ENZYME IMMOBILIZATION ON TITANIA-SILICA-GOLD THIN FILMS FOR BIOSENSOR APPLICATIONS AND PHOTOCATALYTIC ENZYME REMOVAL FOR SURFACE PATTERNING

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

MERVE ÇINAR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
CHEMICAL ENGINEERING

SEPTEMBER 2009

Approval of the thesis:

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ABSTRACT

ENZYME IMMOBILIZATION ON TITANIA-SILICA-GOLD THIN FILMS FOR BIOSENSOR APPLICATIONS AND PHOTOCATALYTIC ENZYME REMOVAL FOR SURFACE PATTERNING

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September 2009, 75 pages

The aim of this study was to investigate the viability of patterning by immobilization, photocatalytic removal, and re-immobilization steps of the enzyme on photocatalytically active thin films for biosensor fabrication purposes. For this aim, TiO_2-SiO_2-Au sol-gel colloids were synthesized and deposited on glass substrates as thin films by dip coating. Cysteamine linker was assembled on gold nanoparticles to functionalize thin films with amine groups for immobilization of model enzyme invertase. Effect of immobilization temperature, enzyme concentration of the immobilization solution and immobilization period on invertase immobilization were investigated. The immobilized invertase activity was found independent from the immobilization temperature in the range tested (4^oC-room temperature). The optimum enzyme concentration and period for immobilization was determined as 10µg/ml and 12 hours respectively. The resulting invertase immobilized thin films showed high storage stability retaining more that 50% of their initial activity after 9 weeks of storage.

Photocatalytic enzyme removal and re-immobilization studies were carried out by irradiating the invertase immobilized thin films with blacklight. Upon 30 minutes of irradiation, immobilized invertase was completely and irreversibly inactivated. Initial immobilized invertase activity (before the irradiation) was attained when invertase was re-immobilized on thin films that were irradiated for 5 hours. Thus it was inferred that with sufficient exposure, enzymes can be completely removed from the surfaces which makes the re-immobilization possible. The possibility of enzyme removal with photocatalytic activity and re-immobilization can pave the way to new patterning techniques to produce multi-enzyme electrode arrays.

Keywords: TiO_2, SiO_2, Au, photocatalysis, thin films, sol-gel, patterning, invertase, immobilization, biosensor

ÖZ

BİYOSENSÖR UYGULAMALARI İÇİN TİTANYUM DİOKSİT-SİLİKON DİOKSİT-ALTIN İNCE FİLMLERE ENZİM İMMOBİLİZASYONU VE FOTOKATALİTİK ENZİM UZAKLAŞTIRILMASI İLE YÜZEY DESENLENMESİ

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Eylül 2009, 75 sayfa

Bu çalışmada, biyosensör uygulamaları için fotokatalitik aktif yüzeylere enzim tutuklanması, enzimin fotokatalitik olarak yüzeyden uzaklaştırılması ve tekrar yüzeye tutuklanması ile desenlemenin uygulanabilirliği araştırılmıştır. Bu amaçla, TiO_2-SiO_2-Au sol-jel kolloid çözeltileri cam yüzeylere ince filmler halinde kaplanmıştır. Sentezlenen ince filmler, sisteamin bağlayıcısının altın nanotanecikler üzerinde kendinden düzenlenmesiyle amin fonksiyonelleştirilmiş ve invertaz tutuklanması sağlanmıştır. Tutuklama sıcaklığının, tutuklama çözeltisi enzim konsantrasyonunun ve tutuklama süresinin, tutuklanan invertaz aktivitesine etkisi araştırılmıştır. Tutuklanan invertaz aktivitesinin denenen sıcaklık aralığında (4^oC-oda sıcaklığı) tutuklama sıcaklığından bağımsız olduğu görülmüştür. Optimum enzim konsantrasyonu ve süresi sırasıyla 10µg/ml ve 12 saat olarak bulunmuştur. Invertaz tutuklanmış enzim yüzeyleri 9 hafta bekleme süresinden sonra ilk

aktivitelerinin 50% den fazlasını korumuş ve yüksek saklama kararlılığı göstermiştir.

Fotokatalitik enzim uzaklaştırması ve tekrar tutuklanması çalışmaları, invertaz tutuklanmış yüzeylerin siyah ışığa (368 nm.) tutulması ile yürütülmüştür. 30 dakika ışığa maruz kalan ince filmlere tutuklanmış invertaz tamamen ve geri dönüşümsüz olarak inaktive edilmiştir. Tutuklanan invertazın başlangıç aktivitesine (siyah ışığa maruz kalmadan önce) 5 saat ışığa maruz kalan yüzeylere tekrar enzim tutuklandığında ulaşılmıştır. Böylece ışığa yeterli süreyle maruz kalan enzimlerin tamamen yüzeyden kaldırılabileceği görülmüş ve tekrar enzim tutuklanması sağlanmıştır. Fotokatalitik aktivite ile enzimlerin yüzeylerden uzaklaştırılması ve tekrar yüzeye tutuklanması olasılığı, çoklu enzim elektrot arrayleri yapımında kullanılabilecek yeni bir desenleme tekniğine zemin hazırlamıştır.

Anahtar Kelimeler: TiO_2, SiO_2, Au, fotokataliz, ince film, sol-jel, desenleme, invertaz, tutuklama, biyosensör

To my family,

ACKNOWLEDGEMENTS

First and foremost, I would like to express my deepest and sincere gratitude to my thesis supervisor, Prof. Dr. Ufuk Bakır for her valuable and encouraging guidance and supervision throughout this study.

I am also grateful to my co-supervisor Prof. Dr. Gürkan Karakaş for his guidance. His wide knowledge has been of great value to me.

I would like to thank all my friends in Industrial Biotechnology and Environmental Catalysis Laboratory; Eda Açık, Serpil Apaydın, Erinç Bahçegül, Eda Bayraktar, Bilal Bayram,Umut Çekmez, Zeynep Eker, Beril Korkmaz Erdural, İrfan Ersöz, Aytaç Kocabaş, Didem Sutay Kocabaş, Gizem Seber, Esra Uçkun, Pelin Yetişemiyen and Alp Yürüm. I will always appreciate to be part of a welcoming, friendly, and helpful group. I am especially grateful to Zeynep Eker for her invaluable friendship and cordiality throughout this study.

I want to extend my appreciation to my all friends who made my undergraduate and graduate years in METU enjoyable and memorable contributing directly or indirectly towards making this study possible.

Scientific and Technological Research Council of Turkey (TÜBİTAK) is also gratefully acknowledged for providing financial means throughout this study.

Finally, I would like to express my deepest gratitude to my family for their unconditional love, support, encouragement and patience throughout my life. I have always felt the privilege of having such a family.

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CHAPTER 1

INTRODUCTION

In general terms, sensor is a device that is able to detect a certain substance and produce a measurable signal. When a biological component (which is usually called as bioreceptor) is present in the recognition site, the sensor is called a biosensor. Performance of biosensors can be improved by using nanomaterials in their construction (Jianrong *et al.* 2004). When diffusional effects are also considered for the performance of biosensor, it follows that the thinner the sensing layer is, the less time this will take and thereby, speed and reversibility of sensor response, may well be improved (Davis *et al.* 2005). Sol-gel type chemical solution deposition technique is a highly flexible method for the fabrication of thin films (Schwartz *et al*. 2004) and it is easy to prepare multicomponent structures to introduce desired properties to the supports on which immobilization is achieved. Sol-gel derived TiO_2-SiO_2 thin films can be used for the immobilization of biomolecules due to their biocompatibility and high surface area.

Considerable attention is devoted to immobilization of enzymes. While all biosensors are more or less selective for a particular analyte, enzyme biosensors represent higher selectivity and specificity for their analytes (Thevenot *et al.* 2001). Enzymes can be immobilized by several techniques. The method for immobilization mainly depends on the type of biosensor, nature of the support and enzyme, required stability and reusability. Covalent immobilization has the advantage of stable coupling although being a complex method.

Covalent immobilization of enzymes to conductive or semiconductive supports can be achieved by the availability of functional groups on the surface of the support.

Inherent surface functions, the monolayers assembled or other functional modifications allow covalent coupling. One of the most commonly used coupling chemistry is carbodiimide chemistry where amine functional groups are introduced to support for coupling with carboxyl group of enzymes (Eggins, 2002).

Covalent coupling of enzymes is also necessary for gradient formation in order to achieve control of enzyme location (Vepari *et al.* 2006). Patterns of enzymes receive interest because parallel testing systems and multisensing for several analytes can be reached with biosensor arrays. To control protein patterning on surfaces there are several methods like photolithography and patterning of monolayers (Reichert *et al.* 1998).

Here, model enzyme invertase was immobilized on TiO_2-SiO_2-Au thin films to investigate the possibility of photocatalytic enzyme removal from the thin films, and re-immobilization which can be further defined as a new methodology for patterning enzymes on biosensor active surfaces. TiO_2-SiO_2-Au colloidal solutions synthesized by sol-gel method were introduced as thin films on glass substrates. Prior to immobilization of invertase on thin films, cysteamine was assembled on Au nanoparticles to functionalize the thin films to achieve covalent immobilization. Immobilized invertase was removed from the surface photocatalytically and re-immobilization of invertase to the photocatalytically treated surfaces was achieved.

CHAPTER 2

LITERATURE SURVEY

2.1 Biosensors

Sensor is a device that is able to detect a certain substance and produces a measurable signal. For some applications, target analyte must be recognized when its concentration is very low and the small changes must be discriminated among many interfering species. This means that the sensor must show a remarkable degree of specificity and sensitivity for the analyte and this combination can be only displayed by biological molecules (Perez, 2004). When a biological component is present in the recognition site, the sensor is called a biosensor. The interaction of the analyte with biological component is designed to yield an effect to be measured by the transducer which converts this information into a measurable signal.

Biosensors can be classified with respect to either their bioreceptor or to their transducer types (Figure 2.1). Most common bioreceptors are based on antibody/antigen interactions, nucleic acid interactions, enzymatic interactions and cellular interactions. Transducers can be classified basically as optical measurements, mass-sensitive measurements and electrochemical measurements (potentiometric and amperometric) (Cullum *et al.* 2000).

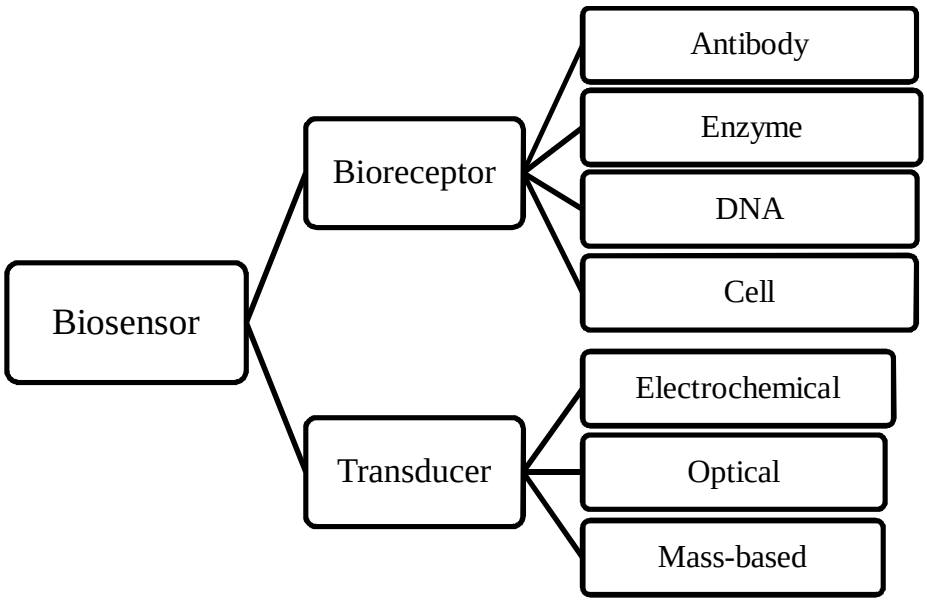


Figure 2.1 Schematics of biosensor classification

2.1.1 Enzyme Biosensors: General Concepts

In the large and expanding area of biosensor technology, great portion of the field is devoted to enzyme biosensors due to their practical applications in clinical diagnostics, analysis of metabolites, process and pollution monitoring. Enzymes have many properties that are advantageous in biosensor applications like

- High specifity for analyte
- Being reusable
- Well-characterized mechanism of action
- Adaptation for conditions with stable forms of enzymes from thermophilic or genetically modified organisms

An enzyme electrode is obtained by immobilizing a thin layer of enzyme. The substrate or substrates which are going to be monitored diffuse into the enzyme layer where the catalytic reaction occurs generating a product and consuming a reactant, which can be detected electrochemically. Depending on the electrochemical property, species are monitored potentiometrically,

conductometrically or amperometrically (Shah *et al.* 2003) and signal can be correlated back to the concentration.

Amperometric biosensors have the advantage of being highly sensitive, rapid, and inexpensive. They monitor a current flow between the working and the reference electrode at a fixed potential. Either reactant or the product must be oxidable or reducible at the electrode surface. In some cases, redox mediators are introduced to allow fast electron transfer reactions (Taylor 1993).

