PREPARATION AND CHARACTERIZATION OF TITANIA-SILICA-GOLD THIN FILMS OVER ITO SUBSTRATES FOR LACCASE IMMOBILIZATION

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF TITANIA-SILICA-GOLD THIN FILMS OVER ITO SUBSTRATES FOR LACCASE IMMOBILIZATION

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The aim of this study was to immobilize the redox enzyme laccase over TiO_2-SiO_2-Au thin film coated ITO glass substrates in order to prepare electrochemically active surfaces for biosensor applications. Colloidal TiO_2-SiO_2-Au solution was synthesized by sol-gel route and thin film was deposited onto the substrates by dipcoating method. The cysteamine was utilized as a linker for immobilization of enzyme covalently through gold active sites. Preliminary studies were conducted by using invertase as model enzyme and Pyrex glasses as substrates.

The effect of immobilization parameters such as immobilization temperature, concentration of enzyme deposition solution, immobilization time for laccase were examined. Leakage studies were conducted and storage stability of immobilized laccase was determined. Highest laccase activity was achieved when immobilization was performed with 50 µg/ml solution at 4°C for 2 hours. Laccase activity decreased after 4 hours of impregnation in enzyme solution. Laccase leakage was observed in the first usage of substrates and 55% activity decrease was determined in the subsequent use which might be attributed to the presence of uncovalently adsorbed enzyme on the fresh samples. In air and in buffer storage stabilities were also tested. It was found that the activity of samples almost

vanished after 6 days regardless of storage conditions. Both enzymes had more activity on ITO substrate.

Keywords: Biosensor, immobilization, thin films, laccase, ITO, TiO_2, SiO_2, Au, sol-gel

ÖZ

LAKKAZ TUTUKLANMASI İÇİN ITO ÜZERİNDE TİTANYUM DİOKSİT-SİLİSYUM DİOKSİT-ALTIN İNCE FİLMLER ELDE EDİLMESİ VE KARAKTERİZASYONU

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Bu çalışmanın amacı, biyosensor uygulamaları için elektrokimyasal aktif yüzey hazırlamak için TiO_2-SiO_2-Au ince filmi ile kaplanmış ITO üzerine redox enzim lakkaz tutuklanmasıdır. TiO_2-SiO_2-Au kolloid solüsyonu sol-jel yoluyla sentezlendi ve ince film substrat üzerine daldırmalı çıkarmalı kaplama yöntemiyle depolandı.Enzimi altın aktif bölgelerine kovalent olarak tutuklamak için sistamin bağlayıcı olarak kullanıldı. Ön çalışmalar invertazın model enzim ve payreks camların substrat olarak kullanılmasıyla yürütüldü.

Tutuklama sıcaklığı, enzim depolama solüsyon konsantrasyonu, tutuklama zamanı gibi immobilizasyon parametrelerin etkisi lakkaz için incelendi. Kayıp çalışmaları yürütüldü ve tutuklanmış lakkazın raf ömrü kararlığı belirlendi. En yüksek lakkaz aktivitesine, tutuklanma 50 µg/ml solüsyonla 4°C'de 2 saat gerçekleştirildiğinde ulaşıldı. Enzim solusyonu içinde 4 saatten fazla tutulduğunda lakkaz aktivitesi azaldı. Substratın ilk kullanımında lakkaz kaybı gözlemlendi ve bir sonraki kullanımda yeni örneklerde nonkovalent adsorplanan enzimin olmasıyla ilişkilendirilebilir %55'lik bir aktivite azalması belirlendi. Havadaki ve tampon

çözeltideki raf ömrü kararlılığı da test edildi. Örneklerin aktivitelerin 6 gün sonra raf ömrü koşullarına bağlı olmaksızın hemen hemen tamamen yok olduğu bulundu. Her iki enzim de ITO substrat üzerinde daha yüksek aktiviteye sahiptiler.

Anahtar Kelimeler: Biyosensör, tutuklanma, ince filmler, lakkaz, ITO,TiO_2, SiO_2, Au, sol-jel

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TABLE OF CONTENTS

ABSTRACT iv

ÖZ……. vi

ACKNOWLEDGEMENTS viii

TABLE OF CONTENTS ix

LIST OF TABLES xii

LIST OF FIGURES xiii

1. INTRODUCTION 1

2. LITERATURE SURVEY 3

2.1. Biosensors 3

2.1.1. Amperometric Biosensors 6

2.2. Immobilization of Biomolecules on the Surface 8

2.2.1. Physical Adsorption 8

2.2.2. Microencapsulation 9

2.2.3. Entrapment 10

2.2.4. Crosslinking 10

2.2.5. Covalent Bonding 11

2.3. Synthesis methods of thin films 13

2.3.1. Sol-Gel Method 14

2.3.2. Coating Techniques 18

2.4. Support Materials 20

2.4.1. Titania Silicate Mixtures 20

2.4.2. Indium-tin oxide (ITO) 21

2.5. Gold Nanoparticles 22

2.6. Redox Enzymes 25

2.6.1. Polyphenol oxidases 28
2.6.1.1. Laccases 28
3. MATERIALS AND METHODS 32
3.1. Materials 32
3.2. Sol-Gel Synthesis 32
3.3. Pretreatment of Pyrex and ITO Glasses 34
3.4. Thin Film Coating 34
3.4.1. Dip-Coating and Thermal Treatment 34
3.5. Enzyme Immobilization 36
3.5.1. Cysteamine Linking on the Surface 36
3.5.2. Immobilization of Laccase 36
3.5.3. Immobilization of Invertase 37
3.6. Analytical Methods 37
3.6.1. Free Laccase Activity 37
3.6.2. Immobilized Laccase Activity 38
3.6.3. Free Invertase Activity 39
3.6.4. Immobilized Invertase Activity 39
3.6.5. Protein Concentration 40
3.7. Contact angle measurement 40
3.8. AFM Measurements 41
3.9. SEM Measurements 41
4. RESULTS AND DISCUSSION 42
4.1. Introduction 42
4.2. Characterization of Thin Films 43
4.2.1. AFM Images of Thin Films 43
4.2.2. SEM Micrographs of Thin Films 44
4.3. Enzyme immobilization on Pyrex and ITO glasses 47
4.4. Optimization of Immobilization Conditions 57

4.4.1. Immobilization Temperature..... 57
4.4.2. The Effect of Enzyme Solution Concentration in Immobilization..... 59
4.4.3. Immobilization Time..... 61
4.5. Storage Stability.....62
5. CONCLUSIONS 64
REFERENCES 66
APPENDICES.....78
A. SPECIFICATIONS OF LUDOX SM-30.....78
B. PREPARATION OF DNSA REAGENT 79
C. BRADFORD METHOD 80
D. BSA STANDARD CURVE.....81
E. EDX SPECTRUM OF TiO_2-SiO_2-Au THIN FILM 82

LIST OF TABLES

Table 2.1. Typical covalent couplings for immobilization 12

Table 2.2.Commonly Available Functional Groups in Proteins and Functionalities of the Required Surfaces 13

Table 2.3. Redox enzyme biosensors modified by gold nanoparticles..... 24

Table 3.1. Laccase immobilization solutions 37

Table 4.1. Contact angle measurement of not cleaned and cleaned Pyrex and ITO glasses 47

Table 4.2. Invertase and laccase activities immobilized on Pyrex and ITO glasses 54

Table 4.3. Immobilized Laccase Activities in leakage experiment 57

LIST OF FIGURES

Figure 2.1. A schematic of an amperometric sensor 6

Figure 2.2. Scheme of direct and indirect electron transduction. a) Direct transduction: the electrons only generate a measurable current if the reaction takes place close to the surface. b) Indirect transduction: mediator shuttles the electrons between the reaction site and the surface.....7

Figure 2.3. Immobilization of biomolecules by adsorption 8

Figure 2.4. Immobilization of biomolecules by encapsulation. 9

Figure 2.5. Immobilization of biomolecules by entrapment 10

Figure 2.6. Schematic representation of crosslinked biomolecules 11

Figure 2.7. Schematic Diagram of solid particle distribution for (a) peptized colloid, (b) aggregated particles and (c) gel 14

Figure 2.8. Routes for sol-gel processed materials 15

Figure 2.9. Schematic of Spin Coating 19

Figure 2.10 .Schematic of Dip-coating 20

Figure 2.11. Energy diagram of In_2O_3 and ITO 22

Figure 2.12. Main electron transfer reaction systems 26

Figure 2.13. Use of redox proteins (a) Sensing the binding of a ligand based on changes of the redox properties, (b) Optical device sensing changes in optical properties of the redox centre upon ligand binding, (c) Detection of enzymatic activity through measurement of catalytic current, (d) protein with multi-redox centres, immobilized on an electrode surface 27

Figure 2.14. Active side structure of laccase from *Trametes versicolor* made of four copper atoms 29

Figure 2.15. Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence (a) or in the presence (b) and of chemical mediators(c) 30

Figure 2.16. Formation of the cation radical and the dication by removal of one and two electrons from ABTS 31

Figure 3.1. Schematic Representation of Sol-gel Preparation 33

Figure 3.2. Dip-coating of substrates with sol-gel 35

Figure 3.3. Structure of cysteamine 36

Figure 4.1. Functionalization and immobilization of enzyme immobilization 43

Figure 4.2 (a) AFM image of etched cleaned glasses, and (b) 3D image 44

Figure 4.3. (a) AFM image of TiO_2-SiO_2-Au thin film, and (b) 3D image 44

Figure 4.4. SEM micrograph of TiO_2-SiO_2-Au thin film 45

Figure 4.5. SEM micrograph of enzyme immobilized TiO_2-SiO_2-Au-Cys thin film surface 46

Figure 4.6. SEM micrograph of TiO_2-SiO_2-Au thin film at 250000 magnification 46

Figure 4.7. SEM image of Pyrex glass coated with TiO_2-SiO_2-Au sol-gel 48

Figure 4.8. SEM image of ITO glass coated with TiO_2-SiO_2-Au sol-gel 49

Figure 4.9. Reaction progress curve of free invertase 50

Figure 4.10. Reaction progress curve of free laccase 51

Figure 4.11. Reaction curve catalyzed by invertase immobilized on conducting side of ITO glass in pairs..... 51

Figure 4.12. Reaction curve catalyzed by invertase immobilized on two sides of Pyrex glass in pairs..... 52

Figure 4.13. Reaction curve catalyzed by laccase immobilized on conducting side of ITO glass in pairs..... 53

Figure 4.14. Reaction curve catalyzed by laccase immobilized on two sides of Pyrex glass 53

Figure 4.15. Two consecutive reactions of laccase immobilized on Pyrex glass at room temperature for 2 h in 0.36 mg/ml enzyme solution 55

Figure 4.16.Two consecutive reactions of laccase immobilized on Pyrex glass at 4°C for 2 h in 0.36 mg/ml enzyme solution 56

Figure 4.17. Two consecutive reactions of laccase immobilized on ITO glass at 4°C for 2 h in 0.36 mg/ml enzyme solution 56

Figure 4.18. Thermal stability of free laccase at different temperatures..... 58

Figure 4.19. Reaction curve catalyzed by laccase immobilized on Pyrex glasses at room temperature and 4°C 59

Figure 4.20. Effect of immobilization enzyme solution concentration on laccase activity..... 61

Figure 4.21. Effect of immobilization incubation period on laccase immobilization on TiO_2-SiO_2-Au-Cys thin film 62

CHAPTER 1

INTRODUCTION

Immobilization is a widely used technique in order to fix biomolecules such as DNA, antibodies and enzymes onto a solid support or into a solid matrix. Enzymes are protein based molecules catalyzing chemical reactions in living organisms and they have unique properties in terms of activity, selectivity and specificity. However, enzymes are very sensitive to environmental parameters and thermally and chemically unstable. They are highly soluble in water and potential to substrate/product inhibition. These facts limit their use in large scale implementation. Immobilization provides reusability by easy separation, better stability, and widens the applications. Solid-phase immobilized enzyme reactors, immobilized enzyme membranes incorporated into sensors such as potentiometric enzyme electrodes and optical sensors and solid-phase immobilized enzyme films are examples of immobilized enzyme forms used in applications (Worsfold, 1995).

Nanostructures such as nanoparticles, nanofibers, mesoporous thin or thick films and sol-gel route encapsulation having special functional properties contribute to the enhancement of immobilization and stabilization. Larger surface area, tunable pore size for biomolecule selectivity (shape and size), surface functionalization, and lowered mass-transfer limitations are some examples of the use of nanostructures in enzyme immobilization (Kim *et al.,* 2006). Sol-gel method is a practical technique for the construction of nanotructured thin films or powders from colloidal solutions and it is widely used.

Titania and silica are the materials that can be sythesized by sol-gel route and they are biocompatible materials that can be utilized as host matrix in variety of immobilization applications of biomolecules. Sol-gel derived silica has high thermal stability and porosity. However the silica structure is fragile and thermal shrinkage is the major drawback for film formation. Titania is a suitable material for enzyme

electrodes and easily produced by sol-gel route. It is a very attractive semiconductor material allowing electron reduction and direct electron transfer of enzymes during the biological processes, reactions and transformations etc. But, it suffers from low porosity and aggregation. Titania-silica provides homogeneous dispersion of titania and better mechanical strength, thermal stability and high surface area than pure titania and it becomes a good candidate for enzyme immobilization with good conductive and large surface area for use in a variety of acid-base catalysis, redox catalysis and photocatalytic processes. Indium-tin oxide (ITO) is a widely used material in enzyme electrode construction. It is a wide band gap semiconductor and has metal like electrical properties. High transparency in visible range and near-IR region, high electrical conductivity, wide electrochemical working window are its superior properties.

In this study, the redox enzyme laccase was immobilized over TiO_2-SiO_2-Au coated ITO glasses for the preparation of electrochemically active thin film surfaces. Immobilization of enzyme was performed by covalent binding method using the thiolamine linker cysteamine. Previous studies were conducted by using model enzyme invertase instead of laccase as it is well-known and abundantly available. Factors, which affect laccase immobilization, such as immobilization temperature, concentration of immobilization enzyme solution and immobilization time were examined. Also, stability studies for immobilized laccase were done. Such an enzyme mediated electrochemically active, conductive and nanosized thin film surface can find a wide range of application like power supplying, environmental remediation, biomimetic systems and biosensors.

CHAPTER 2

LITERATURE SURVEY

2.1. Biosensors

Biosensors are analytical devices to obtain a biological response via biological recognition entity such as enzyme, antibody, bacteria, tissue etc. There are three main components in a sensor. The first one is the responding element which detects the analyte species and produces a signal. It should provide qualitative or quantitative or both qualitative and quantitative analysis of the analyte. The biosensor must fulfill the sensitivity, reproducibility, and selectivity requirements. The second component of a biosensor is an amplifier which increases the signal of the responding element and the third component is the detector that takes the amplified signal and turns it into a value that resembles the analyte species and/or its concentration (Pradeep, 2008).

Linearity, sensitivity, selectivity and response time are the most important parameters that determine a biosensor's performance. The more linearity of the sensor is the better detection of high substrate concentration. Sensitivity is the measure of electrode response per substrate concentration. High selectivity means the minimum contribution of chemicals other than current analyte. Finally, response time corresponds to obtain the time required to 95% of the response (Li, 2007). In the scope of these properties, nanotechnology contributes to high throughput ratio of the biosensors' performance and recently paramount importance is given to nanoparticles in the development of biosensors (Jianrong *et al.*, 2004; Xu *et al*., 2003; Guo *et al.,* 2007). The advantageous of using inorganic nanoparticles can be listed as;

1. Having high surface to volume ratio, they provide extremely high electrochemically-active sites, i.e. the interacting area with the analyte, and resulting in an enhanced sensitivity (Guo *et al*., 2009; Huang *et al.,* 2009, Jianrong *et al.,* 2004).

2. Especially metal nanoparticles can act as promoters in electron transfer between electrode and target molecules and so lead to rapid response time for analyte molecules (Guo *et al*., 2009).
3. It is possible to form desired constructions on the surfaces with the help of superior functional properties of inorganic nanoparticles and modify an electrochemical-sensing interface (Guo *et al.*, 2009).
4. Nanoparticles can be used as support for biomolecules and redox enzymes but also can behave as nano-activators for them and can be electrical labels for biorecognition events (Guo *et al.*, 2009).
5. Nanoparticle assemblies offer lower limits of detection (LOD) compared to conventional macrosized counterparts due to higher ratio between Faradaic and capacitive currents (Guo *et al.*, 2009).

Numerous applications of biosensors are possible with nanoparticles that bind to biological molecules. Acoustic wave, optical, magnetic and electrochemical biosensors are the main types of nanoparticle based biosensors (Jianrong *et al*., 2004).

Acoustic wave biosensors incorporated to a biological component works with the principle of measurement of mechanical acoustic waves. Mass-amplified crystal detectors are sol particles coated with biological reagent (i.e antibody) and when exposed to antigen they resonate at a fundamental frequency. The vibrational frequency of the crystal affected due to the mass change and this is measured. The size of the sol particles influence the detection limit and the preferred size range is reported as 5-100 nm (Jianrong *et al.,* 2004). Au, Pt, CdS, TiO_2 and polymers are also used as support particles. Piezoelectric materials are preferably utilized in most of the acoustic wave biosensors as crystals because they can propagate and convey acoustic waves in a frequency-dependent way. Quartz (SiO_2), lithium niobate ($LiNbO_3$) and lithium tantalate ($LiTaO_3$) are widely used piezoelectric materials. High sensitivity is an important factor and surface of the acustic wave electrode should be chemically stable containing immobilized biological reagents abundantly in a thin coating (Jianrong *et al.,* 2004; Leonard *et al*., 2003).

Optical biosensors are based on the detection of absorbance or fluorescence changes of an indicator or detection of refractive index changes. During the biorecognitive interactions, metal nanoparticles over the surface of the detector reflects enhanced resonance signals. Nanoclusters dispersed over the reflecting conductor surface transducer an optical signal as a result of resonant enhancement. Gold nanoparticles are recently used as novel fluorescence quenchers (Jianrong *et al*., 2004; Ravindra *et al.,* 2007).

Magnetic biosensors are freshly used devices that take the advantage of magnetic properties of nanoparticles. Magnetic nanoparticles provide strong and resourceful analysis in biology and medicine. Single domain or superparamagnetic types that are bound to biorecognitive molecules can be used for the separation and enrichment of the substance to be detected. Magnetic cell separation, magnetic immunoassays are the techniques that uses the magnetic field gradients (Jianrong *et al.,* 2004).

Electrochemical biosensors are based on the reactions that produce ions. An enzymatic reaction in the presence of analyte of interest takes place over the surface of the working electrode and the ions produced cause a potential that can be measured with respect to reference electrode and converted to a signal. There are two types of electrochemical biosensors; amperometric and potentiometric (Ravindra *et al.,* 2007). Recent developments in electrochemical biosensors mostly include metallic nanoparticles. High surface area of them provide high amount of biomolecules immobilized over the electrode that contributed to the sensitivity and lower detection limit. Both enzymatic and non-enzymatic biosensors can be produced with functionalization of electrode surface with metal nanoparticles, which responds to the redox reactions. In enzymatic sensors, enzyme modified metal nanoparticles are sensing parts and nanoparticles acts as mediators. In non-enzymatic sensor, metal nanoparticles directly act as sensing elements (Santos *et al.,* 2002). Transition metals have unique catalytic features for many organic reactions. Metal nanoparticles can also act as labeling agents. Many biological molecules can be labeled by using metal nanoparticles without altering their biological activities that leads to construction of affinity assays (Jianrong *et al.,* 2004).

