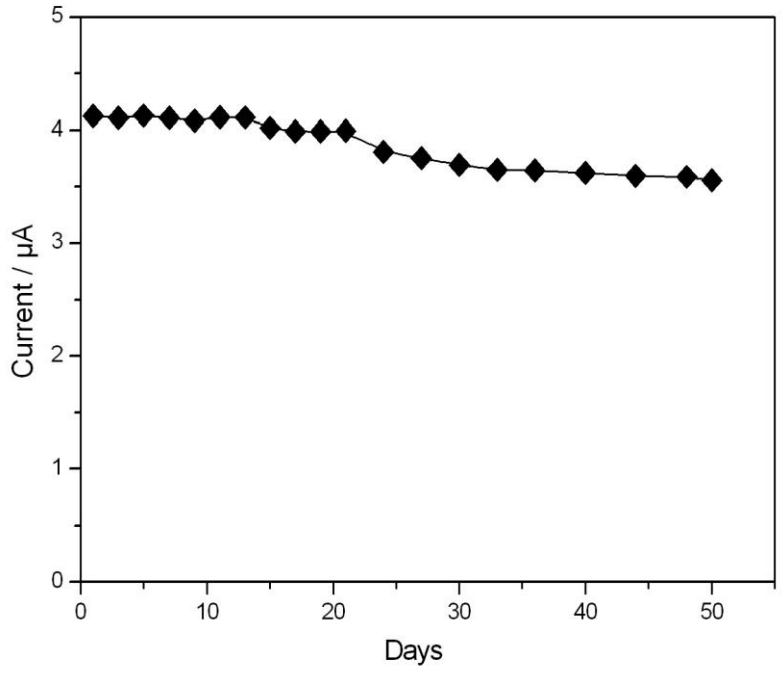


Figure 5.13. Storage stability of phenol biosensor. The measurements were done regularly during 50 days (pH 7,5; ~ 25^{o}C).

Both storage stability and operational stability are important from the practical application point of view, so these two parameters were examined. The storage stability of the biosensor was examined over a 50-day period. When the biosensor was stored in a refrigerator at 4^{o}C and measured intermittently (every 2 days), after 50 days of storage, the biosensor retained about 87% of its original response.(Figure 5.13.)

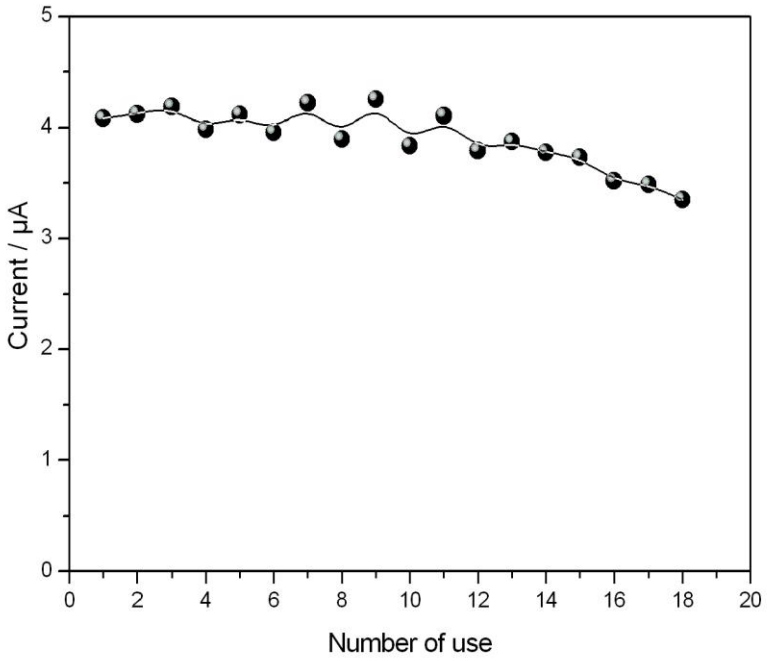


Figure 5.14. Operational stability of phenol biosensor. Each point indicates the average of data collected by three different electrodes (pH 7,5; ~ 25^{o}C).

The reuseability features of phenol biosensor were investigated by performing measurements in a definite time scale. Electrodes were stored at the 4^{o}C (0,1M; pH 7.5 PBS) for 10 min. between each measurement. The response of phenol biosensor gave relatively closed results for first twelve measurements, and activity loss 15% was observed with further application.(in Figure 5.14.)

5.4.6. Real Sample Detection

In order to optimize its applicability, the electrode was used for the detection of the concentration of phenol in the known concentration samples. The electrode responses for the measurement of 3.0–7.0mM phenol were obtained with very high sensitivity between three detections (Table 5.1).

Table 5.1. Determination of phenol concentrations in the stock sample.

Phenol Concentrations	Measured Si(VfcGMA)	Sensitivity (%)
3mM	**2,96**	**98,6**
5mM	**4,94**	**98,8**
7mM	**6,97**	**99,5**

Results indicating that the developed electrodes had high accuracy in measuring the phenol and its derivatives. Three samples were analyzed using three independently prepared electrodes. It was shown that the values measured by the developed biosensor were in good agreement with the stock solutions which they had already prepared before measurement with known concentration. This results showed that the phenol biosensor have great potential for practical application for the analysis in real samples.

CHAPTER 6

CONCLUSION

We have constructed a electrochemical biosensor by immobilization Fe_3O_4 nanoparticles on the surface of Au electrode. The Superparamagnetic Iron Oxide Nanoparticles (Fe_3O_4) could enhance the HRP immobilization. Fe_3O_4 nanoparticles increased the surface area of electrodes and this helped to bind more emzyme onto electrode by covalent binding method. Telomer-modified magnetite nanoparticles with mainly less than 10 nm were prepared and well characterized by different instruments FTIR, TEM, and TGA. In addition to this characterizations electrochemical and UV/vis spectrometer were also used. Cyclic voltammetry graph in figure 5.7 clearly shows the redox peaks decreasing with each electrode modification and same results were proved with UV/vis spectrophotometer. The effect of nanoparticles on the current response well defined and also easily seen Vfc effect of phenol biosensor. Nanoparticles and Vfc both contain iron (Fe) groups, this could enhanced electron transfer rate from reaction medium to the electrode surface. The Vfc ratios were determined according to analytical perfoemance of different enzyme electrodes. During experinets some of different phenolic compounds were used (phenol, catechol, p-cresol, 2,2-aminophenol and pyrogallol) but atechol have the best and high response from 0.5 to 11.0 mM. The sensitivity of the biosensor to the substrates followed the order as: catechol>aminophenol>phenol> pyrogallol> *p*-cresol.

Applied potential was optimized before the batch experiments and showed in figure 5.9. Peroxide concentration is very important for the sensitivity and enzymatic reaction [90]. Optimum peroxide concentration determined as 50mM and this is same as in the literatute (Figure 5.10). In order to optimize enzyme electrodes the optimum working conditions such as; pH, Temperature, Reuseability, Storage and Real Sample were evaluated. Immobilized HRP on the SiP(Vfc-GMA) telomer maintains its activity.

The electrode responses for the measurement of 3.0–7.0mM phenol were gained with very high sensitivity more than 97% for the phenol compound. A reliable, low-cost and sensitive biosensor for phenolic compunds detection is thus developed, possessing a variety of excellent characteristics, including high sensitivity, good repeatability and reproducibility, rapid response and long-term stability.

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