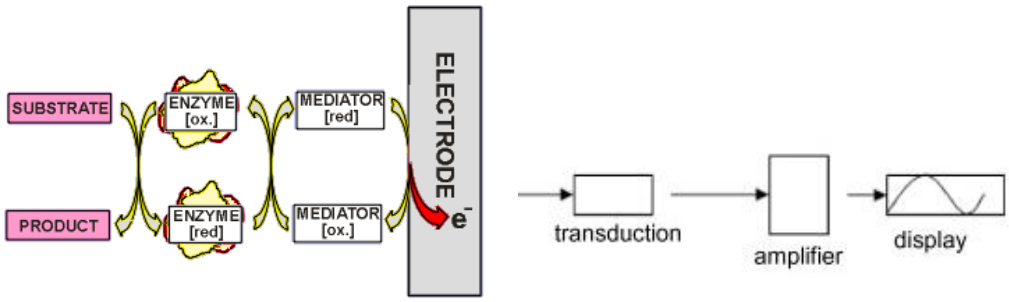


Figure 2.2 A representation of a mediated amperometric biosensor

2.1.2 Application of Nanoparticles in Biosensors

Nanoparticles show unique chemical, physical and electronic properties that are different from those of bulk materials. Many of them of different sizes and compositions are now attainable and can be used to construct improved biosensing devices. Although these nanoparticles play different roles in different sensing systems based on their unique properties, the basic functions can be mainly classified as immobilization of biomolecules, catalysis of electro-chemical reactions, enhancement of electron transfer, labeling biomolecules and acting as reactant. Main functions and advantages of nanoparticles in biosensing area are summarized in Table 2.1.

Table 2.1 Different functions of nanoparticles in sensing (Luo *et al.* 2006)

Functions	Property used	Nanoparticles	Advantages
Biomolecule immobilization	Biocompatibility, large surface area	Metal and oxide nanoparticles (Au,Ag) (SiO_2-TiO_2)	Improved stability
Catalysis of reactions	High surface energy	Metal nanoparticles (Au, Pt)	Improved sensitivity and selectivity
Enhancement of electron transfer	Conductivity, tiny dimensions	Metal and oxide nanopartices (Au,Ag) (ZrO_2-TiO_2)	Improved sensitivity, direct electrochemistry of enzymes
Labeling biomolecules	Small size, modifiability	Semiconductor and metal nanoparticles (CdS, PbS) (Au)	Improved sensitivity, indirect detection
Acting as reactant	Chemical activity	Oxide nanoparticles (MnO_2)	New response mechanism

2.2 Gold Nanoparticles

Gold nanoparticles (AuNP) are the most studied noble metal in biosensors. It is easy to obtain both as a thin film and as a colloid, they are reasonably inert and biomolecules retain their activity when immobilized on AuNPs (Zhang *et. al* 2005). Also their popularity arises from the facts that they can provide direct electron transfer during redox reactions of enzymes and self assembled monolayers can be formed for the immobilization of biomolecules.

2.2.1 Enhancement of electron transfer

Electrical contacting of redox-enzymes with electrodes is important in electrochemical biosensors and direct electrical communication of enzymes with

the electrode is generally a problem due to the thick insulating protein shells blocking the electron transfer (Luo *et al.* 2006). Mostly, nanoscale metal particles enhance this electron transfer. AuNPs can act as tiny conduction centers and are site of electron transfer on the substrate surface allowing direct electron transfer between redox proteins and electrode surfaces without the need of mediators (Kumar, 2007).

In the study of Willner *et al.* (2007), N6-(2-aminoethyl)-flavin adenine dinucleotide functionalized gold nanoparticles assembled on a thiolated monolayer associated with a gold electrode. The resulting enzyme electrode exhibited very fast electron transfer between the enzyme redox center and the electrode due to the gold nanoparticles.

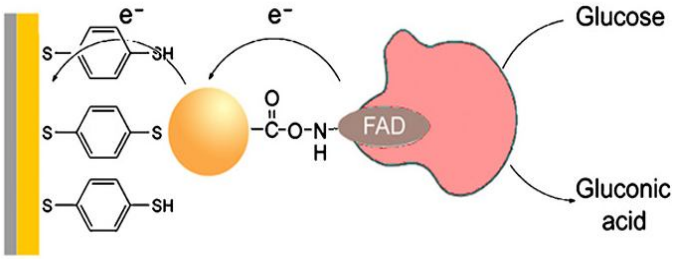


Figure 2.3 Assembly of AuNP-reconstituted GOx electrode (Willner *et al.* 2007)

2.2.2 Self-assembled monolayers

Self-assembly monolayers (SAMs) are used to tailor surfaces with well-defined compositions, structures and thickness that form functionalized surfaces for the immobilization of enzymes. Thiols (R--SH), sulphides (R--S--R) and disulphides (R--S--S--R) all self-assemble on gold. Thiolates depronate upon the adsorption to create strong gold thiolate bonds and under normal conditions they show little tendency to dissociate (Lowe *et al.* 2002).

R--SH + Au → R–S--Au + e-+ H+ (2.1)

Short chain bifunctional molecules like cysteamine (Figure 2.4) can self assemble onto gold nanoparticles for immobilization of enzymes. While the thiol functional group is responsible from the self-assembly, amine functional group takes role in the covalent coupling with carboxyl group of the enzyme.

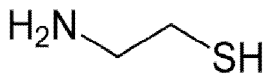


Figure 2.4 Skeleton structure of cysteamine (amine and thiol functional groups)

Thiol carbon chain size influences the immobilization efficiency. Thiols with shorter chains tend to form monolayers with high amount of defects on gold surface resulting in lower coverage whereas longer chains provide more coverage. However, longer chains are not desired for the development of electrochemical biosensors because they passivate the transduction interface, making difficult the electron transfer and, consequently, reducing electrode sensitivity (Mendes *et al.* 2008).

Mendes *et al.* (2008) studied the effects of different self-assembled monolayers on enzyme immobilization for biosensor development. To investigate this effect, monolayers were prepared from thiols with different structures, carbon chain sizes and terminal groups. They showed that $–NH_2$ terminal provided best results for enzyme immobilization on gold surfaces and SAMs formed by cysteamine for horseradish peroxidase showed higher sensitivity.

2.3 Titanium dioxide

Titanium dioxide belongs to the family of transition metal oxides. In the early years, TiO_2 was widely used as a pigment in paints, cosmetics and sunscreens. After the discovery of the phenomenon of photocatalytic splitting of water on TiO_2 electrode under UV light, intense research on TiO_2 led to many areas like photovoltaics and photocatalysis (Carp *et al.* 2004). Also TiO_2 is promising for biosensor applications due to their non-toxicity, chemical and physical stability being biocompatible matrixes for enzyme immobilization and provide enhancement of electron transfer between the redox enzymes and the electrodes.

TiO_2 can be prepared in the form of powder, crystals, or thin films. Both powders and films can be built up from crystallites ranging from a few nanometers to several micrometers. The most suitable method of thin film synthesis is sol-gel methods. It is also widely used for multicomponent structures due to ability of good mixing. Other oxides like silica and various metal ions like Au^{+3} can be introduced to the TiO_2 films with this method (Carp *et. al* 2004).

Two paths can be followed for the production of TiO_2 with sol-gel method, non-alkoxide and alkoxide. Inorganic salts are used in non-alkoxide route while metal alkoxides are used as precursors in alkoxide route. Commonly used titanium precursors are titanium(IV) n-butoxide, titanium(IV) ethoxide and titanium(IV) iso-propoxide. In latter route TiO_2 sol or gel is formed by hydrolysis and condensation of titanium alkoxides. These reactions are followed by a thermal treatment to remove the organic part and to crystallize TiO_2 into desired phase. Titanium dioxide has three crystalline phases; rutile is the most stable phase, anatase is the most photocatalytically active phase and brookite phase does not have any photocatalytic activity (Mills *et al.* 1997). Crystalline phase conversion determines the use of TiO_2 as photocatalyst, catalyst or ceramic material.

Although being an excellent photocatalyst, use of bulk TiO_2 may have some drawbacks. It sinters easily and it is difficult to obtain high surface area, thus

decrease in photocatalytic activity is observed (Schrijnemakers *et al.* 1999). So studies to increase the catalytic efficiency of TiO_2 by modifications with noble metals, transition metals and with other metal oxides attracted great attention. The formation of mixed TiO_2-SiO_2 films improves photocatalytic efficiency through the generation of new active sites and improved thermal stability and surface area of titania (Carp *et al.* 2004). It is shown in many studies that TiO_2-SiO_2 materials have higher photocatalytic activity than pure TiO_2.

2.3.1 Photocatalysis

Photocatalysis is defined as the accelaration of a photoreaction by the presence of a catalyst (Mills *et. al* 1997). A photocatalyst is characterized by its capability to adsorb simultaneously two reactants, which can be reduced and oxidized by a photonic activation through an efficient absorption (Carp *et al.* 2004). Photocatalytic reaction is a series of chain reactions which takes place over the surface of the catalyst initiated by the absorption of photons with appropriate wavelength and generation of electron/hole pair.

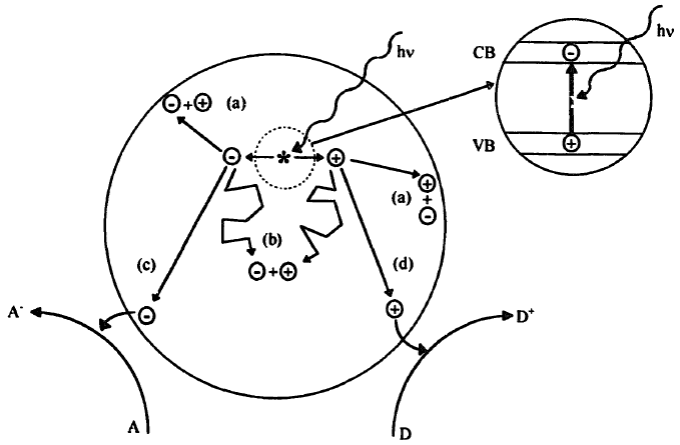


Figure 2.5 Illustration of the major processes occurring on a semiconductor photocatalyst following electronic excitation. Electron-hole recombination can occur at the surface [(a)] or in the bulk [(b)]. Photogenerated electrons can reduce an electron acceptor A [(c)] and photogenerated holes can oxidize an electron donor D [(d)]. (Mills *et al.* 1997)

Ideally, a semiconductor photocatalyst should be chemically and biologically inert, photocatalytically stable, easy to produce and to use, efficiently activated by sunlight, able to efficiently catalyze reactions, cheap, and without risks for the environment or humans (Carp *et al.* 2004). Since as a semiconductor TiO_2 has most of those advantages, it is widely used.

Activation of the semiconductor for photocatalytic reactions is achieved with the adsorption of photon having higher energy than the band gap energy. Band gap energies of rutile and anatase phases of TiO_2 can be seen below.

Table 2.2 Band gap energies of rutile and anatase phases

Crystal phase	Band gap	Wavelength
Anatase	3.2 eV	388 nm
Rutile	3.0 eV	413 nm

Absorption of photon is the first step of photocatalytic reaction and the valence band electrons are excited to the conduction band. This excitation results in the formation of electron and hole pairs (e-/h+) inside photocatalyst and they are transferred to the external surface with diffusion. The water ad-species and hydroxyl species over the surface are reacted with holes and hydroxy radicals are formed which further forms hydrogen peroxide. Similary, the oxygen species react with electrons and active superoxygen radicals are produced (Hoffman *et. al* 1995). Initial steps of TiO_2 photocatalysis can be seen below.

$$TiO_2 + h\upsilon \rightarrow h^+ + e^- \quad (2.2)$$

$$h^+ + OH^- \rightarrow \bullet OH \quad (2.3)$$

$$h^+ + H_2O \rightarrow \bullet OH + H^+ \quad (2.4)$$

$$O_2 + e^- \rightarrow \bullet O_2^- \quad (2.5)$$

$$H^+ + \bullet O_2^- \rightarrow HO_2\bullet \quad (2.6)$$

$$2\ HO_2\bullet \rightarrow H_2O_2 + O_2 \quad (2.7)$$

Those photocatalytic reactions are not specific and therefore the radicals formed at the end of those reactions have potential to oxidize many organic compounds.

2.4 Thin Films Synthesis

For the performance of any biosensor, reversibility, reproducibility and speed of response are very important issues. In any sensor analyte molecules have to diffuse into the sensing component and the products formed must diffuse out. Therefore reversibility and speed of the sensor response can be well improved by having thinner sensing layers (Davis *et al*. 2005) and much effort has been performed for the assembly of thins films to biosensor technology. Self-assembled monolayers, plasma assisted techniques and chemical solution deposition are some of the methods to obtain thin films.

Self-assembled monolayers are formed by strong chemical bond between the building species and the surface of the substrate. It is possible to form ultrathin, stable and ordered monolayers of desired functional groups for immobilizing biomolecules onto the surface (Davis *et al.* 2005).