Enzymes are superior candidates for the use in biosensors. They have outstanding operational properties such as activity, selectivity and specificity resulting in a capability of catalyzing very complex chemical processes under mild conditions. They can also provide a wide range of measurable quantities such as protons, ions, electrons and mass. In addition, they have an amplification effect as they are used more than once during a catalytic reaction (Turner *et al.,* 2002).

2.1.1. Amperometric Biosensors

In amperometric biosensors, detection is based on the measurement of the current response of a redox reaction catalyzed by an enzyme or during a bioaffinity reaction on the electrode surface (Jianrong *et al.,* 2004). Enzyme electrodes are small transducers that are composed of immobilized enzymes that perform electrochemical reactions on the surface. In general, enzymatic detection is carried out by following the rate of formation of a product or disappearance of a substrate. Amperometric detection is possible if the reagent or product is electro active. This class of biosensors mostly depends on the electron donor or acceptor oxidoreductase enzymes (Sarmaa *et al.,* 2009). The working principle of amperometric sensors is illustrated in Figure 2.1.

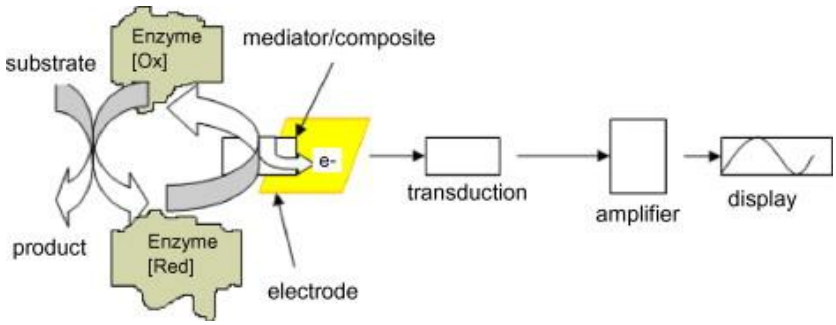


Figure 2.1. A schematic of an amperometric sensor (Sarmaa *et al.,* 2009)

The performance of the biosensor is affected by the structure of the electrode surface including the material choice. Conductivity and hardness of the electrode are the primary considerations and solid supports such as gold, carbon, platinum and their derivatives are generally preferred. Immobilized enzymes over the surface are redox enzymes and alcohol dehydrogenase (Zhao *et al*., 2009), aldehyde dehydrogenase, glucose oxidase (GOx) (Ren *et al.,* 2009), glutaminase, horse radish peroxidase (HRP) (Mateo *al.,* 2008), catalase (Itoh *et al*., 2009), xanthine oxidase (Shan*et al.,* 2009), choline oxidase (Bai *et al.,* 2008), urease (Chen*et al.,* 2008), billirubin oxidase, and lactate oxidase (Huang *et al*., 2008) are just brief examples.

Direct electron transfer between the electrode and the enzyme by metal nanoparticles is another method based on the principle of direct electron transfer between redox proteins and electrode without need for any mediator (Guo *et al.,* 2007; Xu *et al*., 2003). The schematic representation of direct and indirect electron transfer is given in Figure 2.2. Nanoparticle modified electrode surfaces ensure such a native environment and create conducting tunnels to facilitate direct electron transfer by decreasing the insulating effect of protein shell.

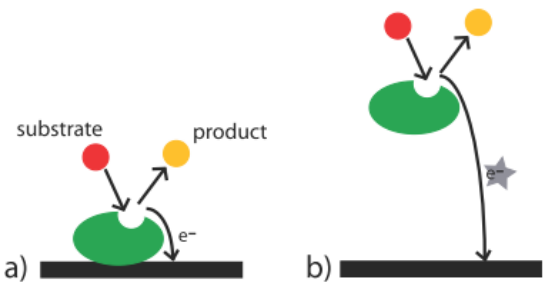


Figure 2.2. Scheme of direct and indirect electron transduction. a) Direct transduction: the electrons only generate a measurable current if the reaction takes place close to the surface. b) Indirect transduction: mediator shuttles the electrons between the reaction site and the surface. (Grieshaber *et al*., 2008)

2.2. Immobilization of Biomolecules on the Surface

Immobilization is the one crucial step to produce biosensor electrodes (Böyükbayram *et al*., 2006; Kim et al., 2006; Gürsel *et al.,* 2003). In general, the enzyme immobilization is carried out by adsorption, microencapsulation, entrapment, crosslinking and covalent bonding techniques.

2.2.1. Physical Adsorption

In physical adsorption (physisorption), proteins are adsorbed to the surface by weak forces such as electrostatic forces, Van der Waals bonds or hydrogen bonds. Physisorption may alter the structure of enzyme and significant conformational changes may take place by adsorption (Drevon *et al.,* 2002; Ahuja *et al*., 2007; Eggins, 2002). The hydroplicity of the support material also affects the thermostability of the enzyme. Enzyme kinetics is not affected much during the immobilization. Adsorption of enzymes and the stability of ad-enzyme species are highly vulnerable to changes in pH, temperature, ionic strength. Another disadvantage is the leakage or leaching of enzyme from the support surface which results in reduction of lifetime and sensitivity of the electrode. There are studies on immobilization of biomolecules via adsorption onto mesoporous silica examining the effects of pore size, surface charge, ionic strength to the rate of adsorption, enzymatic activity (Lee*et al*., 2009; Diaz *et al.,* 1996; Deere *et al*., 2002). A schematic representation of adsorption on the surface can be seen in Figure 2.3.

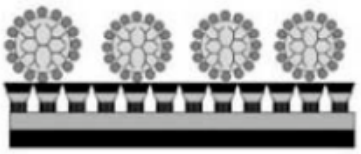


Figure 2.3. Immobilization of biomolecules by adsorption (Zhang *et al*., 2007)

2.2.2. Microencapsulation

Microencapsulation method is the trapping of biomaterial in the electrode surface by the use of an inert membrane (Eggins, 2002). Enzymes can be encapsulated into vesicles, polymersomes or polyelectrolyte capsules. Providing a protective cage in a natural environment, less inactivation is achieved. In addition, encapsulated biomolecules have a good stability against temperature, pH, ionic strength and substrate concentration. Also the immobilized biomolecule concentration might be increased by the microencapsulation technique. The encapsulated biomaterial is very close to the transducer surface and incorporating nanomaterials amplifying signal. Studies on the encapsulation of enzymes in nanopores have revealed higher stability and enhanced detection capability. Many studies have been conducted on encapsulation in polyelectrolyte multilayer capsules such as negatively charged poly(sodium styrenesulfonate) and positively charged poly(allylaine hydrochloride), micelles that are block copolymers with a hydrophobic inner shell such as poly(acrylic acid)or polyester and hydrophilic outer shell such as poly(ethylene glycol), vesicles or liposomes such as palmitoyl-oleoylphosphocholine (POPC), hydrogels and sol-gels such as nanoporous silica matrix (Drevon *et al.,* 2002; Lee*et al*., 2009; Sarmaa *et al.,* 2009, Grieshaber et al., 2008). A schematic representation of encapsulation of biomolecules is illustrated in Figure 2.4.

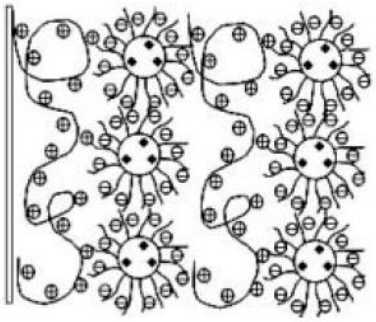


Figure 2.4. Immobilization of biomolecules by encapsulation (Zhang *et al*., 2007).

2.2.3. Entrapment

Similar to encapsulation, biomolecules might also be embedded within a polymeric gel. Widely used membranes are polyacrylamide hydrogels, polyurethane and starch gels, nylon, silastic gels and conducting polymers (Eggins, 2002; Luo *et al.,* 2004). Enzymes can be entrapped into a matrix by electropolymerization of monomer solution in the presence of enzyme (Liu *et al.,* 2007). The diffusion barrier for the substrate through the membrane slows down the reaction. So the diffusion limitation is one of the disadvantages encountered with this technique. Leakage of the enzyme and loss of enzyme activity through the pores depending on the thickness of the membrane is another drawback of entrapment technique. In order to overcome enzyme leakage, enzyme might be crosslinked with polymere matrix by using proper linker (Eggins, 2002; Luo *et al.,* 2004). A schematic representation of entrapment of the biomolecules can be seen in Figure 2.5.

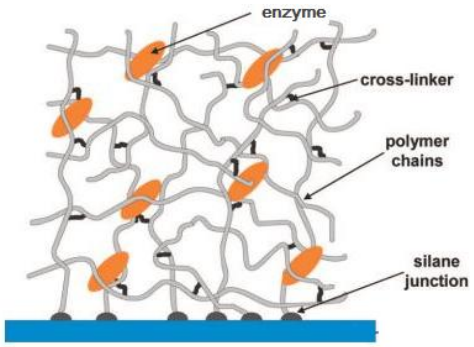


Figure 2.5. Immobilization of biomolecules by entrapment (Klis *et al.,* 2009)

2.2.4. Crosslinking

Bi- and poly- functional reagents might be utilized as crosslinker to fix the protein molecule to support matrix. As a result, inter- and intra-molecular covalent linkages are formed between the proteins. Carbodiiimides and gluteraldehyde are widely known bi-functional reagents that make covalent bond with amino and carboxyl groups. Crosslinking is an effective method to reduce leakage and stabilization of

enzymes by preventing unfolding and protein dissociation. On the other hand, major disadvantages are limited substrate diffusion into the cross-linked enzymes, possible damage to the enzyme, poor mechanical strength and unpredictable progress that is poor control in the aggregate sizes etc. (Drevon *et al.,* 2002; Eggins, 2002; Lee*et al*., 2009). Crosslinking of biomolecules is presented in Figure 2.6.

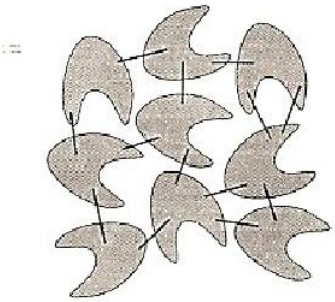


Figure 2.6. Schematic representation of crosslinked biomolecules(Bickerstaff *et. al*, 1997)

2.2.5. Covalent Bonding

Side chains are the nonessential parts of an enzyme for catalytic activity and might be reacted with functional groups at the support surface for the covalent immobilization (Drevon *et al.,* 2002; Eggins, 2002). Covalent bonds are mostly formed between side-chain-exposed functional groups of proteins with suitably modified supports, resulting in an irreversible binding and producing a high surface coverage (Rusmini *et al.,* 2007). Lysine, arginine and histidine are the amine-containing side chain groups of the enzyme and they contain the N-terminal α-amine of the protein chain. Supports are modified with functional groups by chemical treatment and it is accessible to pretreated surfaces commercially. Nucleophilic (amine, thiol) or electrophilic groups (active carboxylic acid, alkyl chloride) are used for the coupling with silanated solid surface. Typical covalent coupling linkages and the potential functional groups of protein side chains are given in Table 2.1 and Table 2.2 respectively. Enzyme might be negatively affected by the coupling conditions. The moderate coupling environment such as low temperature, ionic

strength, proper pH level etc. is essential. Coupling also decreases the enzyme flexibility. (Drevon *et al.,* 2002; Ahuja *et al*., 2007; Eggins, 2002; Lee *et al*., 2009).

Table 2.1. Typical covalent couplings for immobilization (Eggins, 2002)

Reactive group (on surface)	Reactive group (on enzyme)	Coupling linkage
$-R-NH_2$	$HO-\overset{\overset{O}{\|}}{C}-$	$-NH-\overset{\overset{O}{\|}}{C}-$ "Amide"
$-SH$	$HS-$	$-S-S-$ "Disulfide"
$-SH$	$HO-\overset{\overset{O}{\|}}{C}-$	$-S-CO-$
$-COOH$	H_2N-	$-CONH-$ "Amide"
$-CHO$	H_2N-	$-C=N-$

Table 2.2.Commonly Available Functional Groups in Proteins and Functionalities of the Required Surfaces (Rusmini *et al.*, 2007)

Side Groups	Amino acids	Surfaces
- NH_2	Lys, hydroxyl- Lys	carboxylic acid
		active ester (NHS)
		Epoxy
		Aldehyde
- SH	Cys	Maleimide
		pyridyil disulfide
		vinyl sulfone
- COOH	Asp, Glu	Amine
- OH	Ser, Thr	Epoxy

2.3. Synthesis methods of thin films

Response time and reversibility are the most important performance features of a sensor. Diffusion of any reagent through the sensing element to react and diffusion of the product out are essential steps. Thus, the sensing film thickness is an important factor on mass transfer rate, response time and reversibility (Davis *et al*., 2005). Physical vapor deposition, chemical vapor deposition, self–assembled monolayer (SAM), layer-by-layer assembly, Langmuir-Blodgett (LB) films and sol-gel coating techniques can be applied to fabricate thin films.

In the present study, sol-gel technique is employed to produce thin films of TiO_2-SiO_2 mixed oxides and no further information about the other thin film production process will be given.

2.3.1. Sol-Gel Method

Sol- gel process is a colloidal synthesis method for ceramics that consists of an intermediate stage as a sol and/or a gel state (Pierre, 2008). A sol is a stable mixture of a colloidal suspension (Jones, 1984; Brinker *et al.,* 1990). When the solid particles aggregate they constitute a particular volume in solution depending on the microstructure of the particles as can be seen in Figure 2.1.

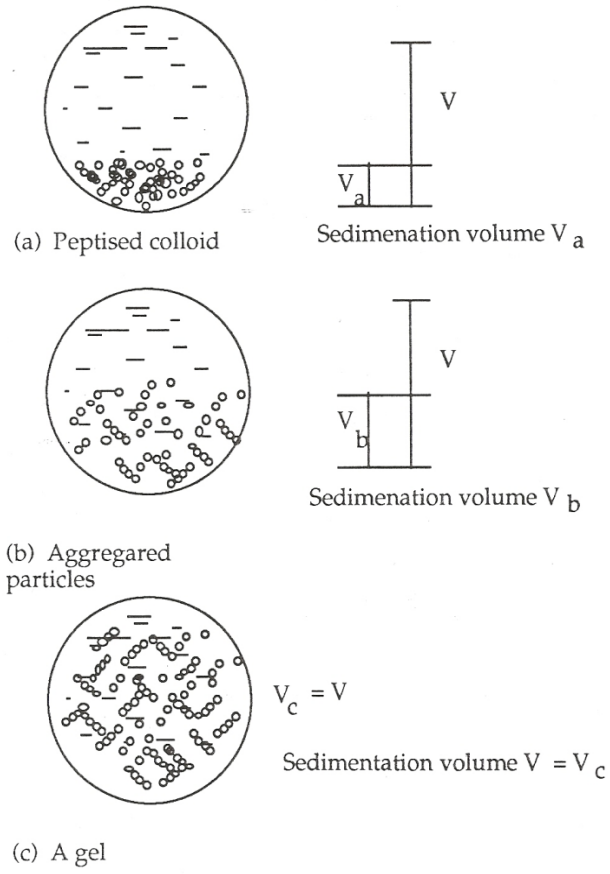


Figure 2.7. Schematic Diagram of solid particle distribution for (a) peptized colloid, (b) aggregated particles and (c) gel (Jones, 1989)

The sedimentation volume is equal to the original solution when the particles are crosslinked and structured being able to stable the solvent. In this case, resultant form exhibits visco-elastic properties and it is called as gel (Jones, 1984).

Sol-gel method is used to fabricate a broad range of products such as ceramics, semiconductors, aerogels, xerogels and etc. Different routes for sol-gel processed materials reported in literature and s illustrated in Figure 2.8. A typical sol-gel process using an alkoxide precursor involves the following steps: (1) formation of stable sols; (2) casting or shape formation; (3) gelation of the sols; (4) aging of the gel; (5) drying of the gel; (6) calcination; (7) sintering if necessary (Lin *et al*., 1998).

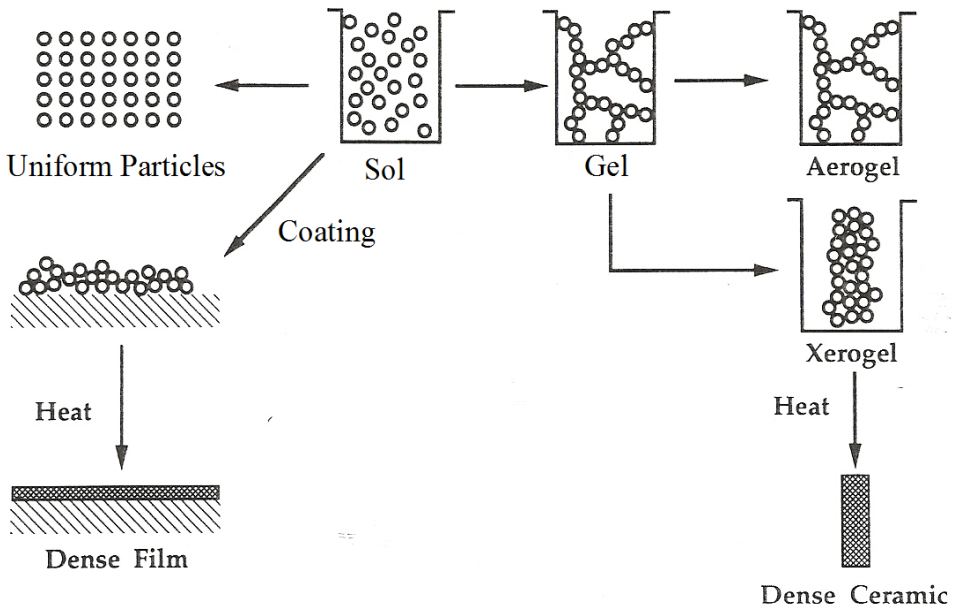


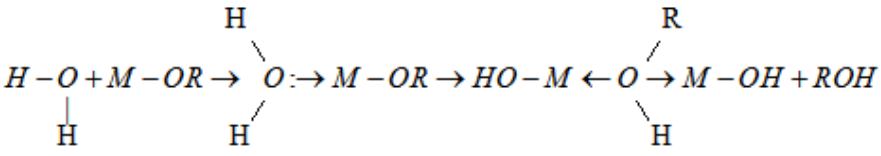
Figure 2.8. Routes for sol-gel processed materials (modified from Brinker *et al.,* 1990)

A precursor material that can be an inorganic salt or metal alkoxide which can be hydrolyzed to form hydrous metal oxide or hydroxide. This monomer suspension (sol) that consist reactive species eager to form higher-order species is peptized by a series of condensation reactions (Lee, 2008). Hydrolysis and condensation

reactions cannot be clearly distinguished and it is difficult to control their relative reaction rates. Depending on pH, water content and structure of the precursors, the rate of hydrolysis and condensation reactions alter. Hydrolysis is the equilibria of aquo (M-(OH_2)), hydroxo (M-OH) and/or oxo (M=O) ligands formed by deprotonation of the solvated metal cation (Pierre, 1998; Brinker *et al.,* 1990) with Equation 2.1

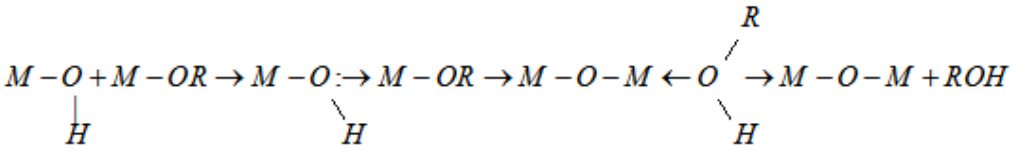
$$[M(OH_2)]^{Z+} \leftrightarrow [M-OH]^{(Z-1)+} + H^+ \leftrightarrow [M=O]^{(Z-2)+} + 2H^+ \quad (2.1)$$

Condensation is a process by which polynuclear complexes are formed by mononuclear complexes (Pierre, 1998). Two types of condensation reaction are possible: olation in which there is a hydroxo ligant between two metal atoms and oxolation in which there is oxo bridge between two metal atoms (Pierre, 1998). Reaction mechanisms of hydrolysis and condensation for metal alkoxide precursors are schematized as Equations 2.2 – 2.6.