Treatment of solid surfaces by different types of plasma such as microwave, radio frequency, corona discharge is often used for modification and plasma surface modifications exhibit complex, multifunctional chemistries; crosslinked and branched structures (Ratner, 1996). Plasma polymerization is a thin film-forming process, where thin and adherent layers are deposited directly on surfaces of the substrates without any fabrication (Sever *et al.* 2009). The resulting films are homogeneous and extremely thin and the sensors produced by using this method exhibits high reproducibility and low noise (Mutlu *et al*. 2008).

Sol-gel type chemical solution deposition technique is a highly flexible method for the fabrication of thin films (Schwartz *et al*. 2004). Since this technique was employed in this study, detailed information will be given.

2.4.1 Sol-gel processing

Sol-gel synthesis proceeds by initially forming a stable suspension of colloidal solid particles, which is defined as sol, followed by a gel phase, which is a porous interconnected solid network (Wright *et al*. 2001). This process have inherent advantages like, no need for expensive equipments, good mixing of multicomponent systems and easy control of particle size, shape and properties. Fibers, powders, dense ceramics and thin films can be produced by sol-gel process. Possible routes for desired products can be seen in Figure 2.6.

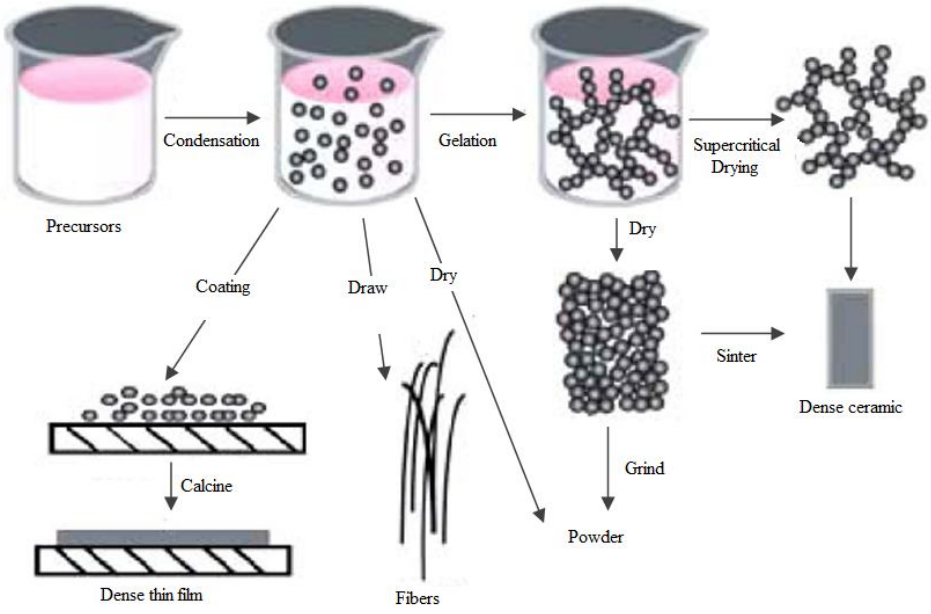


Figure 2.6 Typical sol-gel processing routes

In sol-gel process initially, precursors should be determined which affects the progress of the reactions towards the formation of polymeric gels or colloidal particles. Inorganic salts, metal alkoxides and mixed alkyl/alkoxides can be used as precursors. Basic sol-gel process can be described by the following reactions which start when the metal alkoxide is mixed with water (Schwartz *et al.* 2004).

Hydrolysis

$$M(OR)_x + H_2O \rightarrow M(OR)_{x-1}(OH) + ROH \quad (2.8)$$

Condensation (alcohol elimination)

$$2\ M(OR)_{x-1}(OH) \rightarrow M_2O(OR)_{2x-3}(OH) + ROH \quad (2.9)$$

Condensation (water elimination)

$$2\ M(OR)_{x-1}(OH) \rightarrow M_2O(OR)_{2x-2}(OH) + H_2O \quad (2.10)$$

Hydrolysis is favored when the water to alkoxide ratio is increased. In general, under stoichiometric addition of water, the alcohol producing condensation process is dominant, whereas for excess amount of water, water forming condensation reaction is favored (Brinker *et al.* 1990).

Hydrolysis and condensation steps can be separated by acid and base catalysis. It has been demonstrated that acid catalysis increases hydrolysis rates and ultimately crystalline powders are formed from fully hydrolyzed precursors. Base catalysis is thought to promote condensation that results in amorphous powders containing unhydrolyzed alkoxide (Carp *et al.* 2004). Use of acetic acid may be used to initiate hydrolysis and sols prepared from titanium alkoxides can be stabilized reducing the condensation and precipitation of titania.

2.4.2 Coating Techniques

Coating material should be in sol phase for preparation of good quality thin films (Brinker *et al.* 1990). Once the coating solution is prepared by sol-gel route, films are typically formed by spraying, spin casting and dip-coating as in Figure 2.7.

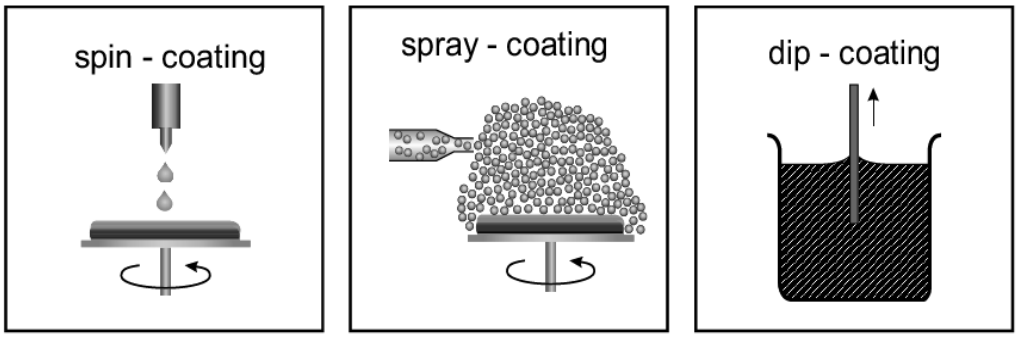


Figure 2.7 Types of coating techniques (Schwartz *et al.* 2004)

In spin-coating, film of coating solution is spreaded on a substrate which is spinned. It is divided into four stages; deposition, spin-up, spin-off and evaporation (Bornside *et al.* 1987). The angular velocity, spin time and viscosity of the solution affect the thickness of the wet film. This technique applies well to disks and cylindrical surfaces.

Spray-coating technique potentially has the advantage of film deposition on non-planar structures and is based on transforming the coating solution to an aerosol with an ultrasonic nebulizer or pressure driven nozzle based systems (Schwartz *et al*. 2004). Coverage can be improved by reducing the droplet size however it is difficult to maintain uniform film thicknesses.

Dip-coating is the mostly used coating technique. It has many advantages over other techniques like thickness can be controlled easily and multilayer coating can be obtained (Brinker *et. al* 1990). It involves the formation of a film through a liquid entrainment process. After the immersion of the substrate into the dip-coating solution, start-up stage comes where the withdrawal of the substrate form the solution begins, followed by film deposition, drainage as the substrate is removed from the solution and solvent evaporation. These steps can be seen in Figure 2.8.

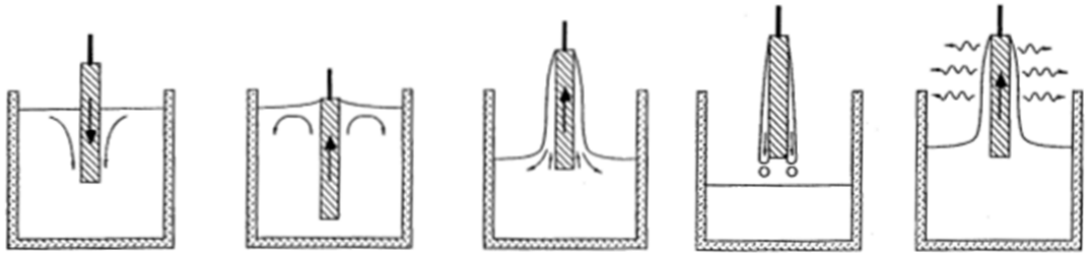


Figure 2.8 Steps of dipcoating; immersion, start-up, deposition, drainage and evaporation, respectively (Brinker *et al*. 1990)

Viscous drag, gravitational forces, surface tension of the curved meniscus affects the thickness of the film that is formed (Brinker *et al.* 1990). Also, sticking probability of the precursor and aggregation plays role in the thickness.

After coating, thermal treatment is the last stage to obtain thin films. Typically, sol−gel-derived precipitates are amorphous in nature, requiring further heat treatment to induce crystallization. To remove the remaining solvent entrapped, thin films are dried and further to obtain the desired oxide phase and the remove the organic moieties, thin films are calcined at appropriate temperatures.

2.5 Enzymes

Enzymes are types of proteins which catalyze biochemical reactions. They increase the rate of reaction by lowering the activation energy without undergoing any permanent change (Horton *et al.* 1996), however enzymes differ from most other catalysts by being much more specific.

Like all other proteins, enzymes are composed of amino acids and the basic properties of them are determined by their protein nature. Amino acids are consist of an amino (NH_2) and a carboxylic acid (COOH) group attached to a central carbon atom and the variation of them is determined by the ‘R’ group, which is also called as side chain (Figure 2.9).

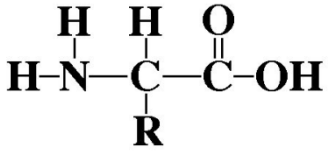


Figure 2.9 General structure of an amino acid

Amino acids are joined together by forming peptide bonds between the amino group of one amino acid and carboxyl groups of the other amino acid resulting in a polypeptide chain and each amino acid in the polypeptide is called a residue. Polypeptide chain, which determines the primary structure, can be coiled into units of secondary structure. Secondary structure refers to the spatial arrangement of amino acid residues that are adjacent in the primary structure, where as tertiary structure includes longer-range aspects of amino acid sequence. Tertiary structure determines the three dimensional arrangement of proteins. Quaternary structure is the last level of structure in which contains more than one polypeptide chain. The function of the enzyme is determined by its three dimensional structure (Branden *et al.* 1999).

Enzymes are classified into six groups according to the reactions that they catalyze; oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. These groups are further subdivided and a four-digit number starting with the letters EC (enzyme commission) is defined for specific identification of all enzymes.

2.5.1. Invertase

Invertase (beta-fructofuranosidase, saccharase, invertin, sucrase; EC 3.2.1.26) is an enzyme which belongs to the group of hydrolases. It is responsible for the hydrolysis of sucrose to an equimolar mixture of glucose and fructose which are

known as reducing sugars. The reaction catalyzed by the invertase is shown in Figure 2.10.

Sucrose + H_2O ↔ β-D-Fructose + α-D-Glucose

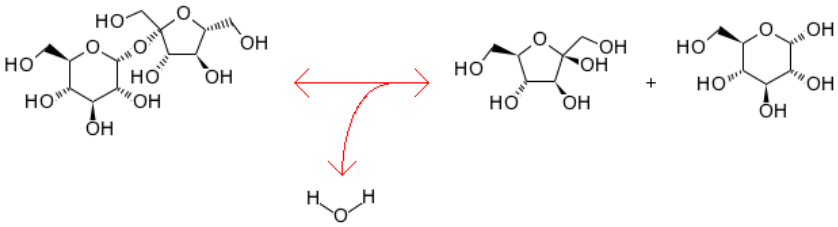


Figure 2.10 Sucrose hydrolysis catalyzed by invertase

Invertase is produced from a wide range of microorganisms and mainly from yeast strains of *Saccharomyces cerevisiae* both in nonsecreted (intracellular) and secreted (extracellular) forms, later being in glycosylated form with approximately 50% of the molecular mass attributed to the carbohydrate moiety (Carlson *et al.* 1983). It can be found in different forms such as dimers, tetramers and hexamers with a molecular weight of nearly 130 kDa for the monomer (Kaplan, 1996).

In contrary to most other enzymes, due to being a glycoprotein, invertase exhibits relatively high activity over a broad range of pH and temperature. It is inactivated at pH 8 and above but it is stable between pH 3 and pH 8 with optimum in the range between pH 4.5 to pH 5.5 (Kaplan, 1996).

Invertase is mainly used in confectionery industry where fructose is preferred over sucrose because it is sweeter and does not crystallize as easily. Sucrose crystallizes more readily than glucose and fructose, so invertase is widely used in the production of non-crystallizing creams, in making jam and artificial honey. Besides the use of invertase in industry, invertase is also being used as a model enzyme in scientific studies to study protein structure, mechanism of enzyme action and enzyme immobilization.

2.6 Immobilization of enzymes

Immobilization of an enzyme is achieved by restricting its mobility via chemical or physical methods. It is mostly used for the enhancement of enzyme properties for the applications in areas like controlled release systems, determination of environmental pollutants and biosensor design. Although the activity of enzyme may decrease during the immobilization process, it has many advantages like simplified separation, easy recovery of immobilized enzymes from the reaction medium and they can be repeatedly used reducing the cost of operation. Also enzymes can be stabilized by immobilization. Support materials can alter parameters like optimal pH and temperature which may be advantageous (Sleytr *et al.* 1993). Although different classifications can be made, there are five main immobilization techniques. The choice of method depends on parameters like the nature of the enzyme, required stability, reusability and cost.

2.6.1 Adsorption

Adsorption, which it is the simplest way of immobilization, involves reversible interactions between the enzyme and the support material. Reversible surface interactions are mostly due to van der Waals forces, ionic bonding, hydrogen bonding and hydrophobic interactions (Bickerstaff *et al.* 1997). Besides being a cheap and quick immobilization technique, active site of the enzyme stays intact and may demonstrate higher activity. However, the most significant disadvantage is the leakage of enzymes from the support. Non-specific binding can be a problem for the cases of the interaction of substrate, product or residual contaminant with the support.

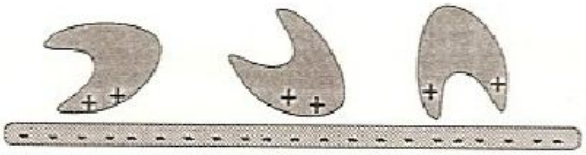


Figure 2.11 Adsorption of enzymes with ionic binding (Bickerstaff *et al.* 1997)

2.6.2 Covalent binding

Covalent method of immobilization involves the formation of a covalent bond between functional groups on the surface and the functional groups of the enzymes belonging to amino acid residues.

Figure 2.12 Representation of covalent binding (Bickerstaff *et al.* 1997)

Amino groups (NH_2) of lysine or arginine, carboxyl group (CO_2H) of aspartic acid or glutamic acid, hydroxyl group (OH) of serine or threonine and sulfydryl group (SH) of cysteine are involved in covalent bond formation (Srere *et al.* 1986) and generally reaction takes place by means of the amino and carboxyl groups. Conditions of covalent immobilization must be determined to be compatible with the stability of enzymes.

Most of the covalent binding procedures involve two steps, activation of the support then coupling of enzyme. It may be advantageous to involve two steps rather than completing the immobilization in a single step (Lowe *et al.* 2002). However, enzyme should be immediately immobilized after the activation of the

support because activated supports have unstable and reactive functional groups (Bickerstaff *et al.* 1997)

There are many methods that can be used in covalent coupling and most common of them can be seen in Table 2.3. It is crucial to decide a method that will not bind to the amino acid active site inactivating the enzyme. If an enzyme has an amine group at the active site, carboxyl group can be chosen for the covalent reaction with the support.

Table 2.3 Typical covalent coupling reactions for immobilization (Eggins, 2002)

Reactive group (on surface)	Intermediate	Reactive group (on enzyme)	Coupling linkage
$-R-NH_2$	$R'-N=C=N-R''$ (Carbodiimide)	$HO-\overset{\overset{O}{\|}}{C}-$	$-NH-\overset{\overset{O}{\|}}{C}-$ "Amide"
$-SH$		$HS-$	$-S-S-$ "Disulfide"
$-SH$	$R'-N=C=N-R''$	$HO-\overset{\overset{O}{\|}}{C}-$	$-S-CO-$
$-COOH$	$-\overset{\overset{O}{\|}}{C}-CN_3$	H_2N-	$-CONH-$ "Amide"
$-CHO$	"Schiff base"	H_2N-	$-C=N-$

2.6.3 Crosslinking

Crosslinking is a type of carrier-free binding of enzymes to each other to form a large, three-dimensional complex structure, and can be achieved by chemical or physical methods (Figure 2.13). Bifunctional agents are used like gluteraldehyde,

dinitrobenzene and diazobenzidin as crosslinkers. Main disadvantage of this technique is crosslinking an enzyme to itself is both expensive and insufficient. Enzymatic activity might reduce relatively.

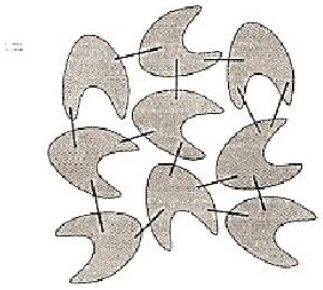


Figure 2.13 Crosslinked enzymes (Bickerstaff *et al.* 1997)

2.6.4 Entrapment

The entrapment method depends on the localization of enzyme in the lattice of a polymer (Figure 2.14). Via entrapment, enzyme movement is restricted by the lattice structure but the enzyme molecules are still free in solution. Widely used lattices are polyacrylamide hydrogels, polyurethane and starch gels, naylon and conducting polymers (Eggins, 2002). The porosity of the gel must be controlled to prevent leakage but allow movement of substrate and product. Entrapment can be enhanced by crosslinking with the lattice and the enzyme. Unavoidably lattice can act as a barrier to mass transfer.

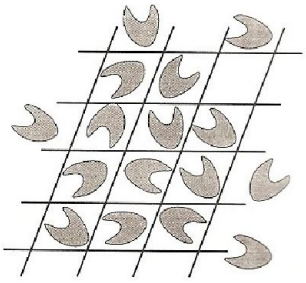


Figure 2.14 Representation of entrapment technique (Bickerstaff *et al.* 1997)

2.6.5 Encapsulation

Encapsulation of enzymes is similar to entrapment but it is achieved by enveloping the biological components within various forms of semipermeable membranes. Many materials have been used to construct microcapsules nylon and cellulose being the most popular ones.

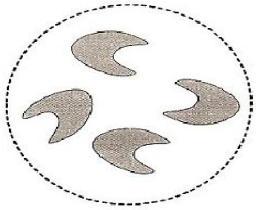


Figure 2.15 Encapsulation type of immobilization (Bickerstaff *et al.* 1997)

2.7 Enzyme Patterning

The area of protein patterning was arised as a critical technology to organize multiple biomolecules on surfaces with resolutions from the micron to the nanometer scale for biological-electronic devices. Array or pattern development begun to receive attention for its broad range of applications from fundamental cell biology to thin-film biosensing like lab-on-a-chip biosensors (Li *et al.* 2008). Current methods capable of providing accurate position and dimension control on patterned proteins include photoresist lithography, photochemistry and self-assembled monolayers.

2.7.1 Conventional photoresist technology

For protein patterning with conventional photoresist technology, chemical linkers with different pendant groups are used. Silane coupling agents are generally preferred to attach enzymes to silica or metal surfaces because they can withstand

the solvents that are used to remove the photoresist. The substrate is cast with photoresist, covered with mask and exposed to ultraviolet irradiation to decompose the photoresist. An adhesion promoting silane is bounded to these exposed sites and then the remaining photoresist is removed with a solvent. The substrate is incubated with an adhesion resistant silane to form mixed monolayers.

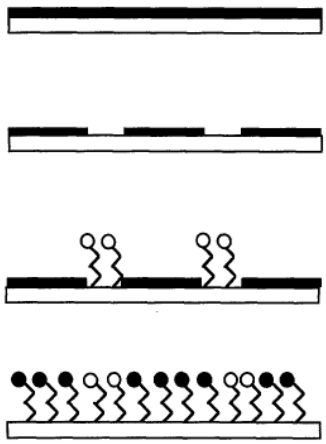


Figure 2.16 Photoresist technique applied to silane SAM's (Reichert *et al.* 1998)

The main disadvantage of the technique is that the proteins can be denaturated with the residual solvent and photoresist. In addition, single incubation of proteins can be achieved with the patterned silane surface and this prohibits it from being used with multiple proteins (Reichert *et al.* 1998).

2.7.2 Photochemical techniques

Photochemical protein patterning methods used chemically labile species which can be activated upon UV irradiation to bind target molecules (Sigrist *et al.* 1995). In order to create enzyme patterns, localized areas of reactivity can be formed by irradiating photochemically derivitized surface using one of the four scenarios

shown in Figure 2.17. Common methods are arlyazide chemistry and nitrobenzyle caging chemistry (Reichert *et al.* 1998).

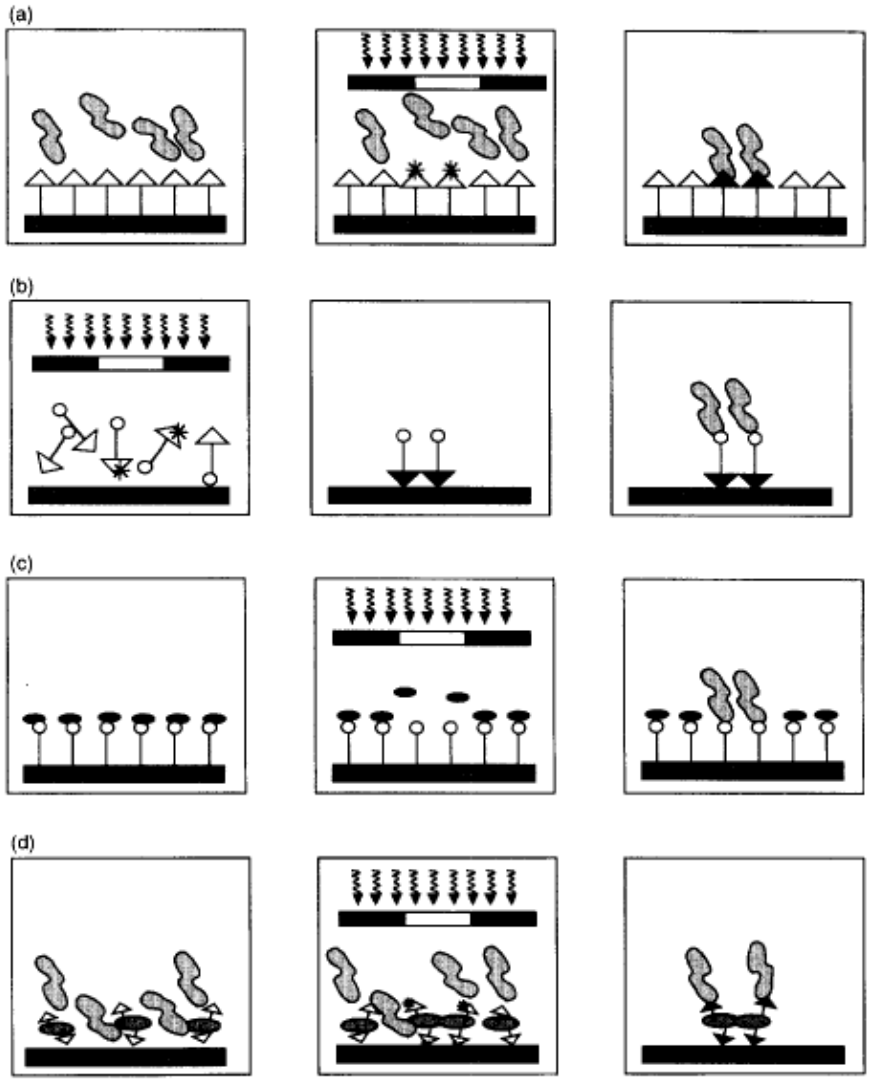


Figure 2.17 Photochemical patterning. (a)Substrate is irradiated and activated regions bind protein in solution. (With deep UV of silane SAMs localized irradiated regions become resistant to protein binding.) (b)Species, activated in localized regions, bind to the substrate, leaving a pendant group for protein binding. (c)Substrate is derivatized with a caged species. Caging group is removed upon irradiation and protein binds to the localized active regions. (d)Substrate is incubated with protein solution and cross-linking polymer, upon irradiation activated polymer binds to the substrate and protein. (Reichert *et al.* 1998)

Arylazide photochemistry is used to selectively immobilize proteins via an azide aromatic group (Pritchard *et al.* 1995). Substrate was incubated with a protein solution and when irradiated with a mask, free azide group was transformed to the active nitrene and bonded to a protein in the incubation solution. The common disadvantage with this chemistry is that the protein must be in contact with the derivitizes surface during the irradiation process. UV range of 265-275 nm is most likely to damage proteins (Pritchard *et al.* 1995) and during irradiation, protein activity can be reduced. However, some substitutions on arlyazide ring enables the compound to be activated at higher wavelenghts.

Nitrobenzyl caging chemisrty involves intorducing a chemical group to a molecule which prevents its activity. Caging group is converted into ketone and carbondioxide with the irradiation of UV light and the released molecule retains its activity. By attaching the caged moiety to molecules which can bind to aminoacids, enzymes can be patterned with photolithography with selectively removing the cage.

2.7.3 Self Assembled Monolayers

As described alkane thiols or alkyl silanes assemble into organized layers when they are exposed to metal surface. One end group remains free while the other end group of the molecular chain binds to the surface. In order to change the binding or surface energy of the monolayer, reactive end groups can be varied. By creating mixed self-assembled monolayers on a surface, proteins can be patterned within the regions of the hydrophilic or adhesion promoting layers (Reichert *et al.* 1998).

Bhatia (1989) examined ultraviolet irradiation of SAMs and the pendant thiol groups are converted to sulfonate groups which inhibit protein adsorption upon deep UV irradiation. Silica surfaces modified with 3-mercaptoproplymethoxy silane were masked and irradiated with 193 nm UV light. The patterned surfaces

were incubated with glucose oxidase enzyme patterns within the thiol regions were observed.