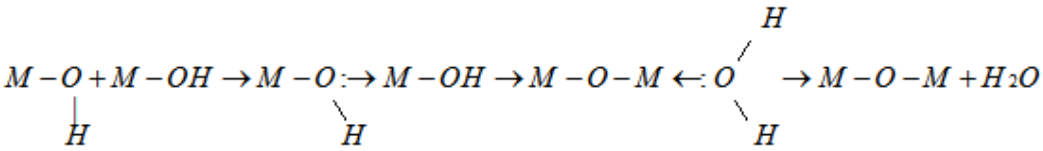
(2.2)

Hydrolysis

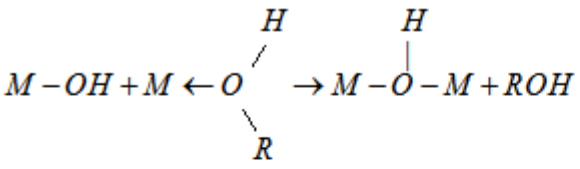


(2.3)

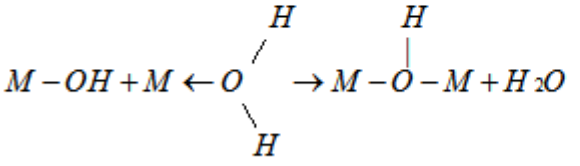
Alcoxolation



Oxolation (2.4)



(2.5)



Olation

(2.6)

Hydrolysis and condensation reactions are catalyzed by the addition of an acid or a base and the nature of the gel is strongly depended on the choice of the catalyst. Hydrolysis occurs by electrophilic attack on alkoxide groups under acidic conditions and by nucleophilic attack on metal ion under basic conditions. Condensation is

directed toward the end groups of the chains in acidic conditions because of the attack on the electron providing ligands resulting in a more linear chains rather than highly branched polymers. Conversely, it is directed toward the middle groups of the chains in basic conditions because of the nucleophilic attack on the positively charged metal ions resulting in highly branched species. It is observed that in acidic conditions hydrolysis is faster and subsequent fast condensation occurs resulting in high polymeric rearrangement. However, in basic conditions, hydrolysis is relatively slow and condensation is favored (Jones, 1984; Brinker *et al.,* 1990; Schwartz *et al.,* 2004).

Applying sol gel technique brings some advantages on the production of metal oxides such as:

- low temperature close to room temperature
- mild conditions, extreme pH values can be avoided
- nanocrystalline and highly porous products
- tunable pore size and porosity
- appropriate for covalent attachment of biomolecules by functionalization
- thin films fabrication without machining and melting
- good optical quality materials (Wright *et al*., 2001)

2.3.2. Coating Techniques

Spin coating and dip coating are mostly used techniques for preparing thin films with sol or solutions. In the spin coating process substrate is placed on a spinner and hold by vacuum. The liquid is dripped on the surface and then spinner is accelerated rapidly to 1000 to 8000 rpm (Brinker *et al.,* 1990; Schwartz *et al.,* 2004). The film formation is explained by a four stage process: deposition, spin-up, spin-off and evaporation. In the deposition stage liquid is spread over the surface, the liquid slips away radially outward by centrifugal force in spin-up stage, the film thickness is reduced by making excess liquid move through perimeter and leave as droplets, and in the final stage, evaporation takes place thinning the film as seen in Figure 2.9(Brinker *et al.,* 1990). Thickness of the film depends on the angular velocity, spinning time and viscosity of the solution (Schwartz *et al.,* 2004).

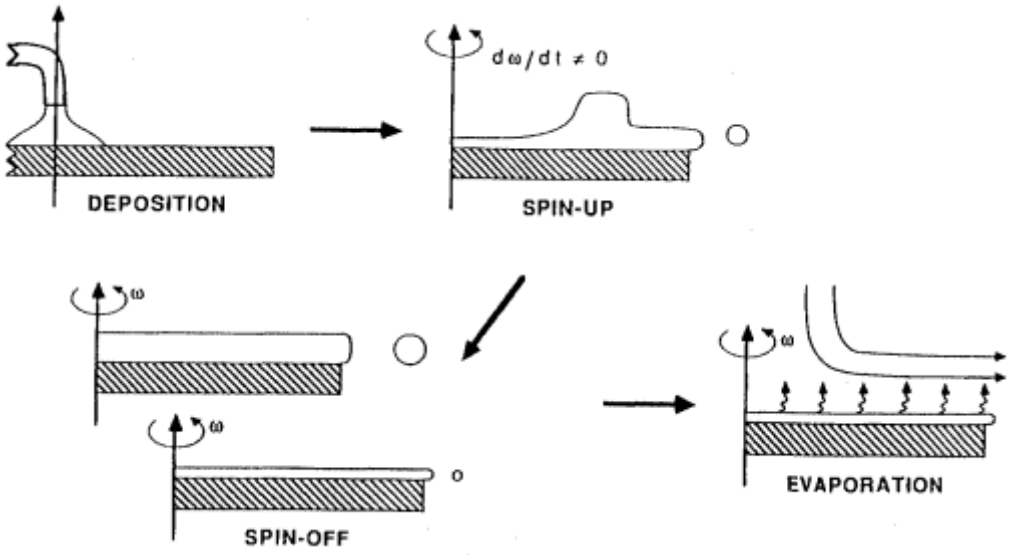


Figure 2.9. Schematic of Spin Coating (Brinker *et al.,* 1990)

In dip-coating substrate is immersed in solution and withdrawn at a constant rate. The process is divided to five stages: immersion, start up, deposition, drainage and evaporation as seen in Figure 2.10. After the immersion, the withdrawal of the substrate from solution starts in start-up, then the substrate is completely removed from solution after deposition, solvent evaporation and drainage steps (Schwartz *et al.,* 2004). In deposition, while moving along the solution, the substrate sweeps some of the liquid toward the deposition region in a boundary layer which is divided into two (Figure 12c) (Brinker *et al.,* 1990). The inner layer moves upward with the substrate where the outer layer moves back to the solution, and the division between the layers affects the film thickness. Viscous drag, gravity force and the surface tension in the concavely curved meniscus are the main factors controlling the film thickness (Brinker *et al.,* 1990). In addition, solution properties such as sticking, aggregation and gelation (Schwartz *et al.,* 2004), and process parameters such as removal rate of substrate and number of the coating layers designate the thickness and the nature of the film.

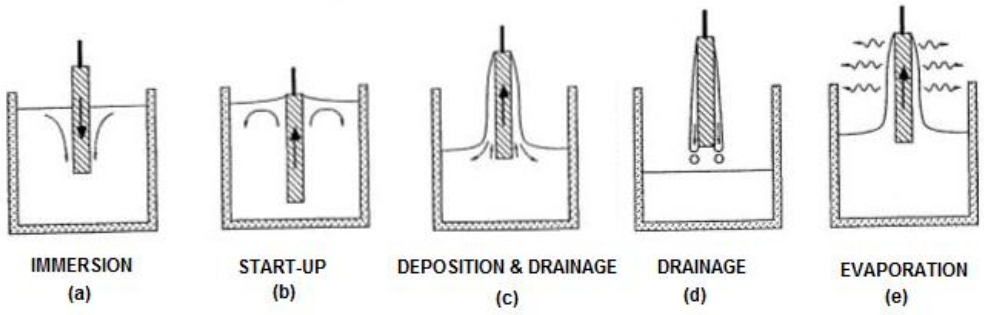


Figure 2.10 .Schematic of Dip-coating (Brinker *et al.,* 1990)

2.4. Support Materials

2.4.1. Titania Silicate Mixtures

The intrinsic chemical and physical properties of high porosity crystallites, together with their high surface areas, open up potential applications in biosensors, magnetics, electronics, mechanics, and micro devices (Qian *et al.,* 2008). Hosts that have good conductivity and large surface area provide good environment for enzyme loading and substrate diffusion, and result in high sensitive and long-term stable biocatalyst. Titania-silica mixed oxides are active solid catalysts or catalyst supports that can be used in a variety of acid-base catalysis, redox catalysis, and photocatalytic processes (Liu *et al*., 2009).

Sol-gel derived silica synthesized under ambient conditions is biocompatible and enzymes can retain their biocatalytic activity. It has some advantages such as unable porosity, high thermal stability and chemical inertness (Sun et al., 2008; Yu *et al*., 2003). However, silica sol-gel matrix is fragile and easily shrinks, cracks and delaminates from the electrode surface (Doong *et al.,* 2006, Yu *et al*., 2003).

Titania is also biocompatible and it has been widely used in solar cell, electronic devices, catalyst support and immobilization of proteins and enzymes because of its remarkable chemical, electronic and optical characteristics, and nano-TiO_2 could be used as a good promoter for the direct electron transfer of enzymes (Cheng *et al*., 2008). However, electrodes and protein immobilization, for which traditional

nanosized crystalline titania is used, have low porosity and titania easily aggregate (Qian *et al.,* 2008).

Titania-silica mixed oxides have a high percentage of the titanium oxides in a uniformly dispersed state and have some advantages such as higher mechanical strength, thermal stability and specific surface area compared to pure TiO_2 (Liu et al., 2009; Jung *et al.,* 1999). Ti–O–Si bonds forms in silica-modified titanium dioxide and the silica-modified titanium dioxides had high thermal stability and the addition of silica in TiO_2 particle could effectively suppress the formation of rutile phase and the growth of the titanium dioxide crystals on calcinations (Li *et al*., 2005) .In addition, binary TiO_2–SiO_2 mixtures have higher photocatalytic activity than traditional TiO_2 (Luo *et al.,* 2009; Anderson *et al.,* 1997; Jung *et al.,* 1999). A broad spectrum of organosilanes are available to modify the silica surface with functional groups to give it desired properties (Claesson *et al.,* 2007) and titanium coordinates with amine and carboxyl groups on the surface of enzymes (Cheng *et al.,* 2008) and with these properties, they are widely used as supports for covalent attachment of enzymes.

2.4.2. Indium-tin oxide (ITO)

Indium-tin oxide is a wide band gap semiconductor having metal like electrical properties (Zhou, 2006). Energy diagram of In_2O_3 and ITO is illustrated in Figure 2.11. As ITO has metal like electrical properties and the ITO-Semiconductor contact is essentially a metal-semiconductor contact (Zhou, 2006).

ITO has high transparency in visible range and near-IR region, high electrical conductivity, wide electrochemical working window, good substrate adhesion and stable electrochemical and physical properties (Lin *et al*., 2007; Zhang *et.al.,* 2005; Sun *et al*., 2006; Moore *et al*., 2006).

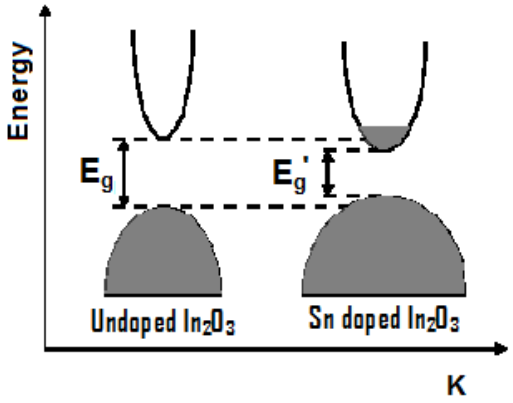


Figure 2.11. Energy diagram of In_2O_3 and ITO (Zhou, 2006)

It has been widely used in development and applications of electronic and optical sensors, direct electron transfer of proteins, electrochemical nucleic acid biosensors, microfluidic on-chip detection, electrochemiluminescence analysis, transparent heating elements, antistatic coatings over electronic instruments, liquid crytal displays, transparent electrodes for various display devices and transparent contact (Lin *et al*., 2007 ; Zhou, 2006).

2.5. Gold Nanoparticles

Recently, nanoparticles that are in 1-100 nm sized have been extensively used as a part of biosensors. These nanoparticles include metal nanoparticles, oxide nanoparticles, semiconductor nanoparticles and composite nanoparticles (Huang *et al*., 2009). Nanoparticles have unique functional properties and each contribute to enhancement of biosensor performance in different manner. Mainly, they have high surface to volume ratio, have high surface energy and they are biocompatible and enhance the electrode conductivity, facilitate the electron transfer and improve the sensitivity, selectivity and stability (Huang *et al*., 2009; Liu et al., 2003; Luo *et al*., 2004; Cai *et al*., 2001; Zhang *et al.,* 2005). Among them, gold nanoparticles are the most stable and one of the most widely used nanoparticles (Zhang *et al.,* 2005). Gold allows easy adsorption of biomolecules to its surface stably without losing their biological activity and stable immobilization (Lin *et al*., 2007; Guo *et al*., 2007; Pingarron *et al*., 2008; Shipway *et al.,* 2000). In addition, gold nanoparticles act as tiny conducting centers and provide direct electron transfer during redox reactions of

enzymes without need of another mediator. There two ways to prepare gold modified surfaces: electrostatically and covalently bond. In the first one, electrostatic stabilization is provided between gold nanoparticles with citrate and negatively charged particles are adsorbed to the electrode surface. In the second one, surface is modified with functional groups (–SH, $–NH_2$, –CN) and gold nanoparticles bind covalently to these groups (Liu *et al*., 2003; Mena *et al.,* 2005) as Equation 2.7 ;

$$RSH + Au \leftrightarrow RS\text{-}Au + e^- + H^+ \qquad (2.7)$$

All of these properties have made gold nanoparticles to be used in a wide range of biosensor applications and some examples of redox enzyme biosensors modified by gold nanoparticles can be seen in Table 2.3.

Table 2.3. Redox enzyme biosensors modified by gold nanoparticles (Pingarron *et al.*,2008)

Enzyme(s)/electrode	Immobilization mode	Detection	Performance	Analyte/sample	Analytical characteristics
Gox/gold	Covalent attachment of Gox to a nAu monolayer-modified Au E	(E=0.3V vs SCE)	K_M^{app} =4.3mM	Glucose	Linear range:$2.0x10^{-5}$ to $5.7x10^{-3}$ M:slope: $8.8\mu AmM^{-1}$ cm^{-2} :LOD:8.2 µM
Gox/GCE	GO and the redox mediator TTF coimmpmilized by cross-linking with glutaraldehyde on gold-modified electrodes with either Cyst or MPA monolayers Gox adsorbed on a colloidal gold modified CPE	(E=0.2v)	Useful lifetime:28days 0.05M PBS, pH 7.4	Glucose	Linear range:0.01-10mM:LOD:$0.7x10^{-5}$M:slope: $1.02\pm0.06mA\ M^{-1}$
Gox/gold	Electrochemical deposition of chitosan-Gox-gold nanoparticles biocomposite	(E=0.7V)	0.1M phosphate buffer, pH 7.4	Glucose /serum sample	Linear range: 0.005-2.4mM LOD:2.7µM
Gox/gold	Layer-by-layer covalent attachment of IO_4^- oxidized Gox and nAu using cysteamine as cross-linker	(E=0.25V)	10mL. 0.1M PBS, pH6.8 containing 0.35mM ferrocenementhane	Glucose	Linear range: $1.0x10^{-5}$-$1.3x10^{-2}$M: slope: $5.72\mu A\ mM^{-1}\ cm^{-2}$
Tyr/CPE	Tyr immob. On a colloiadal gold modified CPE	(E= - 150mV)	K_M^{app} =54±3µM, 0.1M PBS, pH 7	Phenol	Linear range: 4-48µM; LOD: 6.1nM; slope=$12.3\mu A\ cm^{-2}\ \mu M^{-1}$

2.6. Redox Enzymes

Redox enzymes basically catalyze electron transfer reactions that have importance in biological systems such as respiration and photosynthesis and also in biotechnological such as degradation of pollutants and biomass, and drug and food processing (Gilardi *et al.,* 2001). Main reactions catalyzed by redox enzymes are summarized in Figure 2.12.

Wide range of redox enzymes are used for the detection of analytes having medical or environmental importance and it has been developed several routes for electron coupling between the redox enzyme such and the electrode as by electroactivity of substrate or product of the enzyme, by using redox mediators or by direct electron transfer between the electrode and the redox enzyme (Gilardi *et al.,* 2001). In Figure 2.13 some mechanisms of redox enzyme use are shown.

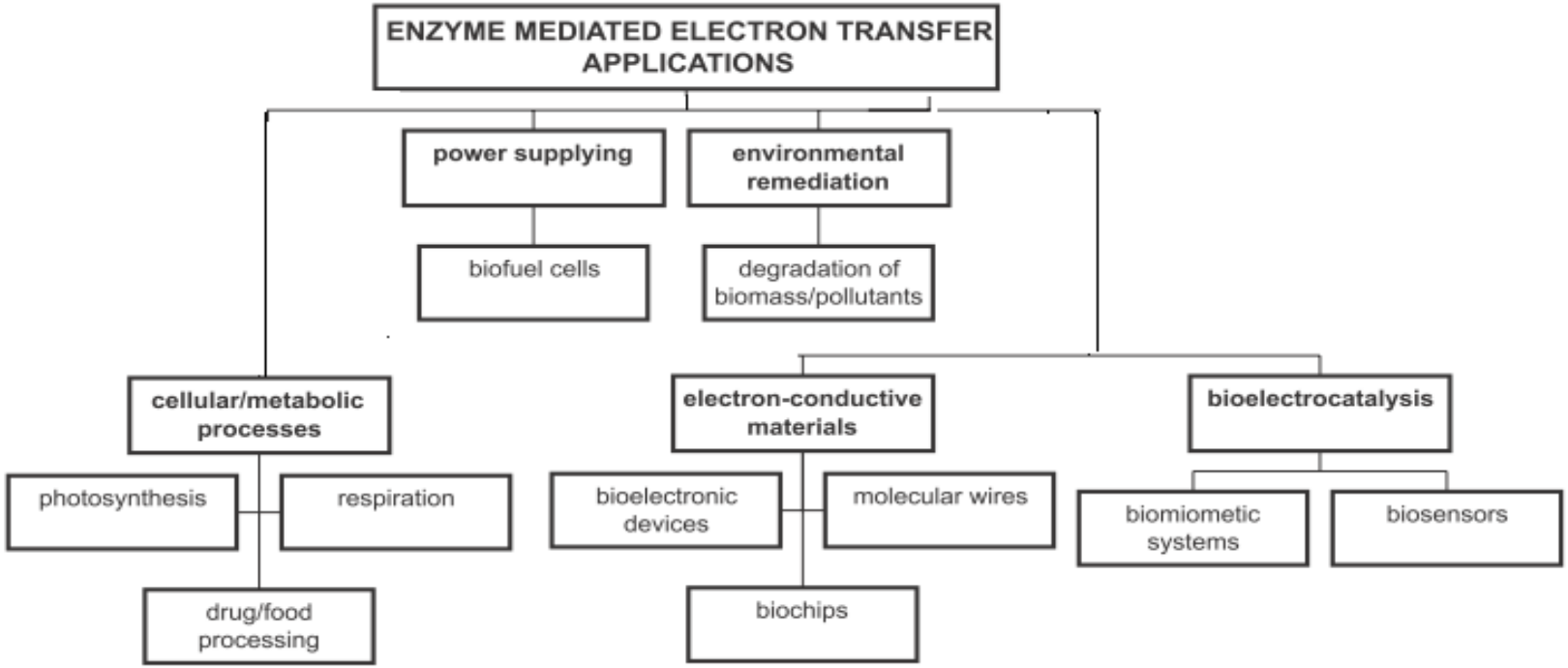


Figure 2.12. Main electron transfer reaction systems (Freire *et al.,* 2003)

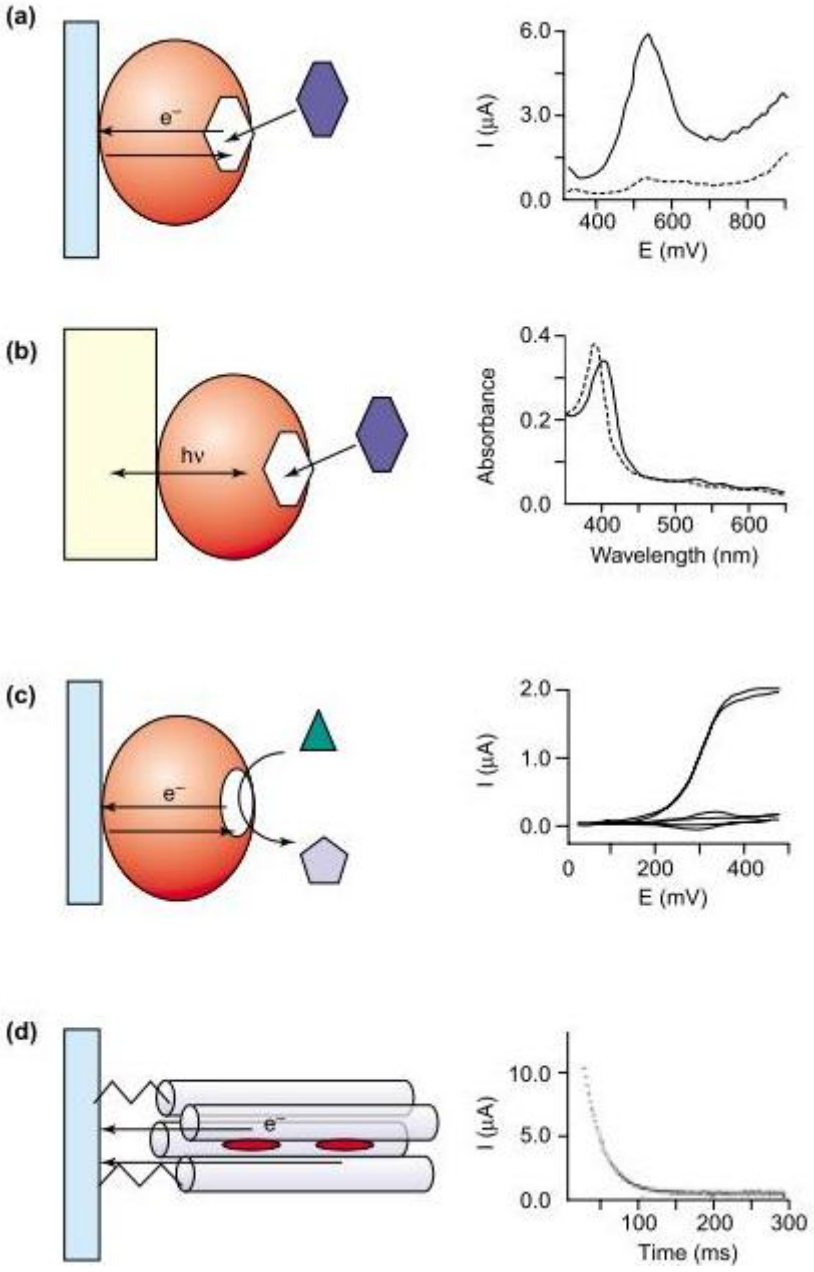


Figure 2.13. Use of redox proteins (a) Sensing the binding of a ligand based on changes of the redox properties, (b) Optical device sensing changes in optical properties of the redox centre upon ligand binding, (c) Detection of enzymatic activity through measurement of catalytic current, (d) protein with multi-redox centres, immobilized on an electrode surface (Gilardi *et al.,* 2001).

2.6.1. Polyphenol oxidases

Polyphenol oxidases are oxidoreductases that catalyze oxidation of phenolic compounds (Durán *et al.,* 2000). Phenolic compounds are widely distributed in nature and their oxidations are important in such processes as cellular oxidation, cellular wall protection, fruit browning, juices and wines processing, pulps delignification, fabrics decoloration, decontamination of soils and water pollution (Durán *et al*., 2000). Laccases and tyrosinases are two groups that catalyze the transformation of a large number of phenolic and non-phenolic aromatic compounds and immobilization of them have been widely studied for synthetic and analytical purposes, bioremediation of contaminated soils, wastewater treatment and beverage treatment (Durán *et al.,* 2002).

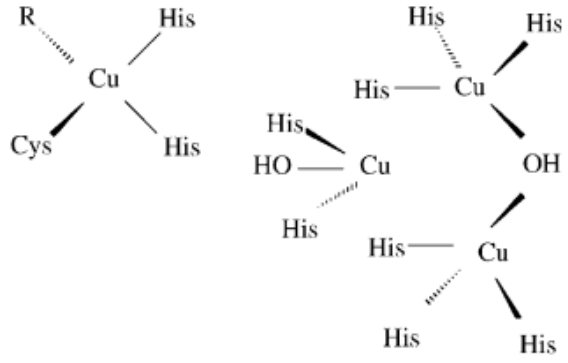
2.6.1.1. Laccases

Laccases (E.C. 1.10.3.2, benzenediol:oxygen oxidoreductase) are copper-containing phenol oxidases that catalyse the oxidation of some inorganic and (e.g., organic compounds phenols and aromatic or aliphatic amines) to their corresponding radicals with the concomitant electroreduction of oxygen to water (Jeon *et al*., 2008; Manole *et al*., 2008) as seen in Equation 2.7;

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O \qquad (2.7)$$

Laccases are produced by plants, fungi, bacteria and even by insects which make redox reactions stimulated by it in ever-present in nature. They exhibit great potential for development as green chemistry model systems as they do not need a heme cofactor, they use molecular oxygen as a final electron acceptor without requiring hydrogen peroxide supply and they have broad substrate specificity (Jeon *et al*., 2008; Bendl *et al.,* 2008). They are industrially relevant enzymes that can be used for a number of diverse applications, e.g. for biocatalytic purposes such as delignification of lignocellulosics and cross-linking of polysaccharides, for bioremediation such as waste detoxification and textile dye transformation (Gianfreda *et al.,* 1999).

Laccase contains four copper atoms that have been classified according to their electron paramagnetic resonance (EPR) features: Type 1 or blue, Type 2 or normal and Type 3 or coupled binuclear copper site where the coppers are antiferromagnetically coupled through a bridging ligand (EPR undetectable) (Dur´an *et al.,* 2002). Schematic model of laccase with active side structure is seen in Figure 2.14. Type I (T1) copper gives the typical blue colour to the protein and is the site where substrate oxidation takes place and Type 2 (T2) and Type 3 (T3) copper form a trinuclear cluster, where reduction of molecular oxygen and release of water takes place (Riva, 2006).The amino acid Histidine (His) has as a positively charged imidazole functional group.



Type 1 Trinuclear center (Type 2I + Type 3)

$Cu(N^{\delta}_{His})_2S^{\gamma}_{Cys}R$ $Cu(N^{\varepsilon}_{His})_2OH.\mu\text{-}OH[Cu(N^{\delta}_{His})_3]_2$

$R = S^{\delta}_{Met}$

Figure 2.14. Active side structure of laccase from *Trametes versicolor* made of four copper atoms (Dur'an *et al.,* 2002)

Laccase oxidizes many substrates: phenolic dyes, phenols, chlorophenols, lignin related diphenylmethanes, benzopyrenes, N-substituted *p*-phenylenediamines, organophosphorus and non-phenolic beta-*O*-lignin model dimer (Durán *et al*., 2000). Simple diphenols such as hydroquinone, catechols, guaiacol and 2,6-dimethoxyphenol, syringaldazine are good substrates for the majority of

laccases, and also nonphenolics such as N-Hydroxybenzotriazol, violuric acid and N-hydroxyacetanilide, and 2,2-azobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) are N–OH compounds capable of mediating a range of laccase-catalyzed biotransformation (Durán *et al*., 2002; Mayer *et al.,* 2002). Schematic representation of different redox catalytic cycles of laccase is shown in Figure 2.15.

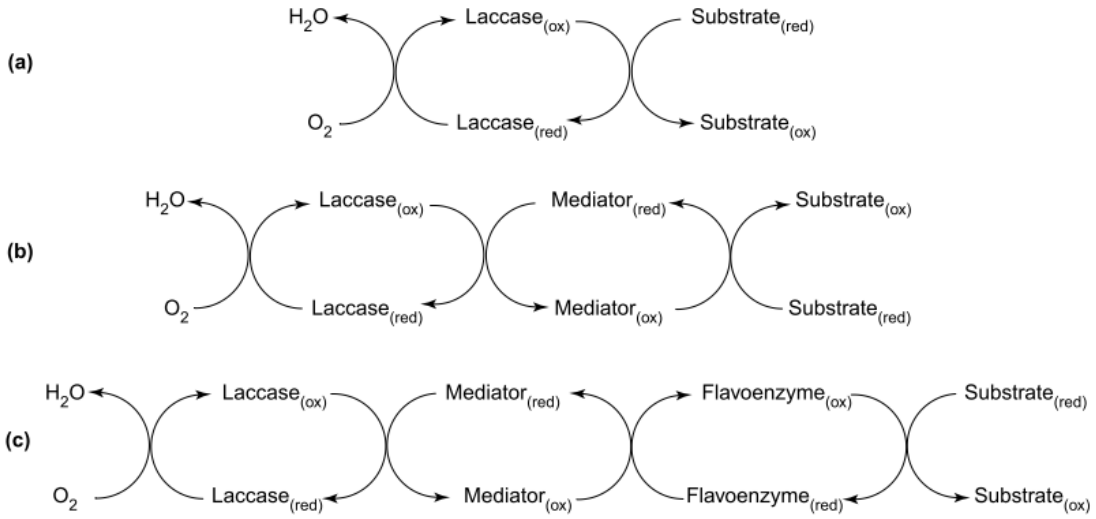


Figure 2.15. Schematic representation of laccase-catalyzed redox cycles for substrates oxidation in the absence (a) or in the presence (b) and of chemical mediators(c) (Riva, 2006).

Beside commonly used mediator compound, ABTS is used for determining enzyme activity directly. ABTS is readily oxidized by free radicals, various peroxidases and laccases to the cation radical $ABTS^{+\bullet}$, and the concentration of the intensely coloured, green-blue cation radical can be correlated to the enzyme activity. Laccase activity can be determined by monitoring the oxidation of ABTS to the stable cationic radical $ABTS^{+\bullet}$ at 420 nm with extinction coefficient ε_{420} = 36,000 M^{-1} cm^{-1} (Wolfenden *et al.,* 1985). It is well known that cation radicals represent an intermediate oxidation step in the redox cycle of azines, and upon extended oxidation and abstraction of the second electron, the corresponding

dications can be obtained (Majchereczyk *et al.,* 1999) and ABTS redox cycle is shown in Figure 2.16.

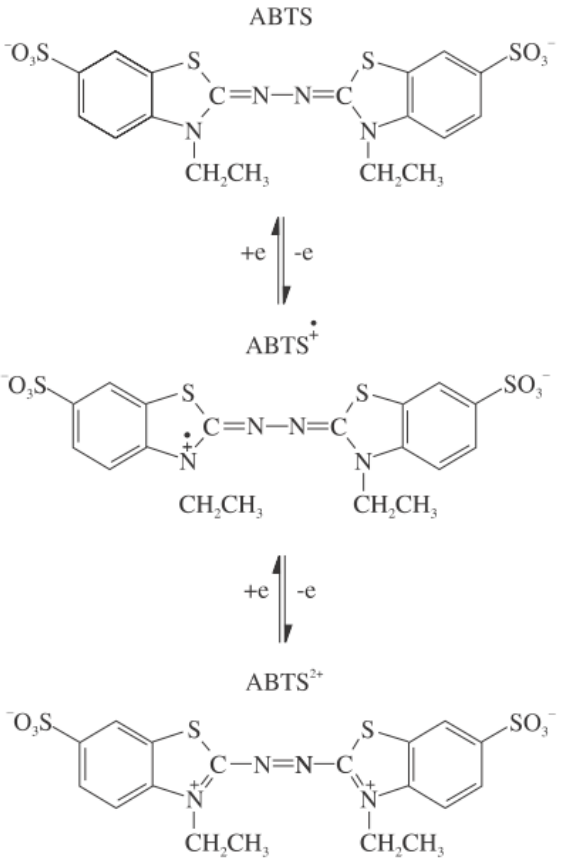


Figure 2.16. Formation of the cation radical and the dication by removal of one and two electrons from ABTS (Majchereczyk *et al.,* 1999)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

Laccase (EC.1.10.3.2) was purchased from Novozymes (USA) and invertase (EC .3.2.1.26) from Novonordisk. Cysteamine was purchased from Fluka (USA). Bovin serum albumin (BSA), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and Bradford reagent were obtained from SIGMA (USA). Titanium isopropoxide (TTIP) were used as titania precursor, the silica colloidal mixture Ludox SM30 was used as silica source (more information is presented in Appendix A) and they were both obtained from SIGMA. PEG 4000 and $AuCl_3$ were also obtained from SIGMA (USA). Other reagents were of analytical grade and obtained from SIGMA or MERCK. Ultrapure water was used throughout this research.

3.2. Sol-Gel Synthesis

Titania-Silica binary colloidal mixture was prepared with the procedure shown in Figure 3.1. 200 ml distilled H_2O and 1 ml acetic acid, which was the catalyst, were mixed and 5 ml TTIP was added dropwise while stirring in order to start hydrolysis. Then, 0.7 ml 65 % (v/v) HNO_3 was added for setting pH 3.5 and the mixture was stirred at 80°C for 30 mins under reflux and continued stirring at room temperature for 2 hours. 6.4 ml Ludox SM 30 was added and after stirring overnight (22.5 hours), 21.3 ml PEG 4000 was added in order to obtain porous surfaces. The mixture was stirred for 24 hours and 18.5 ml $HAuCl_4$, 1 % (w/w) of TiO_2+SiO_2, was added. Finally, the mixture was removed from stirring after 24 hours.

Preparation of $HAuCl_4$ solution: 50 ml dark brown bottle was cleaned with the mixture of 15 ml HCl and 5 ml HNO_3 and subsequently rinsed with distilled water

and dried at 100°C. 0.1 g $AuCl_3$ was added to 32.973 ml distilled water in cleaned bottle and it was dissolved 27 µl HCl was put. Prepared $HAuCl_4$ solution was stored at 4°C.

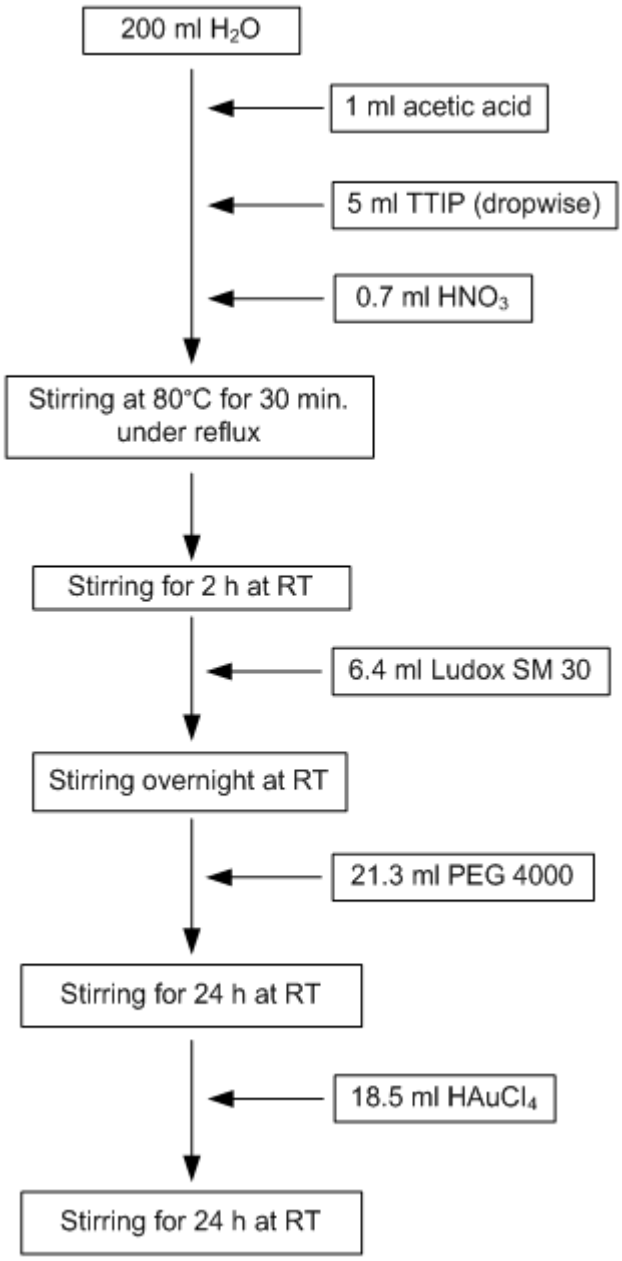


Figure 3.1. Schematic Representation of Sol-gel Preparation

3.3. Pretreatment of Pyrex and ITO Glasses

Microscope slides which were obtained from Industrial Quality and one side ITO coated glasses which were supplied commercially by Şişecam were used as substrates for preparation of thin film surfaces and they were cleaned before being used. Slide surface is usually dirty and contaminated with stains and has scratch marks. Slides can be pretreated with detergent by which dust, dirt and residues are removed or with organic solvents such as ethanol or acetone which remove oils and organic residues. For deep clean, glasses can be etched by inorganic acid such as HCl, nitric acid, aqua regia or base such as ammonium water or NaOH and KOH (Wang *et al.,* 2004).