It is also possible to produce mixed SAMs of alkane thiols on gold surfaces with microwriting, micromachining, stamping and UV microlithography. Delamarche *et al.* (1996) examined photochemistry with alkane thiol SAMs on gold surfaces to create protein patterns. The terminal active ester group of alkane thiols on gold were converted to photoactivatable group benzopheone. Surface was incubated with the protein solution and irradiated with UV light. Biradical formation at the ketyl center of the photolabile group was occured with irradiation and proceeded by a C-C bond with the protein on radical recombination.

This method suffers from the same issues as photoresist technique as it is difficult to have multiple enzyme patterns on a surface. Although the conventional photolithography and SAMs can also be used, photochemical methods are leading for the application for enzyme assays to pattern multiple proteins on a single surface.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Microscope slides which were used as glass substrates were obtained from Industrial Quality. Chemicals used for synthesis of colloidal solution, titanium (IV) i-propoxide (TTIP), LUDOX SM-30 (information can be seen in Appendix A), polyethylene glycol 4000 (PEG 4000) and $AuCl_3$, were purchased from SIGMA-ALDRICH.

Invertase (EC 3.2.1.26) was purchased from NOVO NORDISK. Cysteamine was obtained from FLUKA. D-(+)- glucose were purchased from MERCK. All other reagents were of analytical grade and obtained either from SIGMA or MERCK. Ultrapure water was used during the experiments.

3.2 Preparation of thin film coatings

3.2.1 Pretreatment of glass substrates

Microscope slides, 25mm x 75 mm x 1 mm, were used as glass substrates. KOH solution was utilized for cleaning and etching purposes. Glass substrates were immersed in 1M KOH solution for 48 hours and rinsed with distilled water until pH 7. After rinsing, substrates were ultrasonicated in ethanol for 1 hour and finally they were wiped dried at 100^oC for 1 hour. After pretreatment, glass substrates were stored in a dessicator.

3.2.2 Synthesis of colloidal solution

TiO_2-SiO_2 binary mixtures was prepared using TTIP as TiO_2 precursor and colloidal silica. 5 ml of TTIP, was added dropwise to 1 ml acetic acid and 200 ml distilled water mixture and acetic acid catalysized hydrolysis started. Then 0.7 ml of 65% (v/v) HNO_3 was added to adjust the pH to 3.5. The solution was stirred for 30 minutes at 80^oC in a water bath with a reflux condenser and 2 hours at room temperature. Then 6.4 ml Ludox-SM 30, colloidal silica, was added to introduce silica nanoparticles to the solution and stirred overnight (22.5 hours). 12.5 gr PEG 4000 was dissolved in 25 ml distilled water and 21.3 ml of that PEG 4000 solution was added and stirred for another 24 hours.

Au was introduced to the final TiO_2-SiO_2 solution in the form of $HAuCl_4$. $HAuCl_4$ was derived form $AuCl_3$ by dissolving 0.1 g of it in 32.973 ml distilled water and adding 27 µl HCl. 18.5 ml of $HAuCl_4$ solution was added to the TiO_2-SiO_2 solution and left stirring for 24 hours. Rest of the $HAuCl_4$ solution was stored at 4^oC for further use.

3.2.3 Dip-coating of glass substrates

Pretreated glass substrates were modified with the prepared solution by dip-coating technique as shown in Figure 3.2. 25mm x 60 mm x 1 mm region of the pretreated substrates were immersed in TiO_2SiO_2-Au colloidal solution and withdrawn with a speed of 5 cm/min. In order to have a continuous film, coating is done with 3 successive layers and between each successive layers, substrates are dried at 100^oC for 15 minutes to provide adhesion before the subsequent layer.

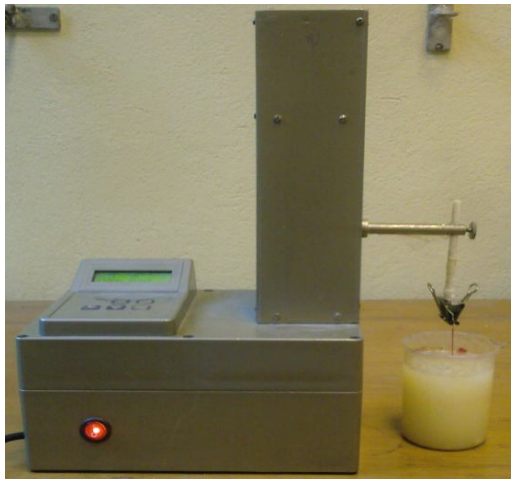


Figure 3.1 Dipcoating equipment

Subsequent to the coating step, heat treatment was done to transform amorphous phase into the crystalline phase and to remove the organics for a porous film. For this aim, thin films are calcined at 500^{o}C for 15 minutes in a preheated tube furnace (Protherm 1000W, PTF 12/50/250) under air flow. Also 15 minutes, 30 minutes and 1 hour of calcinations durations were performed and compared according to the immobilized enzyme activities. The resulted thin film coated glass substrates were stored in desiccators at room temperature.

3.3 AFM Characterization

Pretreated substrates and TiO_2SiO_2-Au thin film coatings were structurally characterized by atomic force microscopy. AFM images were taken with Nanosurf easyScan 2 instrument in Environmental Catalysis Laboratory, Chemical Engineering Department, METU. Measurements were performed in non-contact mode with silicon tip and under air conditions.

3.4 SEM Analysis

Thin films and invertase immobilized thin films were examined by scanning electron microscopy (QUANTA 400F Field Emission) in Central Laboratory, METU with accelerating voltage of 30 kV and at 2000 and 250 000 magnifications. No metal coating was applied to the samples for SEM analysis.

3.5 Immobilization studies

3.5.1 Linker deposition

As a linker, cysteamine was deposited by immersing thin film coated glass substrates in a 200 ml of 0.20 mM aqueous solution of cysteamine. The solution was stirred with a magnetic stirrer at 250 rpm for 3.5 hours at room temperature under dark conditions. Thin films were washed with distilled water to remove the physically bound cysteamine from the surface. Fresh cysteamine solutions were prepared for every deposition process.

3.5.2 Immobilization of invertase

10 μg/ml invertase immobilization solution was prepared in 0.1 M, pH 5.0 sodium-acetate buffer. Cysteamine deposited thin film coated glass substrates were dipped into the 200 ml immobilization solution and stirred with a magnetic stirrer at 250 rpm in order to enhance the mass transfer conditions at room temperature for 12 hours under dark conditions. Finally invertase immobilized films were washed with 100 ml of 0.1 M, pH 5.0 sodium acetate buffer to remove the weakly bound enzymes from the films. Activity assays were immediately performed or invertase immobilized thin films were stored in buffer at 4^{o}C until the assay.

3.5.3 Invertase activity measurements

Activity measurements of immobilized invertase and free invertase were conducted spectrophotometrically with double-beam UV-Visible Spectrophotometer (Thermo Electron Corporation, Nicolet Evolution 100). 50 mg/ml sucrose in 0.1 M, pH 5.0 sodium acetate buffer was utilized as the substrate solution. Reducing sugar concentration, which was produced with the hydrolysis of sucrose, was monitored by DNSA method (Appendix B). For this purpose, 1 ml samples taken from the reaction solution were added to the 1 ml of DNSA at determined intervals and well mixed with vortexing. Samples were treated with 5 minutes of boiling water and with 5 minutes of ice bath. After the samples were reached the room temperature, the absorbance data were recorded at 540 nm.

Activity assays for each sample were repeated at least two times and all activity assays were performed under dark conditions. One enzyme unit (EU) was defined as the amount of enzyme which catalyzes the hydrolysis of 1 µmole sucrose per minute under the reaction conditions (40^{o}C, pH 5.0). Initial reaction rates were calculated from the initial linear part of the reaction progress curve.

The glucose standard curve (Appendix C) was plotted to have the association of absorbance with the concentration of reducing sugar for EU calculations. Standart glucose solutions in the range of 50-150 µg/ml were prepared in 0.1 M, pH 5.0 sodium acetate buffer. 1 ml of glucose samples were mixed with 1 ml of DNSA reagent, kept in boiling water for 5 minutes and ice bath in 5 minutes. After the samples were reached the room temperature, the absorbance data were recorded at 540 nm.

3.5.3.1 Free invertase activity measurements

For free invertase activity, the reaction was started by the addition of 1 ml of 2µg/ml invertase solution to the 24 ml of preheated substrate solution in a 40^{o}C shaking water bath (Nüve ST 102). 1 ml of reaction solution samples were

collected with a time interval of 2 minutes for 10 minutes. Glucose samples were prepared for the glucose standard curve and enzyme activities were calculated as EU/ml. The calculation can be seen below. The equation was divided by 2 due to the fact that two reducing sugars, glucose and fructose, are formed from the hydrolysis of one sucrose.

$$\frac{EU}{ml} = \frac{\Delta OD/\Delta t}{\Delta OD/\Delta c} \times \frac{1\ mmol}{180\ mg\ glucose} \times \frac{1000\ \mu mol}{1\ mmol} \times \frac{25\ ml\ reaction\ mixture}{1\ ml\ enzyme\ solution} / 2 \quad (3.1)$$

ΔOD = change in absorbance at 540 nm

Δt = change in time, min

Δc = change in reducing sugar concentration

$\Delta OD/\ \Delta t$ = slope of the reaction progress curve

$\Delta OD/\Delta c$ = slope of the glucose standard curve

3.5.3.2 Immobilized invertase activity measurements

100 ml of substrate (sucrose) solution was poured into a tube with 4 cm diameter and 16 cm lengh and preincubated in shaking water bath at 40^{o}C until thermal equilibrium was reached. Immobilized enzyme activity assay was started by dipping invertase immobilized film into the sucrose solution. Samples were collected with a time interval of 5 minutes for 40 minutes. Glucose standarts were prepared as explained in invertase activity measurement part. Immobilized invertase activities were calculated as EU/plate where plate indicates invertase immobilized thin film coated glass substrates and the calculation can be seen below. Also, immobilized invertase activity can be expressed in units of EU/mm^2 by considering the area of invertase immobilized glass substrate. (25mm x 60 mm x 2)

$$\frac{EU}{plate} = \frac{\Delta OD/\Delta t}{\Delta OD/\Delta c} \times \frac{1\ mmol}{180\ mg\ glucose} \times \frac{1000\ \mu mol}{1\ mmol} \times \frac{100\ ml\ reaction\ mixture}{1\ plate} / 2$$

(3.2)

ΔOD = change in absorbance

Δt = change in time

Δc = change in reducing sugar concentration

$\Delta OD/\ \Delta t$ = slope of the reaction progress curve

$\Delta OD/\ \Delta c$ = slope of the glucose standard curve

The immobilization was verified by the termination of the increase in reducing sugar concentration when the invertase immobilized films were removed from the reaction solution.

3.5.4 Effect of temperature on immobilized invertase activity

To determine the effect of immobilization temperature on immobilized invertase activity, immobilization was achieved at 4^{o}C and at room temperature by dipping cysteamine assembled thin film coated glass substrates into the 10 µg/ml invertase solution and stirring for 12 hours under dark conditions. At the end of 12 hours, thin films were washed with 100 ml buffer and activity assay was performed as described before.

3.5.5 Effect of enzyme concentration on immobilization

To determine the concentration of the enzyme immobilization solution for the optimum immobilized invertase activity; 5 µg/ml, 10 µg/ml, 50 µg/ml and 100 µg/ml invertase solutions were prepared in 0.1 M, pH 5.0 sodium acetate buffer.

Cysteamine deposited thin films were immersed in enzyme immobilization solutions with determined concentrations and stirred for 12 hours at room temperature under dark conditions. At the end of 12 hours, thin films were washed with 100 ml buffer and activity assay was performed as described before.

3.5.6 Effect of enzyme immobilization period on immobilization

Cysteamine deposited thin films were immersed in 10 µg/ml incubation solution and stirred for 4 h, 12 h, 16 h and 24 h at room temperature under dark conditions. At the end of those time periods, thin films were washed with 100 ml buffer and assayed for activity determination.

3.5.7 Storage stability determination of immobilized invertase

Storage stability of invertase immobilized thin films was investigated for a period of 9 weeks. First invertase activity data is obtained right after the immobilization process and residual immobilized invertase activities were checked every two weeks. When invertase immobilized thin films were not in use, they were stored in 0.1 M, pH 5.0 sodium acetate buffer at 4^{o}C. Residual activities were calculated as:

$$\text{Residual Activity (\%)} = \frac{\text{activity after a determined time period}}{\text{initial activity}} \times 100 \quad (3.3)$$

3.6 Photocatalyzed enzyme inactivation and removal from thin films

3.6.1 Irradiation of free invertase

To decide on the source of irradiation that will be used for photocatalysis, 2μg/ml invertase solution was prepared with 0.1 M, pH 5.0 sodium acetate buffer. 25 ml of invertase solution was poured into the petri plate and exposed to UV-C (peak wavelenght at 254 nm, 9 W) and blacklight (peak wavelenght at 368 nm, 20 W). The distance between the light source and the free invertase samples was 20 cm. Comparison was made between the blank sample, which was kept in dark, and the samples which were irradiated for 30 minutes and 2 hours. Afterwards, activity of free enzyme was determined as in section 3.5.3.1.