Microscope slides in dimesions of 25mm x 75 mm x 1 mm that were used as Pyrex glass substrates were etched and cleaned by 1 N KOH solution by immersing for 48 hours. Then, glasses were rinsed with water till pH 7 and subsequently ultrasonicated in ethanol for 1 hour. Lastly, glasses were wiped with drying towel and dried at 100˚C in for 1 hour.

ITO glasses were cleaned only by using acetone and isopropyl alcohol without etching in order not to damage thin ITO coating. Glasses were ultrasonicated with acetone and isopropyl alcohol for 30 mins each respectively. Then, they were dried at 100˚C for 15 mins.

3.4. Thin Film Coating

3.4.1. Dip-Coating and Thermal Treatment

Sol-gel derived thin films were deposited on Pyrex and ITO substrates by dip-coating method as seen in Figure 3.2. Three layers of sol gel were coated on the substrates. While layer by layer coating, each layer is dried at a temperature and humidity for gelation before next layer in order to provide adhesion and densification. The degree of dryness is where there are no reactive groups that may react with the subsequent layer (*Debsikdar, 1989*). Calcination, relatively high

temperatures than drying, is heat treatment for consolidation of the coatings and crystallization of the film (Yu *et al.,* 2008; Chan *et al.,* 1999). In addition, porosity is provided by solvent evaporation and the organic residuals are removed (Linda *et al*., 2009; Kim *et al*., 2006).

Both Pyrex and ITO glass substrates were withdrawn with the velocity of 0.5 cm/min after dipping into the sol-gel as 25 x 40 mm. After each layer, substrates were dried at 100°C (Nüve FN 055) for 10 mins. Finally, the films were calcinated at 500°C in air for 15 mins in a tube furnace (Protherm 1000W, PTF 12/50/250) in order to accomplish the oxidation and crystallization of the film.

Commercially obtained ITO glasses had one side conducting surface that is one side ITO coating and this side should have been coated with sol-gel. For this purpose, non-conducting side was coated with paraffin in order to prevent being coated with water based sol-gel. Paraffin was dissolved in octane 10 % (w/v). Non-conducting surface was coated with paraffin solution by brush and dried and all process was done under fume exhaust hood. This procedure was repeated between each layer coating after drying at 100°C for 10 min. After the third run, ITO glasses were dried at 100°C for 10 mins same as microscope glass slides and calcinated and there was no paraffin left on the surface. By this way, conducting sides of ITO substrates were coated with sol-gel.

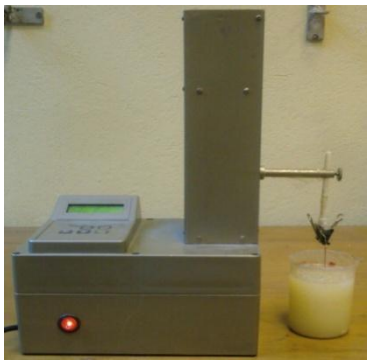


Figure 3.2. Dip-coating of substrates with sol-gel

3.5. Enzyme Immobilization

3.5.1. Cysteamine Linking on the Surface

Enzyme was immobilized on sol-gel derived TiO_2-SiO_2-Au thin film by the thiolamine linker cysteamine (2-mercaptoethylamine) ($HSCH_2CH_2NH_2$) which is shown in Figure 3.3. Cysteamine was expected to covalently bind to gold on the surface with its thiol group as shown in Equation 3.1.

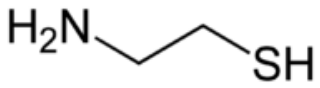


Figure 3.3. Structure of cysteamine

$$RSH + Au \leftrightarrow RS\text{-}Au + e^- + H^+ \quad (3.1)$$

TiO_2-SiO_2-Au sol-gel coated substrates were immersed in 20 mM aqueous cysteamine solution for 3.5 hours at room temperature under stirring in dark at 250 rpm. Then, the substrates were washed with water in order to remove unbound cysteamine.

Immobilization reaction was expected to take place between carboxyl group of the enzyme and amine group of cysteamine with carbodiimide bond formation.

3.5.2. Immobilization of Laccase

Laccase was immobilized on cysteamine modified TiO_2-SiO_2-Au thin film. Different enzyme concentrations from 0.25 to 900 µg/ml and different loading times between 30 min and 12 h were used for immobilization. Laccase solutions of desired concentrations were prepared in 0.1 M pH 5.0 sodium acetate buffer as illustrated in Table 3.1. Substrates were dipped into the enzyme solution at 4 °C under stirring at 250 rpm in dark for a definite loading period. Substrates were withdrawn and each rinsed with 100 ml buffer in order to remove unbound enzyme from the surface.

Table 3.1. Laccase immobilization solutions

	Concentration (µg/ml)									
	2.5	5	10	25	100	180	360	400	600	900
Laccase (µl)	25	50	100	250	1000	2000	4000	4000	6000	9000
Buffer (µl)	975	950	900	750	-	-	-	-	-	-
Buffer (ml)	179	179	179	179	179	198	196	176	174	171
Total (ml)	180	180	180	180	180	200	200	180	180	180

3.5.3. Immobilization of Invertase

Invertase used as model enzyme was immobilized as a preliminary study. Invertase was immobilized on cysteamine modified TiO_2-SiO_2-Au thin film substrates in 10 µg /ml enzyme solution prepared in 0.1 M pH 5.0 sodium acetate buffer. Substrates were dipped into the enzyme solution at room temperature under stirring at 250 rpm in dark for 12 h. Substrates were withdrawn and each rinsed with 100 ml buffer in order to remove unbound enzyme from the surface.

3.6. Analytical Methods

Activity measurements of laccase and invertase were done spectrophotometrically using double-beam UV-Vis spectrophotometer (Thermo Electron Cooperation Nicolet Evolution 100). Both invertase and laccase reactions were carried out in shaking water bath (Nüve ST 402) at 40°C.

3.6.1. Free Laccase Activity

Laccase activity was measured by following the oxidation of ABTS (2.2'.azmobis (3.ethly benzthiazoline-6-suplhonate)) (ε_{420}=36,000 M^{-1} cm^{-1}) (Bendl *et al*., 2008; Bourbonnais *et al*., 1990). 0.5 mM ABTS was prepared in 0.1 M pH 5.0 sodium acetate buffer and preheated to 40 °C. In order to start the reaction, 10 µl 0.045

mg/ml of enzyme was mixed with 990 µl ABTS in cuvette in spectrophotometer. Blank was the mixture of 990 µl ABTS and 10 µl buffer. Reaction was monitored during 90 sec at 420 nm in 5 sec intervals.

One unit of laccase activity (U) was defined as the amount of enzyme that catalyzes oxidation of 1 µmole ABTS per min under operating condition (40°C, pH 5.0). Free laccase activity was calculated using the slope of reaction curve as given in Equation 2.2:

$$\left(\frac{U}{ml}\right) = \left(\frac{\Delta OD}{\Delta t}\right) x \left(\frac{1}{\varepsilon}\right) x \left(\frac{1000\ \mu mol}{mmol}\right) x \left(\frac{1000\ \mu l\ reaction\ mixture}{10\ \mu l\ enyme\ solution}\right) x \left(\frac{60\ sec}{min}\right) \quad (2.2)$$

where;

ΔOD = change in the absorbance at 420 nm
Δt = change in time, sec
ΔOD/ Δt = slope of reaction curve
ε = extinction coefficient, (M^{-1} cm^{-1})

3.6.2. Immobilized Laccase Activity

Activity of the laccase immobilized on the substrate was determined with the modification of free enzyme activity procedure. Reaction was carried out in dark and shaking water bath was used in order to provide good mixing and increase substrate/enzyme interaction. 100 ml 0.5 mM ABTS was used as substrate for enzyme immobilized glass. Substrate was prepared in 600 ml beaker for increasing the liquid-gas interface area for O_2 diffusion. A small piece of glass was placed on the base of the beaker in order to provide an inclined position for the enzyme immobilized glass without entirely lying on the base of the beaker. Immobilized glass was immersed in the substrate that preincubated at 40°C and reaction was monitored by taking 500 µl of reaction mixture and recording the change of absorbance at 420 nm during a minimum 20 min in 5 or 10 min intervals. Blank consisted of only substrate solution.

Immobilized laccase activity per glass plate (U/plate) was determined by using slope of the reaction curve with the formula in Equation 2.3;

$$\left(\frac{U}{plate}\right) = \left(\frac{\Delta OD}{\Delta t}\right) x \left(\frac{1}{\varepsilon}\right) x \left(\frac{1000\ \mu mol}{mmol}\right) x \left(\frac{100\ \mu l\ rxn\ mix}{1\ plate}\right) \quad (2.3)$$

where;

ΔOD = change in the absorbance at 420 nm
Δt = change in time, min
ΔOD/ Δt = slope of reaction curve
ε = extinction coefficient, (M^{-1} cm^{-1})

3.6.3. Free Invertase Activity

Invertase activity was determined with DNS (dinitrosalycylic acid) method (Miller, 1959) following the hydrolysis reaction of sucrose to glucose and fructose. Reaction was followed by mixing 1 ml 2 µg/ml invertase and 24 ml 50 mg/ml sucrose at 40°C. Both solutions were prepared with 0.1 M pH 5.0 sodium acetate buffer.

3.6.4. Immobilized Invertase Activity

Immobilized invertase activity was also determined with DNS method. 50 mg/ml sucrose in 100 ml 0.1 M pH 5.0 sodium acetate buffer was incubated at 40°C in tube having 4 cm diameter and 16 cm length. Reaction was started by immersing invertase immobilized glass and 1ml reaction mixture was taken in every 5 or 10 min for minimum 30 min and mixed with 1 ml DNS in test tubes and vortexed. Test tubes were boiled for 5 min and cooled for 5 min. Glucose concentration during reaction was followed by reading absorbance at 540 nm. Glucose standard was prepared in between 0-150 µg/ml concentrations with 1 mg/ml glucose solution. DNS preparation was given in Appendix B.

One unit of invertase activity (U) was defined as the amount of enzyme which catalyzes the hydrolysis of 1 μmole sucrose per min under the reaction conditions (40^{o}C, pH 5.0). Immobilized invertase activity per glass plate (U/plate) was determined by using slopes of the reaction curve and glucose standard curve in the formula in Equation 2.4;

$$\left(\frac{\text{U}}{\text{plate}}\right)=\left(\frac{\Delta\text{OD}/\Delta t}{\Delta\text{OD}/\Delta\text{C}}\right)\text{x}\left(\frac{1\text{ mmol}}{180\text{ mg glucose}}\right)\text{ x }\left(\frac{1000\text{ μmol}}{\text{mmol}}\right)\text{x}\left(\frac{100\text{ μl rxn mix}}{1\text{ plate}}\right)\text{ x }\left(\frac{1}{2}\right) \quad (2.4)$$

ΔOD = change in the absorbance at 540 nm
Δt = change in time, min
ΔC = change in reducing sugar
ΔOD/ Δt = slope of reaction curve
ΔOD/ ΔC = slope of the glucose standard curve

As glucose and fructose were two reducing sugars, the equation was divided by 2 for conversion to hydrolyzed sucrose.

3.6.5. Protein Concentration

Protein concentration was determined by Bio-Rad Dye Reagent Concentrate microassay which is a Bradford dye binding method. BSA served as the standard protein. The procedure and standard curve are given in Appendices C and D respectively. 1/25 and 1/50 dilutions of pure laccase were used in Bradfrod method.

3.7. Contact angle measurement

Surface wettabilities of the Pyrex and ITO glass substrates were studied with contact angle measurements of CAM (Contact Angle Meter) in Environmental Catalysis Laboratory, Chemical Engineering Department, METU. Pyrex glass and ITO glass were cleaned and etched before coating as explained in section 2.3. Contact angles of substrates before and after cleaning were measured by

dropping distilled water on the surface and hydrophobicity changes of Pyrex and ITO glasses were examined.

3.8. AFM Measurements

Surface topography of bare substrate and sol-gel derived TiO_2-SiO_2-Au thin film was analyzed by Atomic force microscopy (AFM). Images were obtained in non-contact mode with silicon tip from Nanosurf easyScan 2 in Environmental Catalysis Laboratory, Chemical Engineering Department, METU.

3.9. SEM Measurements

Surface was characterized also by Scanning electron microscopy (SEM). SEM images of thin film coated ITO and Pyrex glasses were collected on Quanta 200 scanning electron microscope (FEI, USA) in Gazi University. The accelerating voltage was 15 kV and 1000 magnification was applied. Also, SEM images of the thin film and enzyme immobilized thin film were obtained from QUANTA 400F Field Emission SEM in METU Central Lab with accelerating voltage of 30 kV and at 2000 and 250 000 magnifications.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Introduction

In this study, the immobilization of redox enzyme laccase over semiconductor thin film coated Pyrex® glass and ITO (indium-tin oxide) substrate was studied. The enzyme immobilization over sol-gel derived TiO_2-SiO_2-Au thin film was achieved by using linker molecule with two functional groups. Cysteamine is thiolamine group molecule comprising of thiol (SH) amine (NH_2) functional groups forming a covalent bond between the gold–thiol (Au-S) and amine-enzyme (NH-COOH) as seen in Figure 4.1. Gold-amine bond formation is another possibility as it is reported in literature (Liu *et al*., 2003). Previously, immobilization studies were examined by using invertase as a model enzyme since invertase is commonly studied enzyme being easily available and cheap.

In the experimental studies the effect of immobilization parameters such as immobilization temperature, enzyme concentration of immobilization solution, immobilization time for laccase were tested and leakage and storage stability of immobilized laccase were determined.

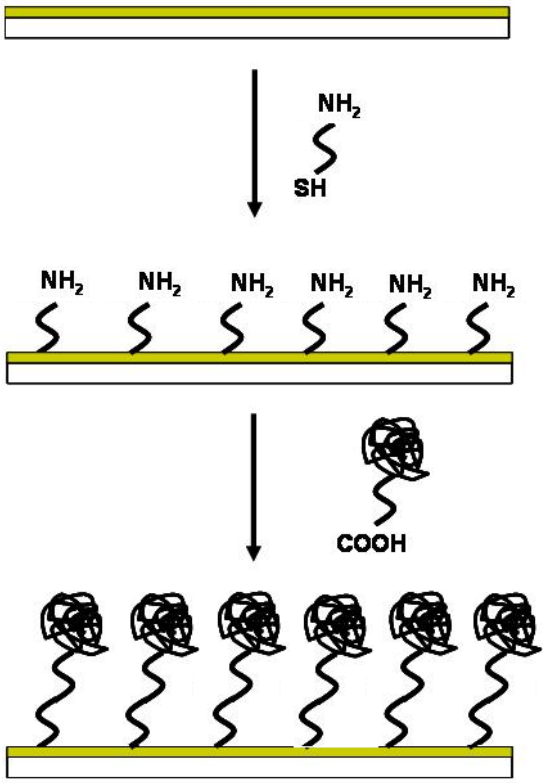


Figure 4.1. Functionalization and immobilization of enzyme immobilization

4.2. Characterization of Thin Films

4.2.1. AFM Images of Thin Films

Surface topology of thin films was characterized by AFM. Figure 4.2 is the AFM image of etched and cleaned glass. Surface change can be seen when looking at Figure 4.3 which is AFM image of TiO_2-SiO_2-Au thin film coated surface. It was achieved to form homogeneously dispersed spherical nanosized particles as thin film as seen in Figure 4.2 (a). Comparison of the Figure 4.2 (b) and Figure 4.3 (b), the 3D images, indicates that produced TiO_2-SiO_2-Au thin film was porous.

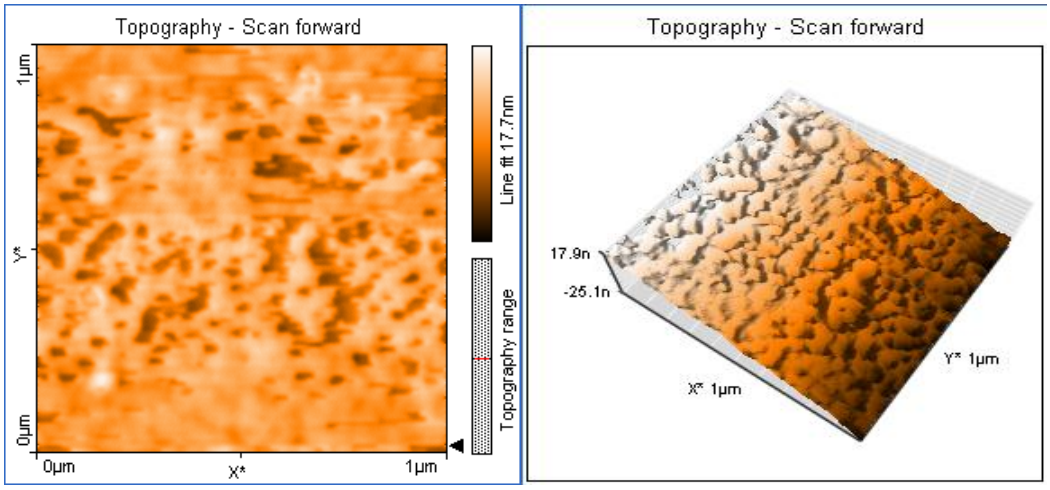


Figure 4.2 (a) AFM image of etched cleaned glasses, and (b) 3D image

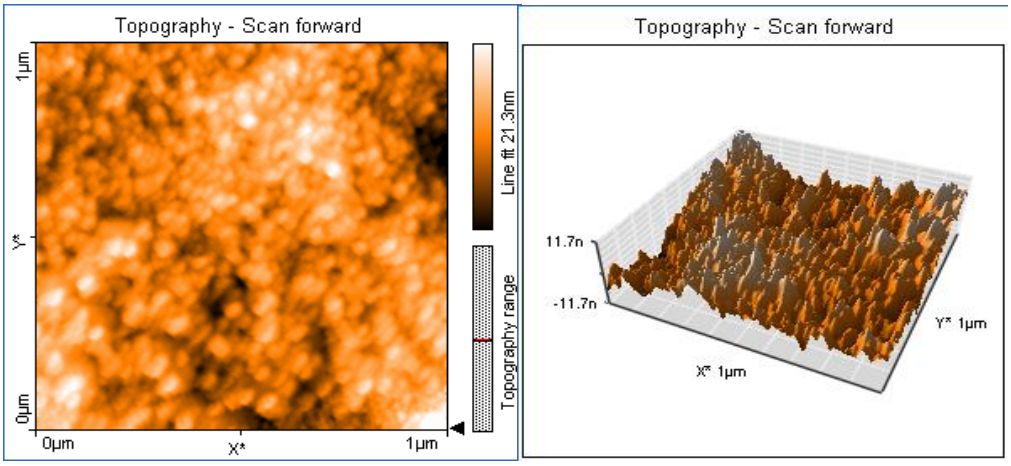


Figure 4.3. (a) AFM image of TiO_2-SiO_2-Au thin film, and (b) 3D image

4.2.2. SEM Micrographs of Thin Films

Surface morphology of thin films was characterized by SEM. Figure 4.4 is the SEM image of TiO_2-SiO_2-Au thin film and Figure 4.5 is the image of enzyme immobilized TiO_2-SiO_2-Au-Cys thin films. Both images were taken at 30 kV and 2000

magnification and enzyme was the model enzyme invertase. The SEM images verify there is a homogeneous thin film coating on the surface. Figure 4.6 that is the 250000 magnified SEM image shows there is nanosized particles on the surface.