3.6.2 Irradiation of invertase immobilized thin films

The photo-induced enzyme removal studies were conducted by irradiating thin films with blacklight (General Electric, 20 W, fluorescent, peak wavelenght at 368 nm). Since the invertase immobilized thin films were not stable in air, they were exposed to blacklight in a petri plate containing 25 ml 0.1 M, pH 5.0 sodium acetate buffer. The thickness of the buffer layer was shallow enough and it did not block irradiation to reach the thin films. Both sides of the glass substrates, on which invertase immobilized thin films are present, were subjected to blacklight for the half of the total time. The distance between the light source and the samples were kept constant at 20 cm.

After irradiation, thin films were washed with 100 ml buffer. Subsequently, invertase activity assay was performed as described in section 3.5.3.2.

In addition to those, to understand the effect of blacklight is reversible or not, irradiated thin films were stored in buffer at 4^{o}C for a day, till the next activity assay.

3.6.3 Reimmobilization of invertase to the irradiated thin films

In order to see the possibility of enzyme immobilization to the irradiated thin films, invertase was reimmobilized to those films with following the steps described in Section 3.5.1 and 3.5.2.

3.6.4 Determination of irradiation exposure time

Irradiation time was adjusted by optimizing the reimmobilized invertase activity. Invertase immobilized thin films were irradiated for 30 minutes, 2 hours, 5 hours, 8 hours and 12 hours as described in section 3.6.2. Their enzymatic activities were determined to ensure complete inactivation of thin films. For activity recovery studies, invertase is re-immobilized to the films as in section 3.5.1 and 3.5.2. Results were compared with the immobilized invertase activities before the irradiation process.

CHAPTER 4

RESULTS AND DISCUSSION

The aim of this study was to immobilize the model enzyme invertase on sol-gel derived TiO_2-SiO_2-Au thin films and to achieve photocatalytic enzyme removal and re-immobilization which can be used to create patterns of enzymes for biosensor arrays.

In this context, surface morphology of thin films was investigated by atomic force microscopy (AFM) and scanning electron microscopy (SEM). After the achievement of immobilization on thin films, immobilization conditions were optimized by considering the effects of calcination period of thin films, enzyme immobilization temperature, enzyme concentration of the immobilization solution and immobilization incubation period. In addition to those, storage stabilities of invertase immobilized thin films were also determined. Photocatalytic enzyme removal studies were based on the effect of blacklight on immobilized invertase over the TiO_2-SiO_2-Au thin films and finally the degree of invertase re-immobilization was determined.

4.1 Thin Film Synthesis

TiO_2-SiO_2-Au thin films were synthesized for the immobilization of enzymes and photocatalytic removal of immobilized enzymes from the surface. TiO_2 and SiO_2 based thin films and nanoparticles are widely used for immobilization of biomolecules due to their high surface area, biocompatibility and easy preparation (Luo *et al.* 2006). Au nanoparticles also have the advantage of being biocompatible

and Au nanoparticles can act as tiny conduction centers to facilitate electron transfer and provide self assembly of thiolates to functionalize the surface for covalent immobilization.

TiO_2-SiO_2-Au colloidal solution for thin film coating was prepared by sol-gel route. Before the TiO_2-SiO_2-Au thin films were introduced to the surface, glass substrates were pretreated in order to etch the surface, to remove the organics and to have hydroxylation for improved coating. Titanium (IV) isopropoxide was used as a TiO_2 precursor and mixed TiO_2-SiO_2 colloids were prepared by addition of colloidal SiO_2. With the use of alkoxy groups (e.g. isopropoxides), small colloidal clusters with uniform particle size can be formed by reducing the rate of hydrolysis and condensation. Also with high ratio of water in reaction medium and acid catalysis, small clusters can be achieved by complete hydrolysis (Kwon *et al.* 2003). Au was introduced in the form of auric acid ($HAuCl_4$) to the mixed TiO_2-SiO_2 colloidal solution. PEG was added to the solution and during calcination step, this organic polymer was removed from the thin films resulting in a porous structure for enzyme immobilization.

Besides the aim of preparing a surface for enzyme immobilization, photocatalytic function of the synthesized thin films were also important. It is known that the photocatalytic activity of TiO_2 is greatly influenced by its crystallinity, grain size, surface area and surface hydroxyl content and TiO_2–SiO_2 composites has many advantages over pure TiO_2 (Hoffman *et al.* 1995; Yu *et al.* 2001; Guan, 2005; Yurana *et al.* 2006). The presence of SiO_2 stabilizes the anatase phase, which is the most photocatalytically active phase, by hindering grain growth. Also smaller grain size means larger surface area which can be correlated with the increased hydroxyl content of the surface. In addition to the enhancement of photocatalytic activity of TiO_2, SiO_2 can be used as a binding material to improve the adhesion property of TiO_2 films on glass subtrates (Kwon *et al.* 2003). In addition to those, effect of Au addition to the colloidal solution should not be ignored from the point of photocatalysis although the main objective of Au doping was to enhance the biosensor performance. There is an optimum dosage level below which noble

metals can act as electron-hole separation centers enhancing the photocatalytic efficiency of TiO_2. However when it is exceeded, noble metals can act as electron-hole recombination centers reducing the photocatalytic activity (Han *et al.* 2009). Li *et al.* (2001) found that a molar content of 0.5% of either gold or gold ion doped in TiO_2 was the best dosage to achieve the highest methylene blue photodegradation efficiency.

Calcination temperature is another important parameter that has an effect on crystalline structure of the thin films and determines transformation between the amorphous and crystalline phases. It is known that anatase is the most photocatalytically active phase. Kim *et al.* (2002) investigated the effect of calcination temperature on structural properties of titanium dioxide and found that thin films of titanium dioxide are in amorphous phase when calcined at 300^oC while they are in anatase phase after being calcined at 400^oC and rutile phase formation occurs at the calcination temperature of 1000^oC. Calcination temperatures of 400 ^oC -500 ^oC are widely used for thin films of TiO_2 or TiO_2-SiO_2 on glass substrates (Bennani *et al.* 2009; Ersöz, 2009; Pourmand *et al.* 2008).

TiO_2-SiO_2-Au colloidal solutions were prepared considering the points discussed above, however optimization of those parameters were out of the scope of this study. Sythesized TiO_2-SiO_2-Au colloidal solutions were deposited on pretreated glass substrates as thin films by dip-coating. Coating was done with 3 successive layers in order to have a continuous film and complete coverage of the glass substrates. Thin films were calcined at 500^oC for 15 minutes to remove the organic moieties and to have the desired oxide phase. TiO_2-SiO_2-Au thin films were characterized morphologically with AFM and SEM. In addition to morphological characterizations, analysis such as X-RAY, FTIR and BET can be performed for detailed analysis of thin films. FTIR analysis was tried to be utilized however, the results were unsuccessful. It was reported that the thickness of the synthesized thin films were about 800 nm, (Koç, 2009) which made them inconvenient for those kinds of techniques.

4.1.1 AFM characterization of thin films

Pretreated glass substrates and thin films of TiO_2-SiO_2-Au over pretreated glass substrates were structurally characterized by AFM in non-contact mode under air conditions. In Figure 4.1, representative AFM images of pretreated glass substrates and TiO_2-SiO_2-Au thin films can be seen for $1\mu m^2$ scanned area.

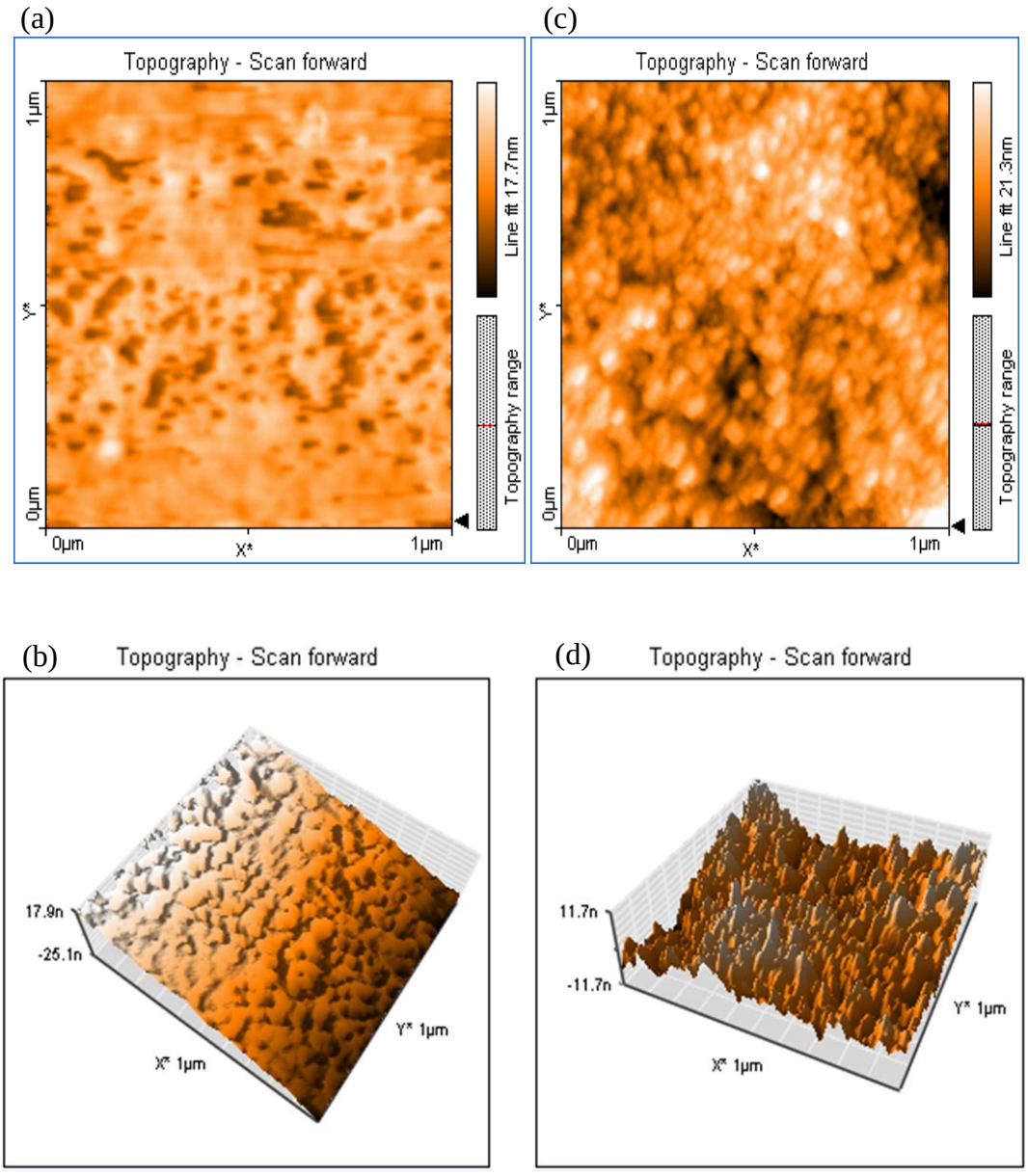


Figure 4.1 AFM images of (a) pre-treated glass substrate, (b) corresponding 3-D view; (c) TiO_2-SiO_2-Au thin film, (d) corresponding 3-D view

As can be seen in (a) and (b), surface of the glass substrates were successfully etched where holes represent the etched areas. Images (c) showed a typical sol-gel derived thin film coating; substrate was completely and uniformly coated with spherical nanoparticles having dimensions approximately between 10nm and 30nm. According to image (d), it was inferred that a reasonable roughness was obtained.

4.1.2 SEM Analysis of thin films

The surface morphologies of TiO_2-SiO_2-Au thin films which were calcined at 500^oC for 15 minutes were also examined by SEM imaging. In Figure 4.2 and Figure 4.3 representation of them can be seen under x2000 magnification and x250000 magnification respectively.