Surface images given in Figure 4.4 and Figure 4.5 depict that sol-gel derived TiO_2-SiO_2-Au thin film is well adhered to surface and enzyme immobilization has no adverse effect on coating. Enzyme on the surface cannot be seen in SEM images even in large magnifications. Qiu *et al.* (2009) compared the SEM images of surface with and without enzyme and interpreted that the reason of darker and more blurred image of surface with enzyme was that enzyme was much less electron dense than the substrate gold in their study.

Higher magnifications reveal that surface is comprised of particles with 10nm size with very sharp particle size distribution. EDX analysis verified the presence of Ti and Si. However, no proof was evidenced for gold (Au) because of high instrumental background (Appendix E).

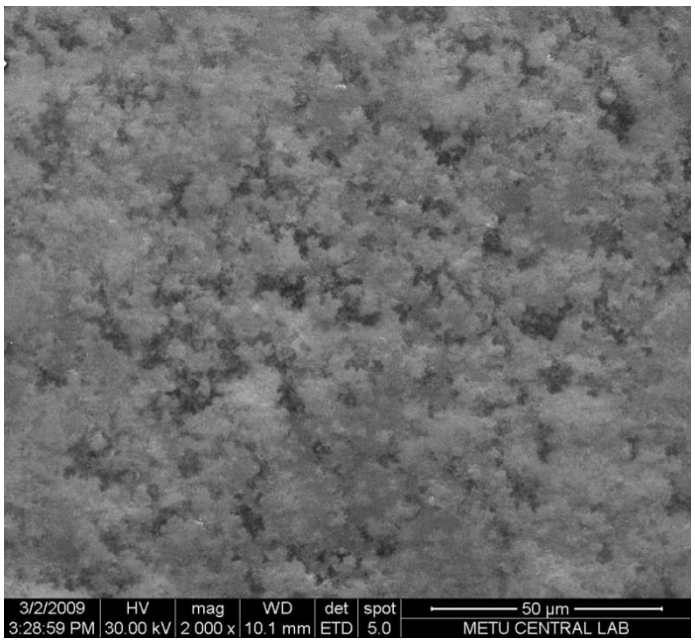


Figure 4.4. SEM micrograph of TiO_2-SiO_2-Au thin film

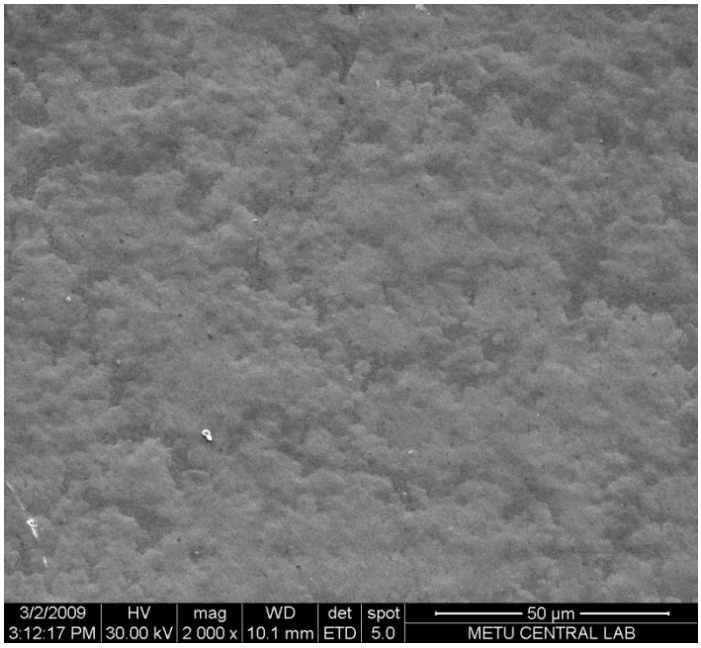


Figure 4.5. SEM micrograph of enzyme immobilized TiO_2-SiO_2-Au-Cys thin film surface

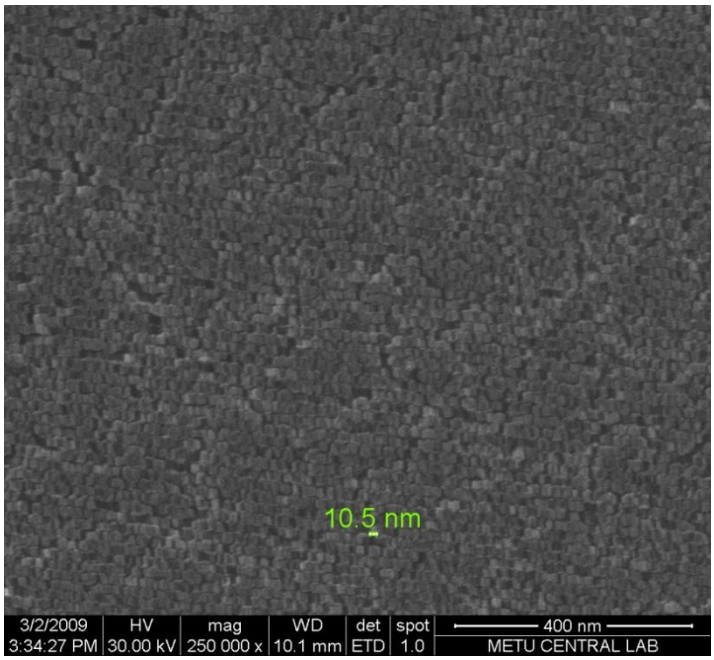


Figure 4.6. SEM micrograph of TiO_2-SiO_2-Au thin film at 250000 magnification

In fact, such kind of a sol-gel material should be analyzed in detail by using X-ray, FTIR, BET and porosimetry. However, synthesized thin films were about 800 nm in this study with SEM results (Koç, 2009) and it is not possible to characterize such thin films with these kinds of techniques. FTIR was tried but, it was unsuccessful. Analysis can be done with powders synthesized with the same sol-gel, but it will not give equivalent results. Thus, no further study was done on this.

4.3. Enzyme immobilization on Pyrex and ITO glasses

The adhesion of the colloidal solution depends on the wetting properties of the surface. The amount and success of enzyme immobilization on the surface is directly related with the thickness and porosity of the resulting film. ITO glass surface is more hydrophobic than the Pyrex glasses and contact angle measurements with water over substrate surfaces are presented in Table 4.1. As it is clearly seen from Table 4.1, Stock Pyrex glasses are much hydrophilic than the stock ITO coated substrates. However, the wetting properties of ITO surfaces enhanced with cleaning pretreatments with acetone and isopropyl alcohol solutions significantly. Similarly, the Pyrex glasses become slightly more hydrophobic after etching and cleaning with KOH and ethanol solutions. Davenas *et al.* (2008) performed wettability studies of ITO substrates to examine the surface properties and found that elimination of organic contaminants upon the cleaning treatment (ultrasonic bath in organic solvents) lead to an increase of the free energy of the ITO surface.

Table 4.1. Contact angle measurement of not cleaned and cleaned Pyrex and ITO glasses

Not Cleaned		Cleaned	
Pyrex	ITO	Pyrex	ITO
35°	92°	44°	51°

Pyrex and ITO glass substrates were coated with three successive layers of TiO_2-SiO_2-Au sol-gel by using dip coating method and calcinated at 500 °C for 15 min under air flow. The both sides of the glass substrates are coated with the dip

coating technique and in order to make a better comparison with the Pyrex substrates, the bare side of ITO coated glass substrates are masked with paraffin. The Surface morphologies of Pyrex and ITO substrates were obtained by SEM at 15 kV and 1000 magnification. Figure 4.7 and Figure 4.8 show that both substrates were successfully coated with a homogenous layer of TiO_2-SiO_2-Au sol-gel. The thin film was well-adhered to the Pyrex and ITO glasses and similar surface morphologies were observed.

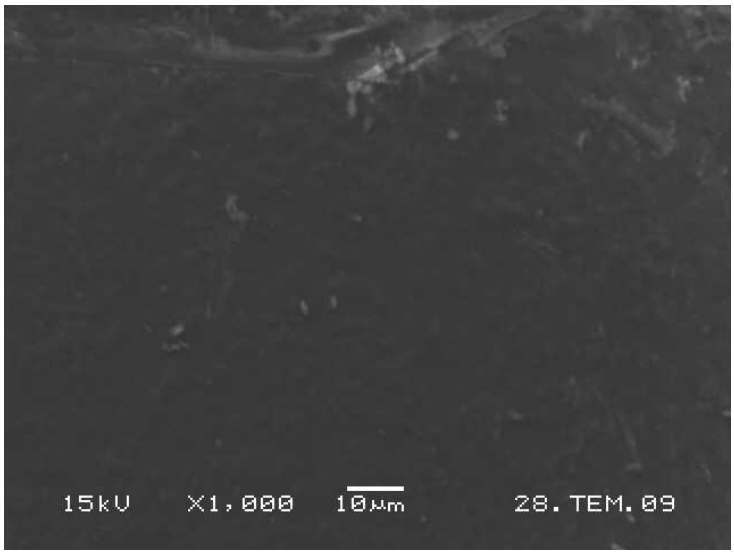


Figure 4.7. SEM image of Pyrex glass coated with TiO_2-SiO_2-Au sol-gel

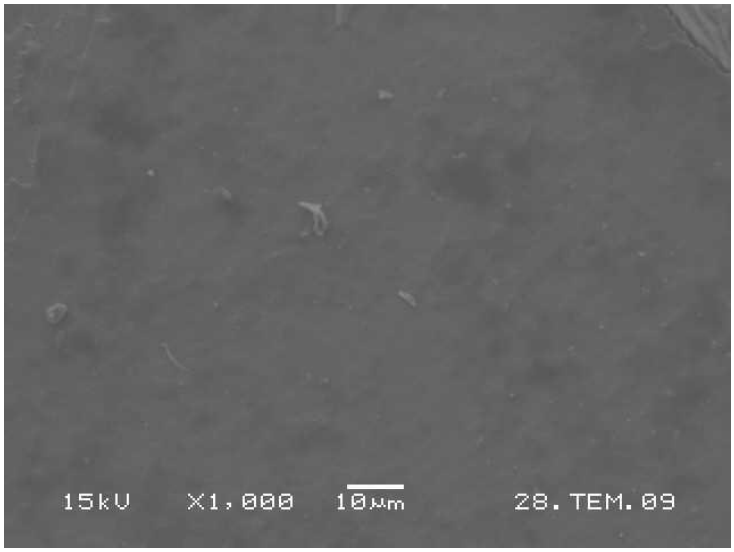


Figure 4.8. SEM image of ITO glass coated with TiO_2-SiO_2-Au sol-gel

Both the model enzyme invertase and laccase were immobilized on Pyrex glass and ITO substrates and their enzymatic activities were tested. In this study, the enzyme immobilization steps and enzymatic activity tests were carried out under dark conditions in order to prevent any photocatalytic reaction. (The samples with same composition TiO_2 were prepared and their photocatalytic activities were studied separately (Çınar, 2009)). Reaction progress curves of free invertase and laccase are given in Figure 4.9 and Figure 4.10 respectively. Reaction progress curves of immobilized enzymes are given in Figure 4.11 – 4.14. Invertase was immobilized at room temperature by dipping in enzyme solution for 12 h in dark and laccase was immobilized also in dark at 4°C by dipping in enzyme solution for 2 h. It was verified that the enzymes that catalyze the hydrolysis reaction (invertase) of sucrose and oxidation reaction (laccase) of ABTS were successively immobilized over the surface and reaction progress is continuous.

The reaction of invertase converts sucrose to glucose and fructose and laccase oxidizes ABTS to $ABTS^{++}$ as shown in Equations 4.1 and 4.2;

sucrose → beta-D-fructose + alpha-D-glucose (Yun *et al.,* 2007) (4.1)

$4ABTS^{2-} + O_2 \rightarrow 4ABTS^{+\bullet} + 2H_2O$ (Nogala *et al.,* 2008) (4.2)

The enzyme leakage (leaching of enzyme into solution) is very important concern and tested by measuring the enzymatic activity. The stability of enzyme on the thin film samples were tested and during the reaction, the enzyme coated substrate was withdrawn and the activity measurements was continued. As it is shown in the Figure 4.11 and 4.12, the enzymatic activity of invertase is vanished after the withdraw of the Pyrex and ITO coated substrates. These results indicate that the invertase is successfully immobilized over SiO_2-TiO_2-Au-Cys thin films.

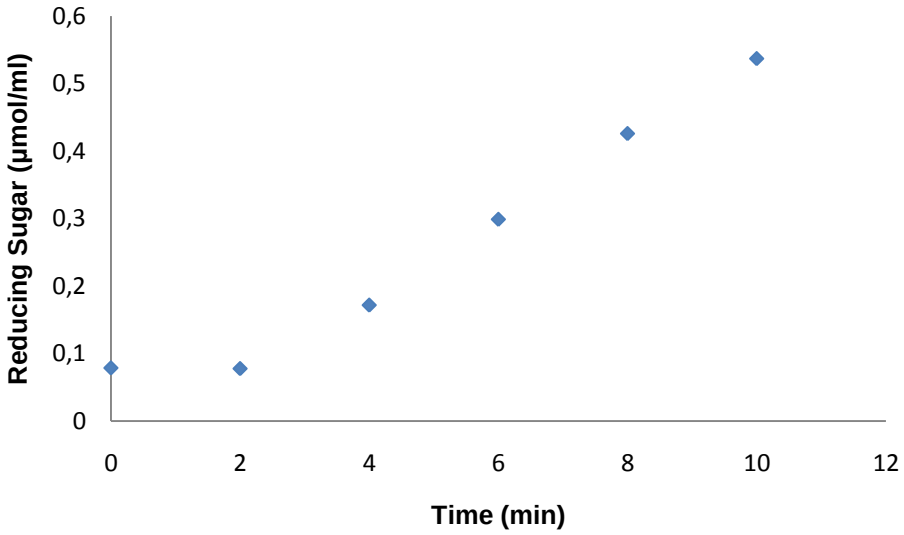


Figure 4.9. Reaction progress curve of free invertase (at 40°C, pH 5.0, 0.145 M sucrose, 2µg/ml)

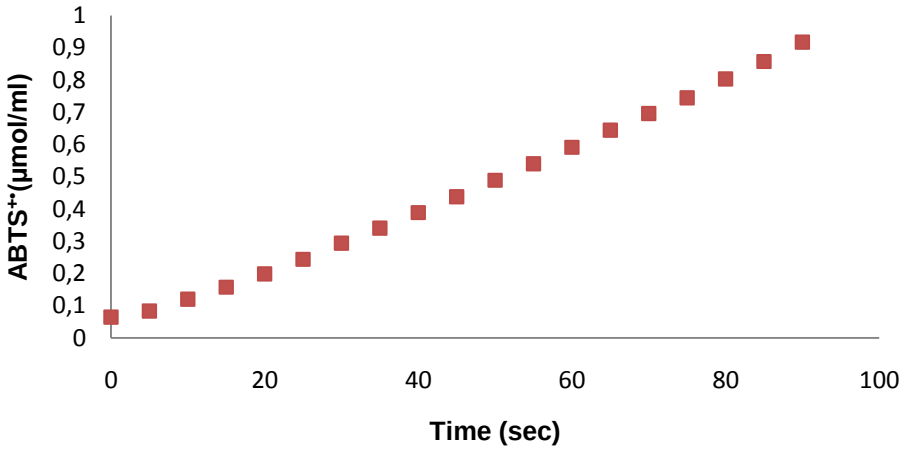


Figure 4.10. Reaction progress curve of free laccase (at 40°C, pH 5.0, 0.5 mM ABTS, 45 µg/ml laccase)

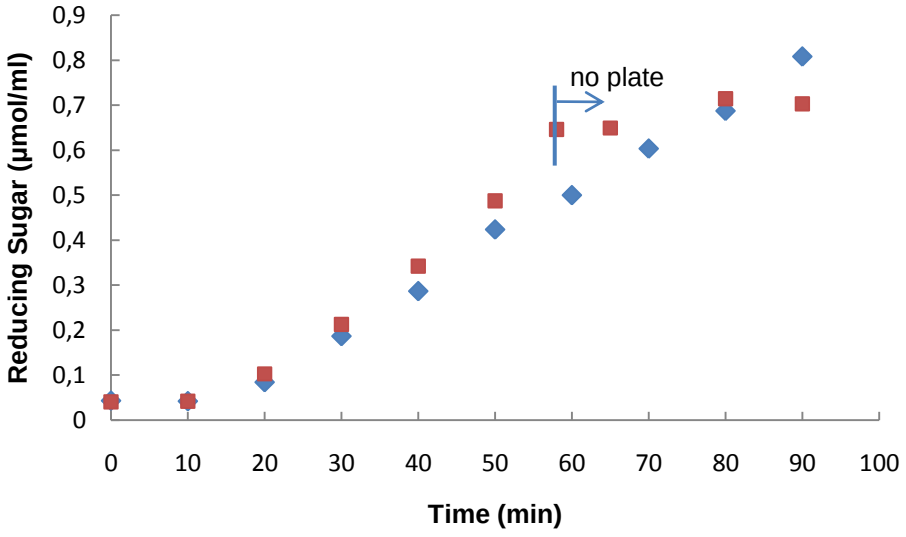


Figure 4.11. Reaction curve catalyzed by invertase immobilized on conducting side of ITO glass in pairs (2.5x4 cm) (at 40°C, pH 5.0, 0.145 M sucrose)

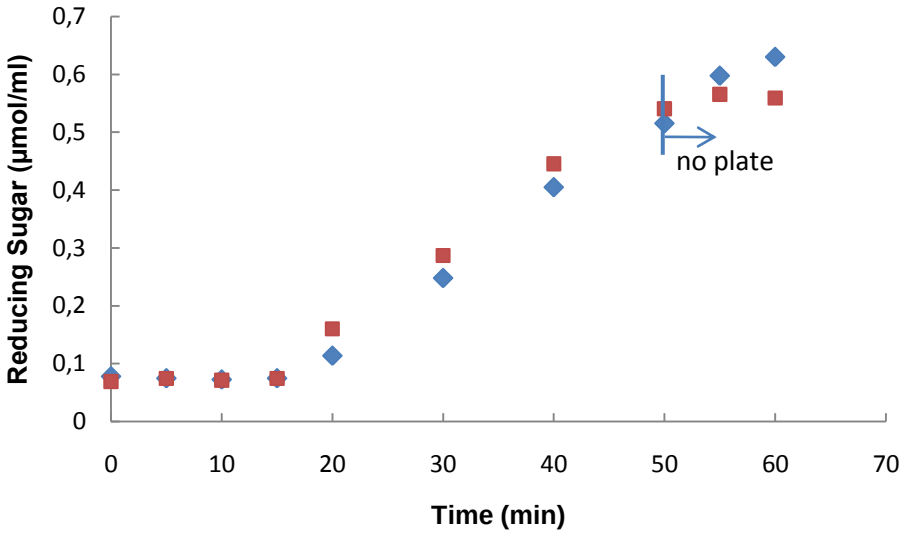


Figure 4.12. Reaction curve catalyzed by invertase immobilized on two sides of Pyrex glass in pairs (2.5x6 cm) (at 40°C, pH 5.0, 0.145 M sucrose)

Similarly, the same experiments were performed with the Pyrex and ITO coated substrates to test laccase activity. As seen in Figure 4.13 and Figure 4.14, experiment showed that the reaction continued with smaller rate when there was no glass substrate in the reaction medium. However, reaction rate increased again when the substrate was resumed into the reaction medium. The partial decrease in activity might be explained by two possibilities. The first possibility is the the autocatalytic reaction of the produced radicals in solution after the withdraw of the sample and the second as more undesirable case is the leakage of enzyme to the solution. These possibilities were further tested.