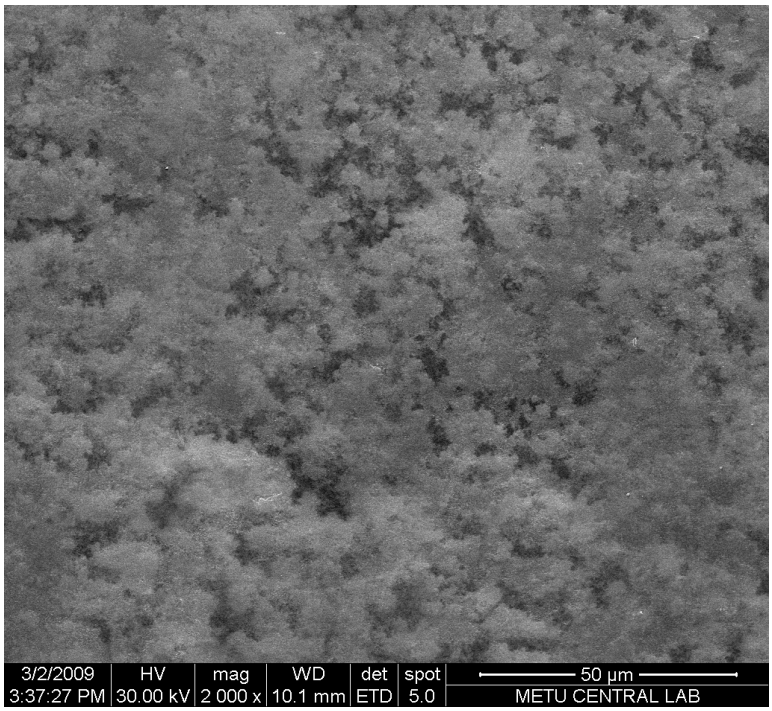


Figure 4.2 SEM images of TiO_2-SiO_2-Au thin films with x2000 magnification

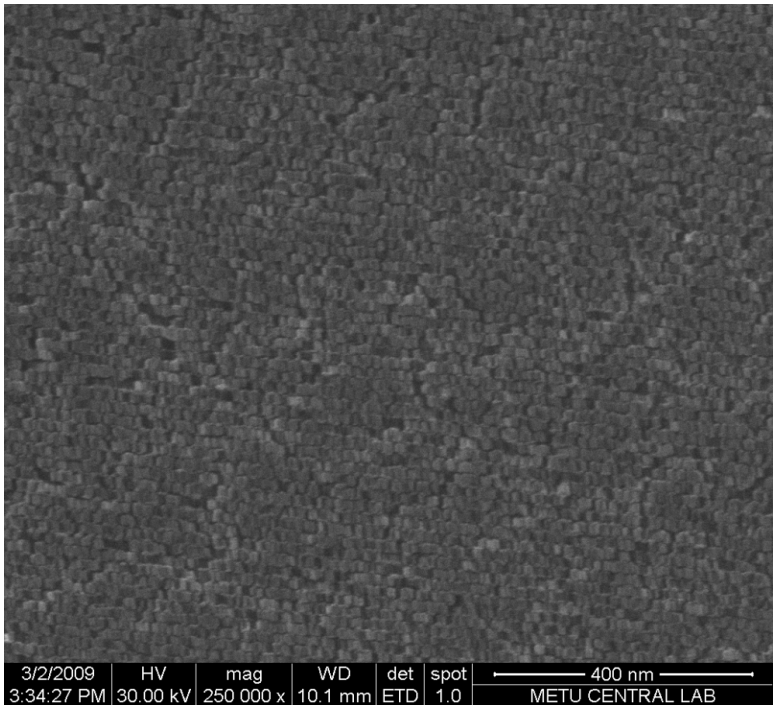


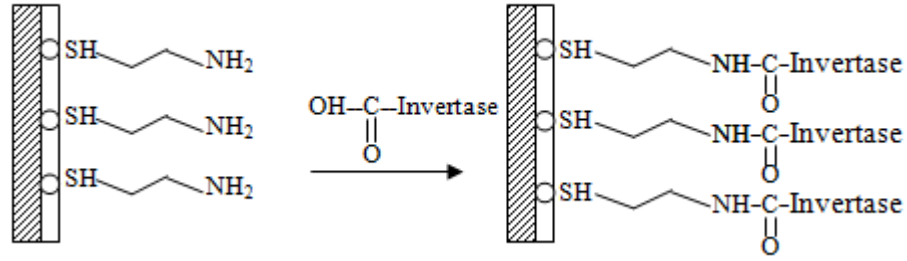
Figure 4.3 SEM images of TiO_2-SiO_2-Au thin films with x250000 magnification

As a general appereance, complete coverage of glass substrates with uniform and crack free TiO_2-SiO_2-Au thin films was observed (Figure 4.2). Figure 4.3, which is the higher magnification image of thin film coatings, coincides with AFM results in the sense that surface is comprised of nanoparticles which exhibited similar dimensions. Further, the presence of Ti and Si was verified by energy dispersive X-ray spectroscopy (EDX) (Appendix D). However, the presence of Au was not verified due to high instrumental background and low Au loadings which was below the detection limits.

4.2 Enzyme immobilization

TiO_2-SiO_2-Au thin films were functionalized with cysteamine for the covalent immobilization of the model enzyme, invertase. Thiol compounds such as cysteamine are known to form self-assembled monolayers on gold. With the help

of the cysteamine linker, amine functional groups were introduced to the surface which takes role in the covalent immobilization of invertase through the carboxyl group of the enzyme. Steps for proposed enzyme immobilization scheme can be seen below in Figure 4.3.



Thin film coated glass substrates and assembly of cysteamine on Au nanoparticles

Covalent immobilization of invertase on thin films with cysteamine linker

Figure 4.4 Immobilization of invertase on cysteamine and TiO_2-SiO_2-Au thin film assembly

Immobilization of invertase on TiO_2-SiO_2-Au thin films was verified by following the enzymatic reaction spectrophotometrically. The reaction was started by dipping enzyme immobilized thin film coated glass substrates into the sucrose (reactant) solution. Reducing sugar (product) concentration in reaction mixture was measured spectrophotometrically with respect to time to follow the enzymatic reaction. In Figure 4.5, reaction progress curves for two different invertase immobilized thin films can be seen. To check the success of immobilization, one of the glass supports was removed from the substrate solution after the 50^{th} minute however the other reaction was continued till the 80^{th} minute. Consistent data points were obtained by mixing the reaction solution with the help of shaking water bath.

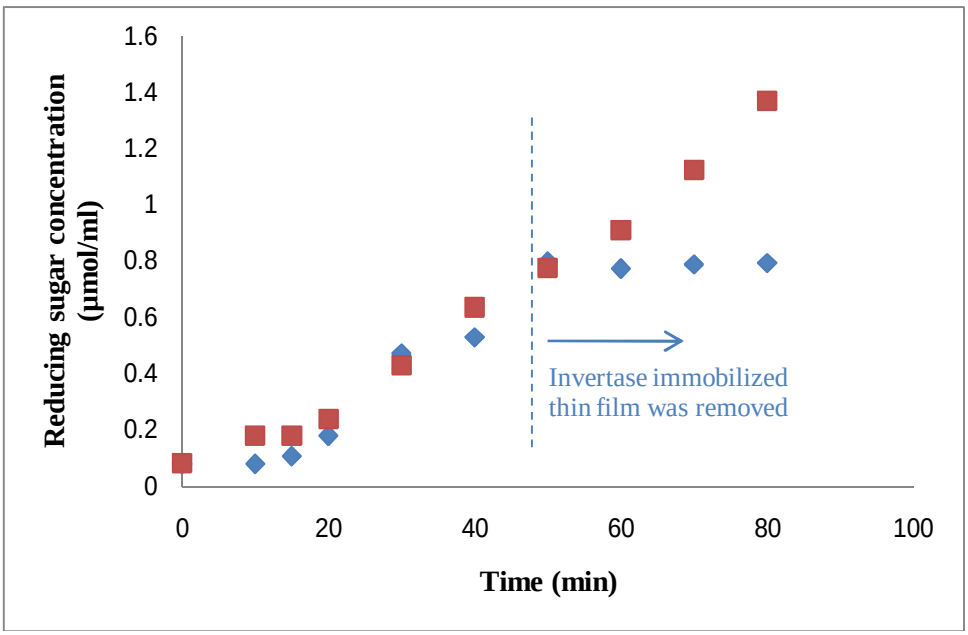


Figure 4.5 Reaction progress curves showing the termination of enzymatic reaction with the removal of invertase immobilized thin film coated glass substrates (assay conditions at 40°C, pH 5.0; 0.145 M sucrose concentration)

As it can be understood from the reaction progress curves, enzymatic reaction has stopped immediately by removing invertase immobilized glass substrate while the reaction was still proceeding for the case which the glass substrate was kept inside the solution. Generally, 10-15 minutes of lag time was encountered in reaction progress curves, which was related with the time needed for the accumulation of reducing sugar to be detected. The termination of the enzymatic reaction with the removal of glass substrate verified the immobilization of invertase onto cysteamine deposited TiO_2-SiO_2-Au thin films successfully without any enzyme leakage to the reaction solution.

4.2.1 SEM Analysis of enzyme immobilized thin films

After the achievement of enzyme immobilization, the surface morphologies of the invertase immobilized thin films were examined together with TiO_2-SiO_2-Au thin

films by SEM imaging. Although, it is not expected to observe the enzyme molecules on the surface, this characterization can give information about the nature of the thin films.

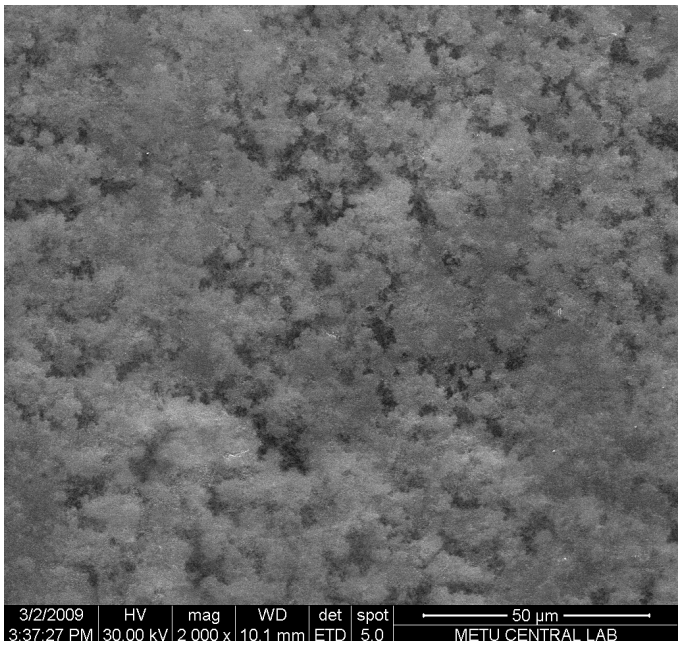


Figure 4.6 SEM images of TiO_2-SiO_2-Au thin films with x2000 magnification

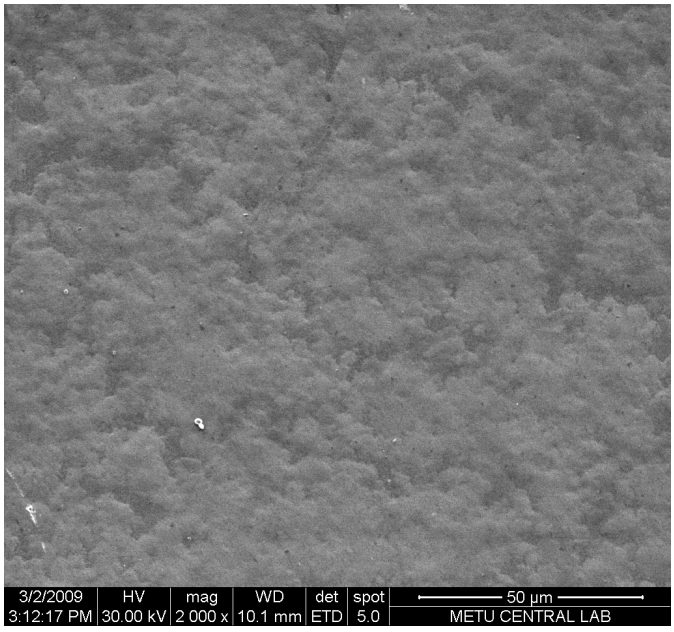


Figure 4.7 SEM images of invertase immobilized TiO_2-SiO_2-Au thin films with x2000 magnification.

When Figure 4.6 and Figure 4.7 were compared, it was seen that the surfaces have the same nature (dissimilarities are related with the contrast difference of the images). Thus, it was inferred that immobilization had no adverse effect on thin films and coating was well adhered to the glass substrates.

4.3 Optimization of enzyme immobilization conditions

Optimized conditions for the operation of the enzyme biosensors are obtained upon investigation of the effect of factors such as enzyme loading and solution conditions like temperature and pH (Sleytr *et al*. 1993; Daigle *et al*. 1997). These factors have an influence on the efficiency and life-time to a great extent. In this study, conditions were optimized by considering the effect of calcination duration of thin films, enzyme immobilization temperature, immobilization period and concentration of the immobilization solution on immobilized invertase activity.

4.3.1 Effect of calcination period on enzyme immobilization

Surface area of the support is important for immobilization because higher surface area means possibility of higher enzyme loading (Wang, 2006). Two of the parameters that can affect surface area of the support are calcination temperature and duration of the thin films. As calcination temperature and duration increases, porosity and surface area decreases resulting in a denser film.

In order to see how the enzyme immobilization is effected by the calcination duration, thin films were calcined for 15 minutes, 30 minutes and 1 hour keeping calcination temperature constant at 500^{o}C. Reaction progress curves and the apparent activities of the immobilized invertase for those samples can be seen in Figure 4.8 and Table 4.1 respectively.