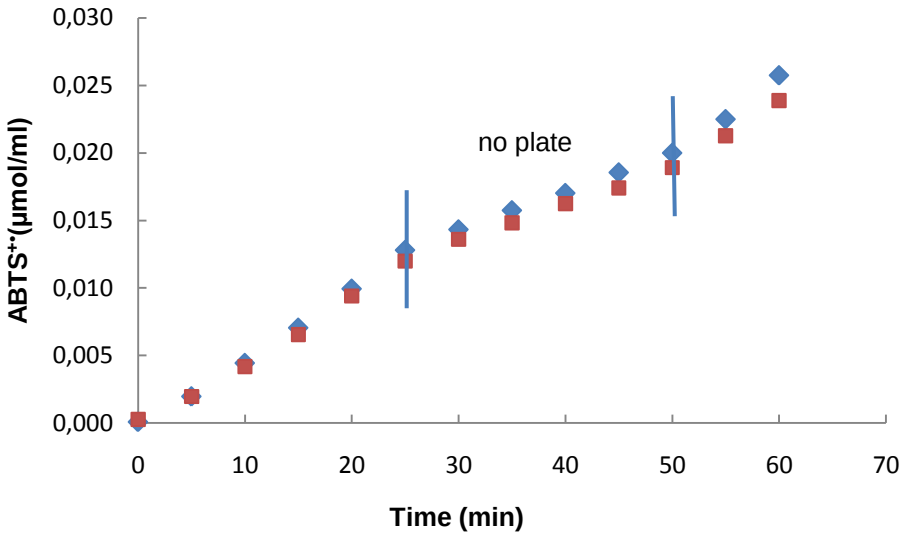


Figure 4.13. Reaction curve catalyzed by laccase immobilized on conducting side of ITO glass in pairs (2.5x4 cm) (at 40°C, pH 5.0, 0.5 mM ABTS)

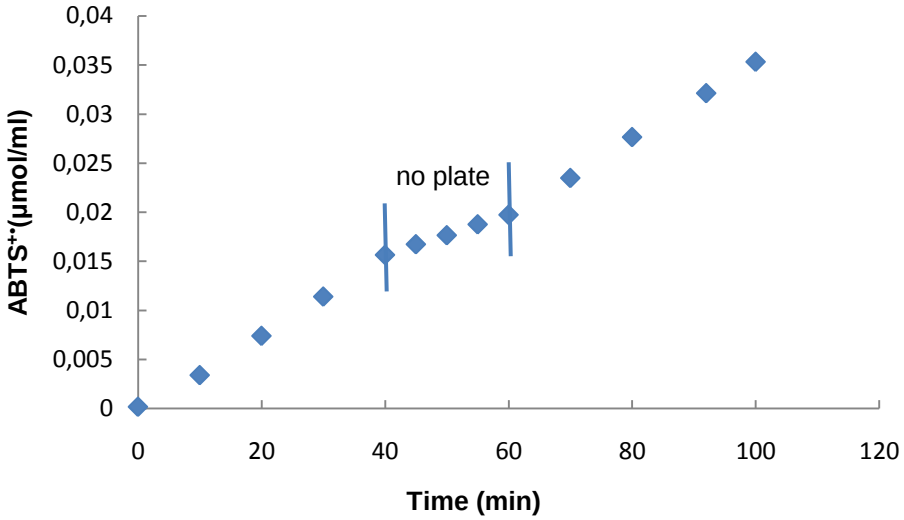


Figure 4.14. Reaction curve catalyzed by laccase immobilized on two sides of Pyrex glass (2.5x4 cm) (at 40°C, pH 5.0, 0.5 mM ABTS)

The activity tests were also analyzed kinetically and the enzymatic reaction rates of the samples were calculated. As it is seen from Table 4.2, the invertase reaction

is much faster than the laccase. Since laccase and invertase are different enzymes, their characteristics such as molecular weight, structure, activity values are different from each other, comparison should not be made between these two. Extracellular invertase from *Saccharomyces Cerevisiae* has molecular weight 80-300 kDa while laccase from *Trametes Pubescens* has a max 65 kDa. When the invertase and laccase activities were compared over the Pyrex and ITO substrates, it is clearly seen that both enzyme has more activity over the ITO substrate in spite of only one side of ITO substrates were coated. This might be explained by the higher amount of immobilization over the ITO surface, higher surface area or porosity.

Table 4.2. Invertase and laccase activities immobilized on Pyrex and ITO glasses (one side is 2.5x4 cm)

Immobilized Invertase Activity (U/plate)*		Immobilized Laccase Activity (U/plate)**	
Pyrex	ITO (one side)	Pyrex	ITO (one side)
0.43 ± 0.02	0.56 ± 0.03	0.039 ± 0.002	0.049 ± 0.002

* One unit of invertase activity (U) was defined as amount of enzyme required for hydrolyze 1 µmole sucrose per min under the reaction conditions (40°C, pH 5.0).
** One unit of laccase activity (U) was defined as amount of enzyme required for oxidize 1 µmole ABTS per min under operating condition (40°C, pH 5.0).

In order to elucidate the possibilities of autocatalytic reaction during the withdrwal of substrate or enzyme leakage to a solution, another set of experiment was conducted. For this purpose, two cycles of reactions were carried out consecutively with the same enzyme immobilized glass substrate. The test substrates were prepared by the immobilization of 0.36 mg/ml laccase at 4°C for 2 hours. As it is shown in Figure 4.16, during the first reaction cycle, the enzyme immobilized substrates were withdrawn and the reaction progress was followed by activity measurements. It was observed that, the reaction proceeds with %68 loss of activity in the absence of substrate compared to reaction with substrate. In the second cycle, the same substrate was used again and when it is withdrawn, no

activity was detected. This fact clearly revealed that the presence of activity followed after the withdrawal of substrate is because of the leakage of enzyme to the reaction medium other than the autocatalytic activity.

The similar trends were also observed with the samples immobilized at room temperature and ITO substrates immobilized at 4°C (Figure 4.15 - 4.17). The results of these experiments are illustrated in Table 4.3. In a sense, consecutive reaction cycles give information about operational stability. As it is seen, there is about 45 percent decrease in second cycle that means low operational stability of immobilized laccase.

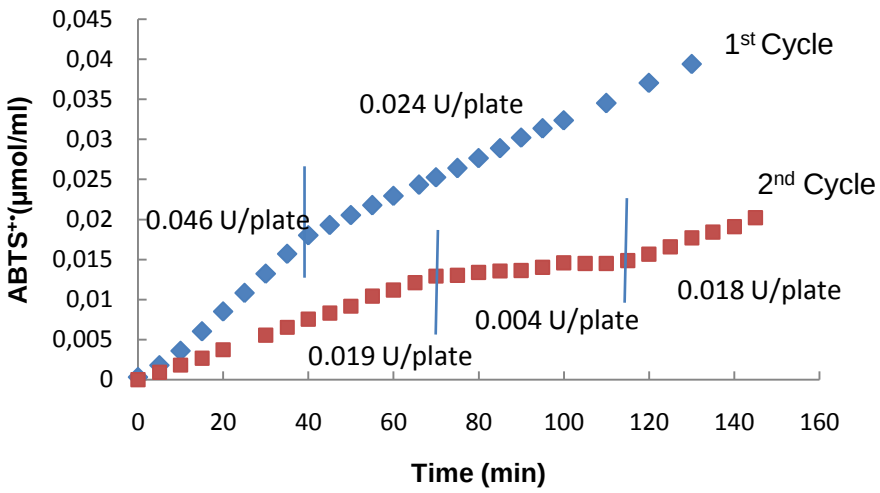


Figure 4.15. Two consecutive reactions of laccase immobilized on Pyrex glass at room temperature for 2 h in 0.36 mg/ml enzyme solution (40°C, pH 5.0, 0.5 mM ABTS)

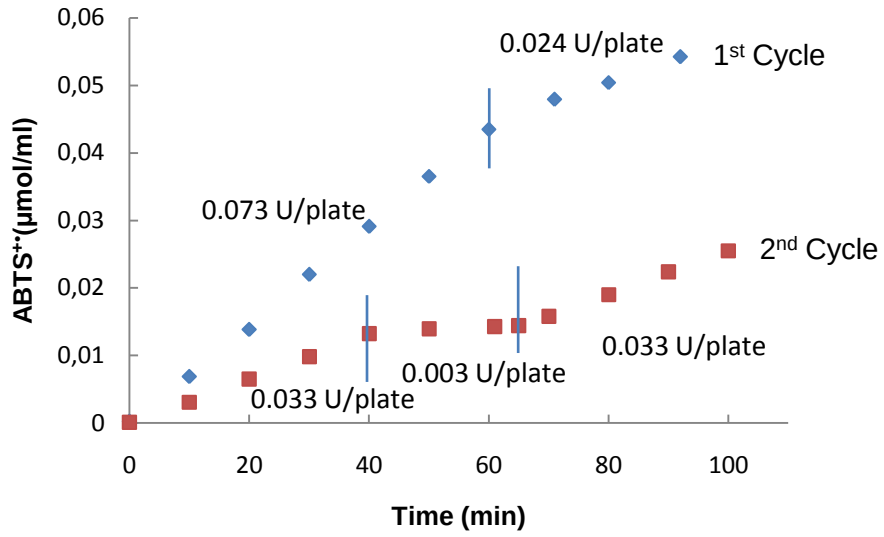


Figure 4.16.Two consecutive reactions of laccase immobilized on Pyrex glass at 4°C for 2 h in 0.36 mg/ml enzyme solution (40°C, pH 5.0, 0.5 mM ABTS)

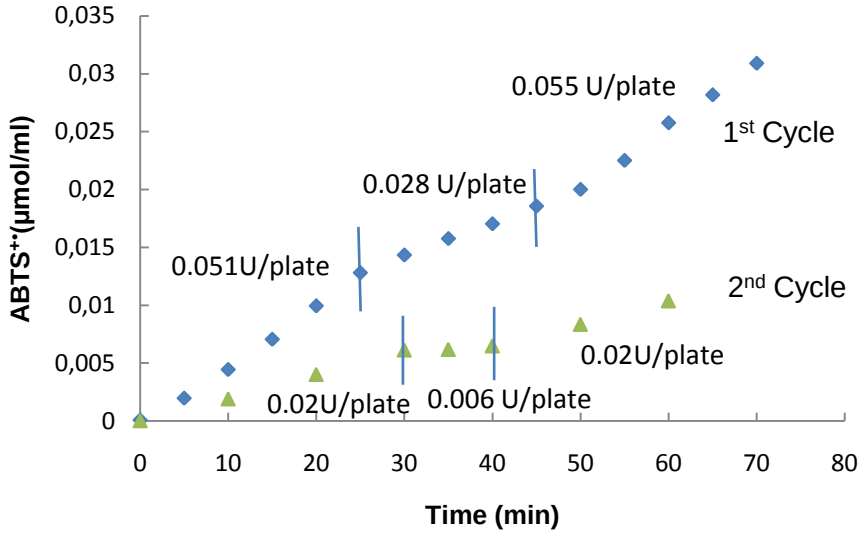


Figure 4.17. Two consecutive reactions of laccase immobilized on ITO glass at 4°C for 2 h in 0.36 mg/ml enzyme solution (40°C, pH 5.0, 0.5 mM ABTS)

Table 4.3. Immobilized Laccase Activities in leakage experiment

Immobilized laccase activities (U/plate)									
	Pyrex glass R.T.			Pyrex glass 4°C			ITO 4°C		
Cycle	with glass	without glass	with glass	with glass	without glass	with glass	with glass	without glass	With Glass
1st	0.046	0.024		0.073	0.024		0.051	0.028	0.055
2nd	0.019	0.004	0.018	0.033	0.003	0.033	0.02	0.006	0.02
Decrease	41 %			45%			40%		

4.4. Optimization of Immobilization Conditions

The results of enzymatic activities showed that the methods applied were successful for both model enzyme invertase and laccase. However more detailed studies were continued with laccase for the production of active surface to detect phenolics by measuring electrochemical signals. In this part of the study, the enzymatic activities were tested by using different immobilization parameters. Immobilization temperature, immobilization enzyme solution concentration and immobilization time were some of the parameters that were studied for this purpose.

4.4.1. Immobilization Temperature

Laccases can be purified from various sources. However the specific activity, stability and working conditions change drastically depending on the source and method. Vianello *et al.* (2004) reported specific activities of laccases from different sources showing that there was even two orders of magnitude specific activity change between them. In another study, Koschorreck *et al.,* (2008) cloned and expressed different genes of laccase and determined specific activities, optimum pH and temperatures and thermostabilities differing in a wide range.

In the present study commercially obtained laccase was identified in terms of thermostability with respect to time as indicated in Figure 4.18. For this purpose, the enzymatic activity of free laccase was tested at room temperature, 4°C and 40°C in solution for 4.5 h. As it is seen from Figure 4.18, the laccase activity decreases with respect to time at all temperatures tested because of the denaturation. The activity loss was more pronounced with increasing temperature, and the most decrease in activity was observed at 40°C as expected.

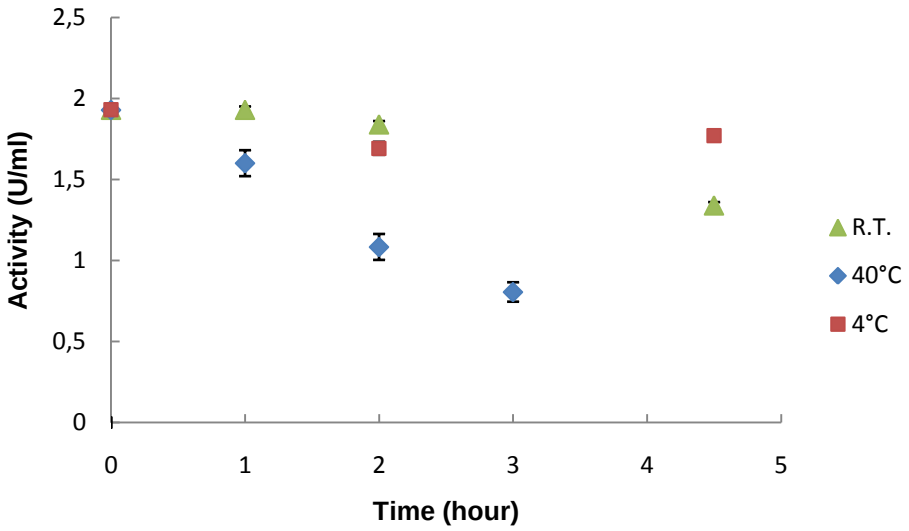


Figure 4.18. Thermal stability of free laccase at different temperatures

In the light of the results of preliminary experiments, the laccase immobilization tests were performed at 4°C and room temperature. For this purpose, commercially obtained laccase was diluted 50 times which corresponds to a concentration of 0.36 mg/ml. The laccase concentration was determined by using Bradford method (Appendix C). Enzyme immobilization was carried out by dipping cysteamine modified TiO_2-SiO_2-Au thin film coated Pyrex glass substrates at room temperature and 4°C for a 2 h period. During the immobilization, in order to enhance the mass transfer conditions, the enzyme solution stirred by using magnetic stirrer. The reaction curve catalyzed by TiO_2-SiO_2-Au-Cys-Laccase thin films immobilized over Pyrex glass at 4°C and room temperature were presented

in Figure 4.19. As it can be seen from the Figure 4.19, the enzyme immobilization at 4°C results better activity than the one at room temperature. The laccase activities of the thin films were determined as 0.073 ± 0.003 U/plate and 0.041 ± 0.003 U/plate for 4°C and room temperature respectively. The temperature effect is much more pronounced in immobilized laccase activity when the results were compared with the free enzyme activities. The limited activity of immobilized enzyme at room temperature might be attributed to the partial denaturation of enzyme and equilibrium kinetics between the surface and the enzyme molecules (Equation 4.3).

$$\text{Au-Cys} + \text{Lac} \leftrightarrow \text{Au-Cys-Lac} \qquad (4.3)$$

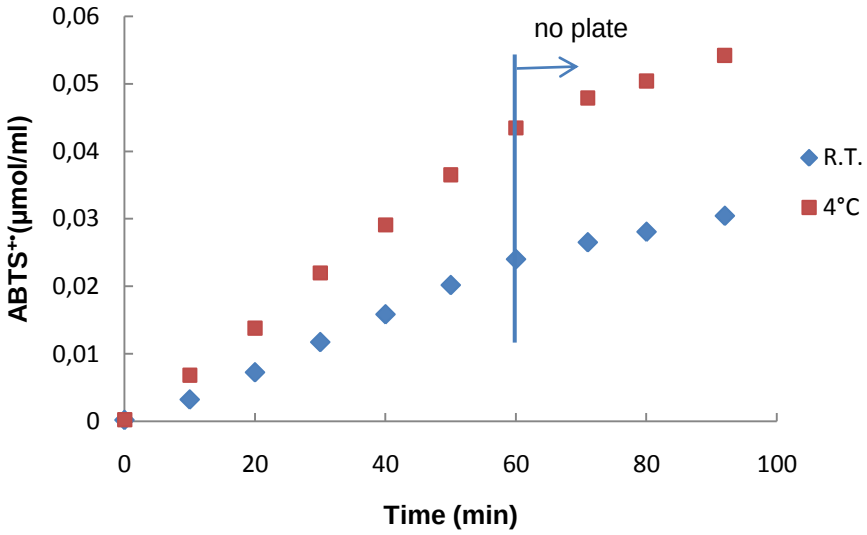


Figure 4.19. Reaction curve catalyzed by laccase immobilized on Pyrex glasses at room temperature and 4°C (pH 5.0, 0.5 mM ABTS)

4.4.2. The Effect of Enzyme Solution Concentration in Immobilization

The effect of solution concentration on laccase activity was studied by using different laccase solutions. When the laccase immobilization kinetics considered, the higher laccase concentration supposed to yield thin films with higher enzyme

loadings. However, the hindrance effects, mass transfer limitations are the other effects that have to be considered.

Laccase was immobilized on TiO_2-SiO_2-Au-Cys thin film coated Pyrex glass at different immobilization concentrations in the range of 2.5 – 900 µg/ml. Immobilization solutions were 180 ml and Pyrex glass substrates were dipped for 2 h in dark at 4°C and the solution stirred by magnetic stirrer. As it is shown in Figure 4.20, steep increase in activity was observed with thin films coated with laccase solution with low concentration. Further increase in laccase solution concentration does not yield thin films with better activity. It has been verified in various enzyme immobilization studies that there is an optimum loading where further increase in concentration do not yield higher activity. Salis *et al.* (2009) reported the optimum enzymatic activity of laccase over SBA-15 as 217 kU $g_{SBA\text{-}15}^{-1}$ and the loss of activity at higher loadings. Campuzano *et al.* (2002) obtained an optimum enzyme loading after which response of glucose oxidase electrode response does not change. Gao *et al*. (2005) also found that increasing lipase loading resulted in faster formation of alkyl esters but further enzyme loadings lead to slower rate. The higher activity of lower loadings might be explained as the good dispersion of laccase over the active sites and the contrary formation of clumpy overcrowded surface with higher loadings. Salis *et al.,* (2003) explained that the decrease in the activity at high enzyme loadings indicated that the system was subjected to mass transfer limitations and the chemical reaction may have been limited by the mass transfer of the reagents towards the surface of the catalyst by internal diffusion. Similarly, in this study, full coverage was achieved even with 2.5 µg/ml immobilization enzyme concentration and enzyme activity was nearly same up to 100 µg/ml enzyme concentration. Higher loadings resulted in decrease in the immobilized laccase activity. The reasons may be surface pore plugging, substrate diffusion limitation to/from active site of the enzyme because of high packing, steric hindrance of enzyme molecules and binding of enzyme in wrong orientation.

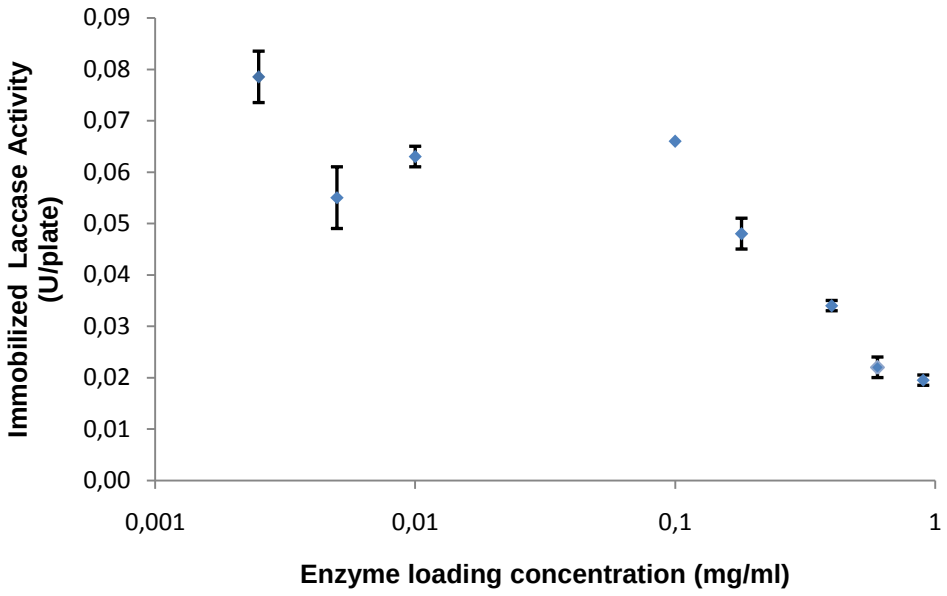


Figure 4.20. Effect of immobilization enzyme solution concentration on laccase activity

4.4.3. Immobilization Time

Enzyme immobilization time was examined to test the effect of impregnation time on enzymatic activity. For this purpose, TiO_2-SiO_2-Au-Cys samples were impregnated with 180 ml of 0.1 mg/ml laccase solution at 4°C and kept in solution between 30 mins to 12 hours. Immobilization time up to 4 h resulted in almost the same activities of laccase and then activity decreased by further treatment as shown in Figure 4.21. According to Jiang *et al*. (2005), longer treatment of magnetic chitosan microspheres with laccase results with lower accessibility of the substrates to the active sites. Cordek *et al*. (1999) commented that deposition of additional enzyme layer could be one of the reasons for the decline of glutamate dehydrogenase activity in their system after 25 h enzyme immobilization. So, decrease in the activity for higher incubation periods might be attributed to high enzyme loading causing limited substrate diffusion towards the enzyme molecules, steric effects and binding in wrong orientation as in the case with previous section.

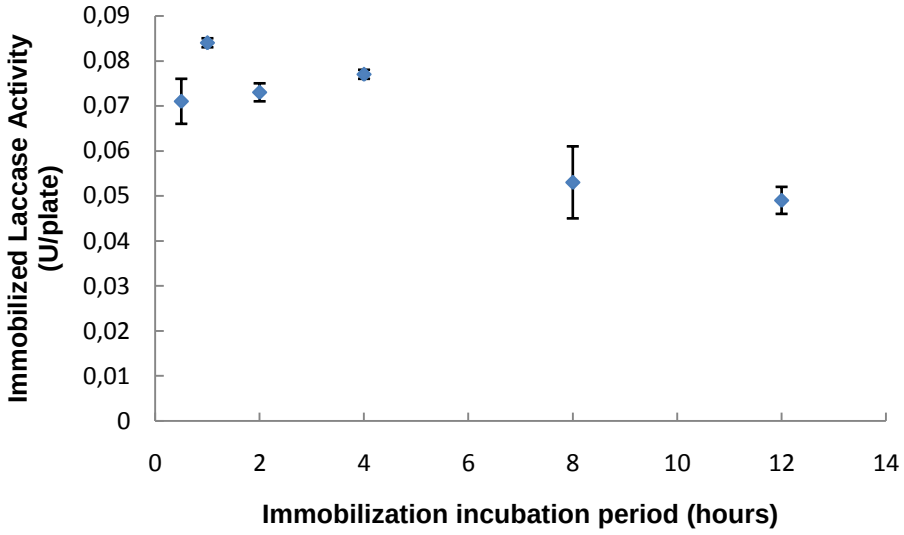


Figure 4.21. Effect of immobilization incubation period on laccase immobilization on TiO_2-SiO_2-Au-Cys thin film

4.5. Storage Stability

Storage conditions and stability are important criteria and have to be characterized carefully. Storage temperature and dry or wet storage were taken into consideration with that respect. Laccase immobilized on TiO_2-SiO_2-Au-Cys thin film coated Pyrex glasses were stored at 4°C in accordance with the result in Section 3.3.1 that free enzyme retained its activity at 4°C. In addition, there are several studies that immobilized laccase should be stored at 4°C (Vianello *et al.,* 2004; Qiu *et al.,* 2008; Rahman *et al.,* 2008; Cracknell *et al.,* 2008; Leontievsky *et al.,* 2001; Jiang *et al.,* 2005).

Immobilized laccase was stored either in 0.1 M pH 5.0 sodium acetate buffer or in air. Storage stability was analyzed with immobilized enzyme on Pyrex glasses. In one experiment, enzyme was immobilized on four glass substrates for 2 h at room temperature in 0.36 mg/ml immobilization enzyme solution. Two of them were used for measuring fresh immobilized enzyme activity while other two were stored at 4°C for 16 in buffer and in air. Immobilized enzyme activites were determined as 0.046 U/plate, 0.029 U/plate and 0.027 U/plate for fresh (as prepared), 16 h storage in buffer and 16 h storage in air respectively. In another experiment,

enzyme was immobilized on a pair of glass substrates for 2 h at 4°C in 0.1 mg/ml immobilization enzyme solution and then the same glasses were stored at 4°C for 6 days, in buffer and in air. Immobilized enzyme activity was 0.066 U/plate, 0.0047 U/plate and 0.0039 U/plate for fresh use, 6 day storage in buffer and 6 day storage in air respectively. As a result of these experiments, activity of immobilized laccase almost vanished after 6 days. Storage of immobilized laccase in buffer or air did not have significant difference with respect to store stability but, storing in air is advantageous for easy use and low cost.

CHAPTER 5

CONCLUSIONS

In this study, invertase (model enzyme) and laccase were immobilized covalently on Pyrex and ITO glass substrates which were previously coated with porous TiO_2-SiO_2-Au thin film and modified with cysteamine. Both enzymes had more activity on ITO substrate. Immobilization of main enzyme laccase was examined deeply.

Laccase immobilization at 4^oC resulted better activity than the one at room temperature. Consecutive reaction experiments showed there was leakage into the reaction medium. About 55 % decrease in laccase activity was observed in two consecutive reactions which might be ascribed to leaching of physically adsorbed enzyme through the solution or unstability of enzyme in immobilized form.

The laccase immobilization experiments showed that the enzyme activity of the substrates does not depend on the impregnation concentration during deposition however, the use of concentration enzyme solution higher than 50 µg/ml cause the loss of activity which might be attributed by accessibility, contamination and pore mouth plugging. Immobilization time up to 4 h resulted in almost the same activities of laccase and then activity decreased by further treatment. The reason might be again overcrowding of the surface by enzyme. Immobilized laccase activity almost vanished after 6 days storage. Storage of immobilized laccase in buffer or air did not have significant difference with respect to store stability but, storing in air is preferable because of easy use.

In the lights of these facts, the surface structure used is not suitable for laccase immobilized sensor materials because of low stability and leakage. Increasing gold amount or increasing porosity might enhance enzyme immobilization resulting in higher activity but, focusing on covalent linking with other functional groups or

immobilization technique might be more reasonable for producing stable and electrochemically active surface with laccase.

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APPENDIX A

SPECIFICATIONS OF LUDOX SM-30

Product Name	LUDOX® SM-30 colloidal silica, 30 wt. % suspension in H_2O
Product Number	420794
Product Brand	ALDRICH
CAS Number	7631-86-9
Molecular Weight	60.08

TEST	SPECIFICATION
Appearance (Color)	Cloudy White
Appearance (Form)	Liquid
ICP: Confirms Silicon Component	Confirmed
Specific Gravity	1.209 - 1.227 at 60 Degrees Fahrenheit
pH	9.7 - 10.3 at 25 Degrees Celsius
Viscosity	4.8 - 6.8 cps at 25 Degrees Celsius
Silica	29.0 - 31.0 %
Ratio of SiO2/Na2O	45 - 56
Surface Area (m2/g)	320 - 400
Sulfate (as Na2SO4)	≤0.06 %
Transmittance	≥74 %
Vendor Information	Confirmed Product of Grace Davison

APPENDIX B

PREPARATION OF DNSA REAGENT

Chemicals used for preparing DNSA reagent are given in Table 1.

Table B.1. Chemicals used in DNS reagent in percentage

Chemicals	% (w/v)	Weight (g) (for 500 ml)
3.5 dinitrosalyclic acid	1	5
NaOH	1	5
Sodium sulfite	0.05	0.25
Phenol	0.2	1
Na-K-tartarate	36.25	181.25

Na-K-tartarate was dissolved in 300 ml distilled water (A) and 3.5 dinitrosalyclic acid was dissolved in distilled water in dark (B) separately. B was mixed with A and dissolve under stirring in dark. NaOH was added slowly. Finally, sodium sulfite and phenol was added and the volume was 500 ml adding by water . The mixture was stirred for about 2.5 - 3 h in order to dissolve totaly and stored in dark brown bottle.

APPENDIX C

BRADFORD METHOD

The assay is performed in test tubes. 0.1 ml of the protein sample and 3 ml of Bradford Reagent are mixed as follows. Bradford Reagent is brought to room temperature. Protein Standard and sample are prepared by diluting 2 mg/ml or 1 mg/ml BSA protein standard as seen in Table 2. After mixing 3 ml Bradford Reagent with samples, they are vortexed immediately. Samples are incubated at room temperature for 10 mins. Brillant blue and protein binding gives an absorbance at 595 nm. Absorbances of samples are measured at 595 nm and protein concentration of unknown sample is determined by using the standard curve.

Table C.1. Preparation of BSA standard and sample tubes

Tube No	BSA Sample (ml)	BSA Standard (mg/ml)	Bradford Reagent (ml)
1	0.1	0	3
2	0.1	0.25	3
3	0.1	0.5	3
4	0.1	1	3
5	0.1	1.4	3
6	0.1	unknown	3

APPENDIX D

BSA STANDARD CURVE

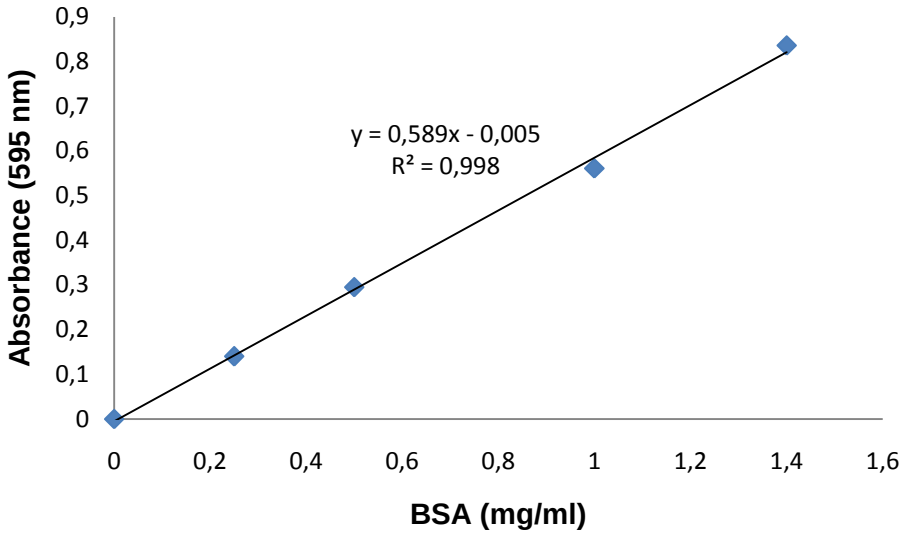

Figure D.1. BSA standard is prepared in the range from 0.1-1.4 mg/ml

APPENDIX E

EDX SPECTRUM OF TiO_2-SiO_2-Au THIN FILM

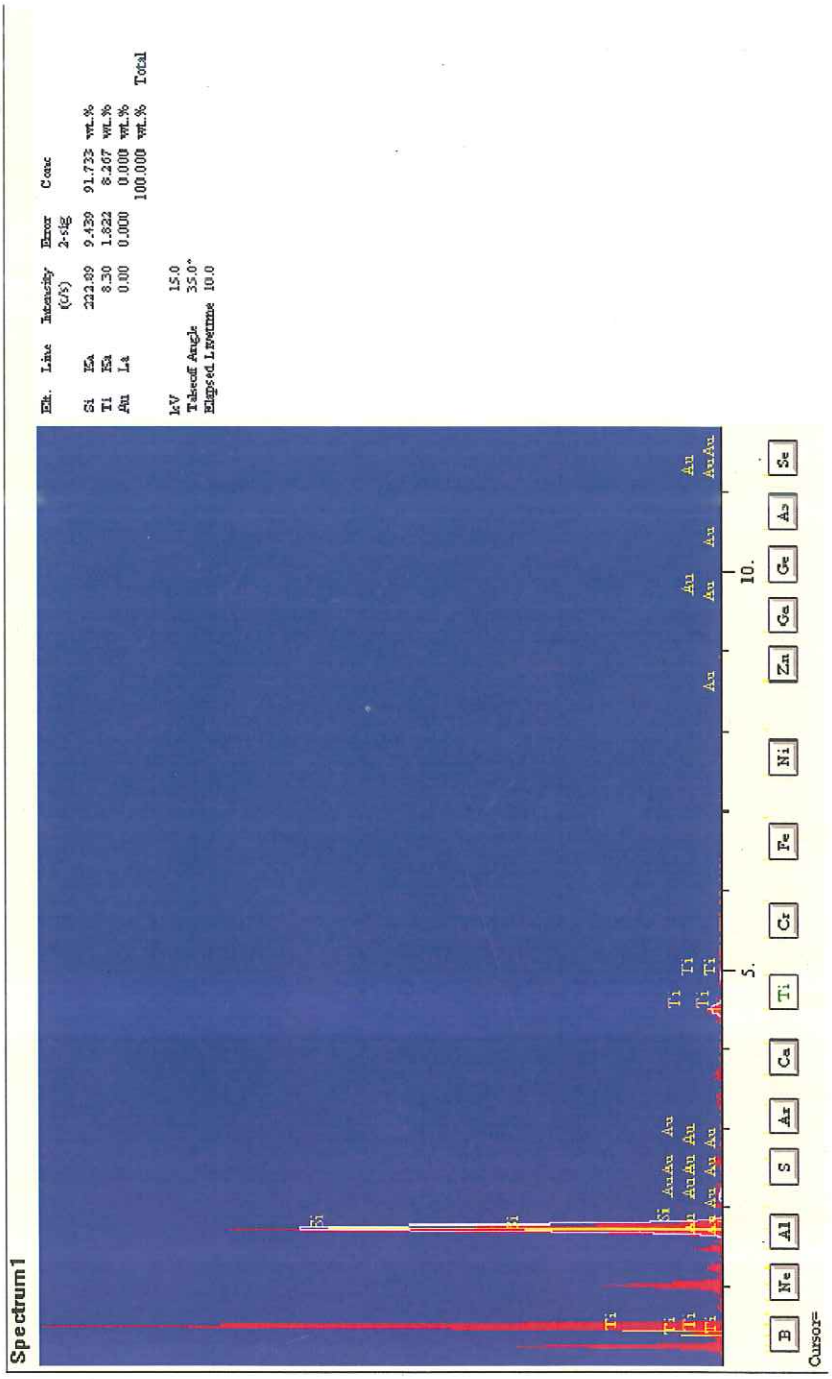

Figure E.1. EDX Spectrum of TiO_2-SiO_2-Au Thin Film