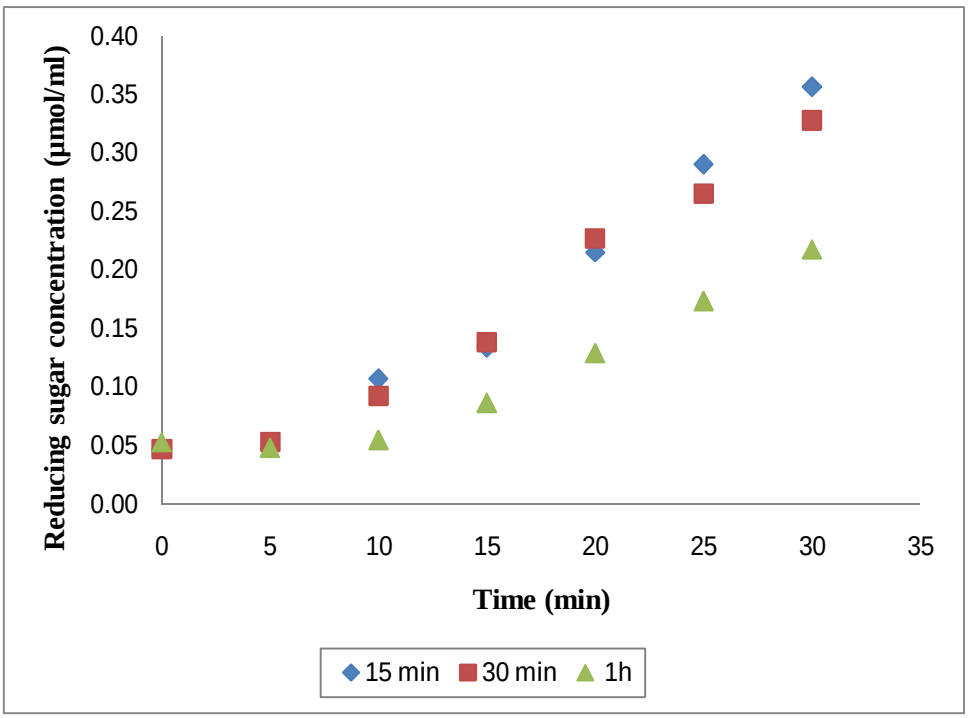


Figure 4.8 Effect of calcination duration on reaction progress curves of immobilized invertase (assay conditions at 40^{o}C, pH 5.0, 0.145 M sucrose)

Table 4.1 Apparent activities of the immobilized invertase on thin films with respective calcination duration

Calcination duration	Apparent activity (EU/plate)
15 minutes	0.61±0.01
30 minutes	0.57±0.01
1 hour	0.36±0.01

According to those results, invertase immobilized thin films which were calcined for 1 hour showed the lowest apparent activity. This may be due to the decrease in surface area with prolonged heat treatment and as a result, amount of enzyme loading on the thin films were lowered. Although there is a slight difference, maximum activity was achieved when invertase was immobilized on thin films which were calcined for 15 minutes.

4.3.2 Effect of immobilization temperature on enzyme immobilization

Since invertase was covalently immobilized, rate of immobilization may depend on temperature. At lower temperatures, immobilization may require longer periods. However at higher temperatures activity of the enzyme might be lost during immobilization period due to enzyme denaturation so this parameter can have both positive and negative effects on enzyme immobilization. In order to see the effect of immobilization temperature on apparent invertase activity, immobilization was achieved at room temperature (RT) and at 4°C. Reaction progress curves can be seen in Figure 4.9.

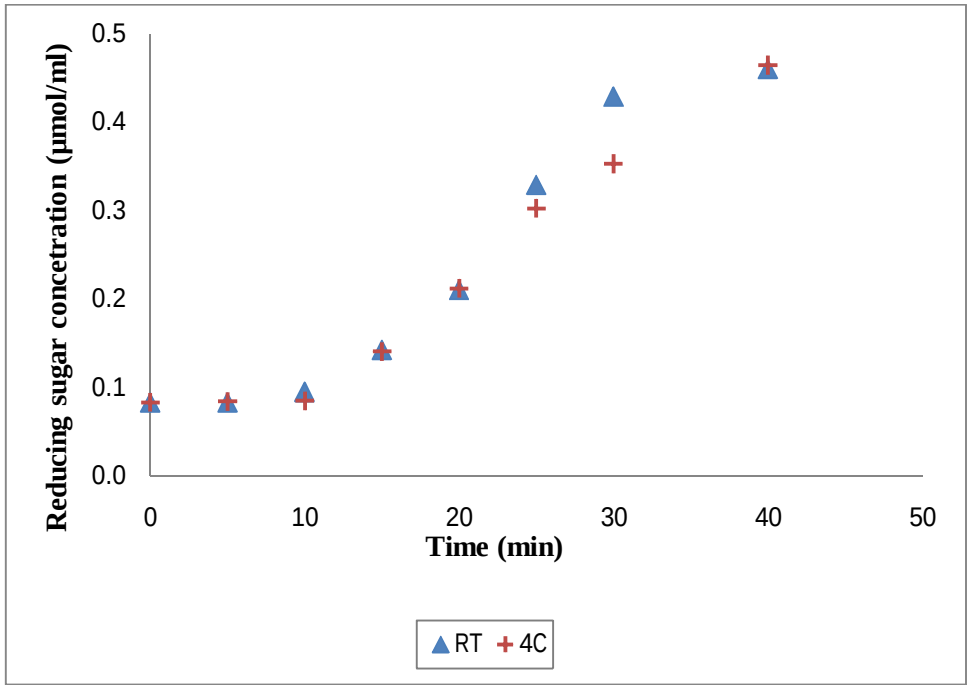


Figure 4.9 Reaction progress curves for invertase, immobilized at room temperature and 4°C (assay conditions at 40°C, pH 5.0, 0.145 M sucrose)

Apparent activities of invertase, immobilized at room temperature and at 4°C, were calculated to be 0.68±0.01 and 0.64±0.01 EU/plate respectively, thus it was concluded that in the range tested, immobilization temperature does not have any

influence on immobilized invertase activity. This can be attributed to the inherent stability of invertase at room temperature which is not surprising considering the highly glycosylated nature of invertase (Carlson *et al.* 1983).

4.3.3 Effect of enzyme concentration of the immobilization solution on enzyme immobilization

There are several studies on the effect of enzyme loading on biosensor performance and concentration of the immobilization solution is one of the factors that affect the enzyme loading. Rate of product formation is controlled by the rate of reaction or the rate of substrate diffusion to the enzyme layer. When the enzyme loading is high, substrate diffusion to the enzymatic layer determines the rate, limiting the overall reaction rate (Sleytr *et al.* 1993). Salis *et al.* (2009) studied the effect of loading on enzyme activity by using different enzyme concentrations and it is seen that enzymatic activity increases linearly with the loading up to a maximum. Higher loadings resulted in a decrease of enzyme activity.

As a part of optimization, the same effect was considered by immersing thin film coated glass substrates in immobilization solutions with different enzyme concentrations. In Figure 4.10, apparent activities can be seen for various enzyme concentrations of immobilization solutions.

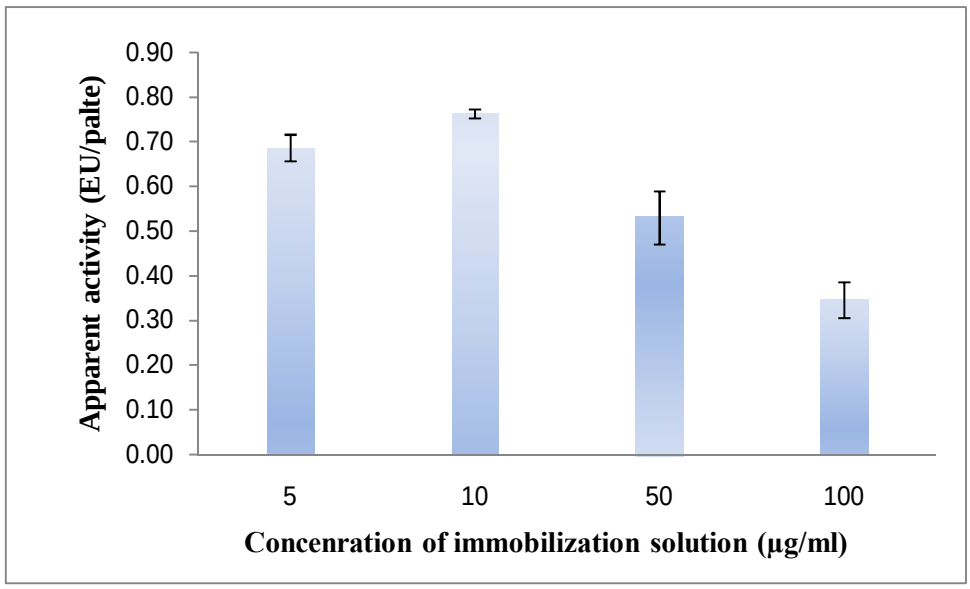


Figure 4.10 Effect of enzyme concentration on apparent activities of immobilized invertase

According to the data, optimum enzyme concentration was determined to be 10 µg/ml. Increase in concentration from 5 µg/ml to 10 µg/ml increased the immobilized invertase activity form 0.69(±0.03) EU/plate to 0.76(±0.01) EU/plate. However, the results showed that further increase in enzyme concentration did not provide an increase in apparent enzyme activity and when thin films were incubated in 100 µg/ml immobilization solution, activity reduced to 0.35(±0.04) EU/plate. This can be related with the fact that high enzyme loading limits the diffusion of the substrates to the enzyme active sites causing a loss in activity. Also with high loading, steric effects may arise between neighboring enzyme molecules and active sites can be hindered resulting in a decreased apparent activity of the immobilized invertase.

4.3.4 Effect of enzyme immobilization period on enzyme immobilization

Effect of immobilization period on immobilization is similar to the effect of concentration of enzyme in the immobilization solution, which can be correlated

with enzyme loading. Immobilized enzyme activity may increase as time course of enzyme immobilization increased and after an optimum value, immobilized enzyme activity may decrease due to high enzyme loadings or enzyme inactivation. Based on this knowledge, different immobilization periods were analyzed to achieve the highest immobilized enzyme activity. Results can be seen in Figure 4.11.

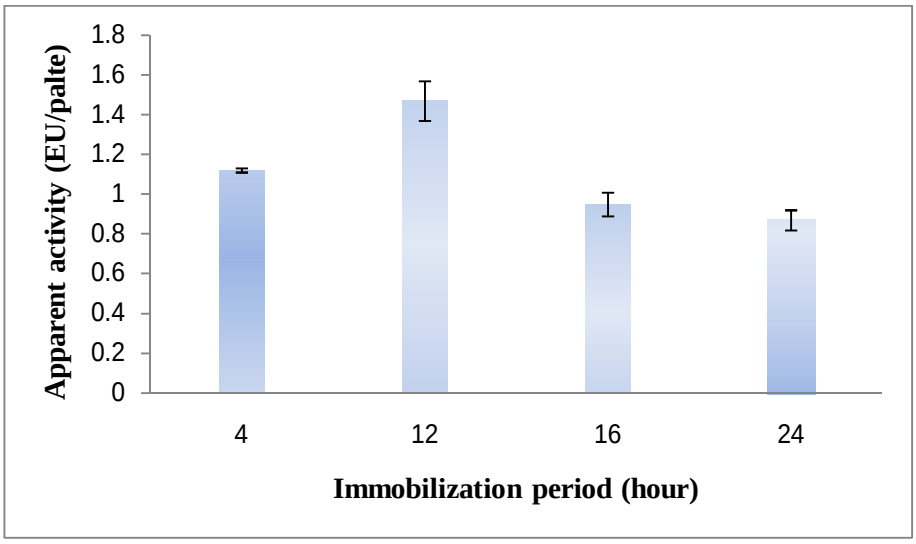


Figure 4.11 Effect of immobilization period on apparent activity of immobilized invertase

Relation between the immobilization period and corresponding immobilized invertase activities showed that there is an optimum value of immobilization period. When immobilization period was increased from 4 to 12 hours, immobilized invertase activity was also increased, however further increase in immobilization period did not result in increased activity. The activity of free invertase did not changed during the 24 hour at immobilization conditions, so the reduction in enzymatic activity cannot be related with the loss of invertase activity in immobilization solution. Apparently, prolonged immobilization period resulted in high enzyme loadings which may cause limited diffusion of substrates to the enzyme active sites and steric effects reducing the apparent activity.

4.4 Storage stability of invertase immobilized thin films

Enzyme stability issues are always of high significance in the production of a stable and reproducible biosensor because practical applications can be hindered by poor stability (Spain *et al.* 2008). In Figure 4.12, residual enzyme activity can be seen with respect to storage time. When stored at 4^{o}C in 0.1 M pH 5.0 sodium acetate buffer, it was seen that invertase immobilized thin films retained 85% of their initial activity after 4 weeks and more than 50% of their initial activity retained after 9 weeks and free invertase lost its activity in two weeks. When compared with similar studies from literature (covalent immobilization of invertase and the same storage conditions) (Chen *et al.* 2000, Danisman *et al.* 2004), it was seen that invertase immobilized on TiO_2-SiO_2-Au thin films showed quite good storage stability. However, it is observed that when they were stored in dry conditions, immobilized invertase activity diminished immediately.

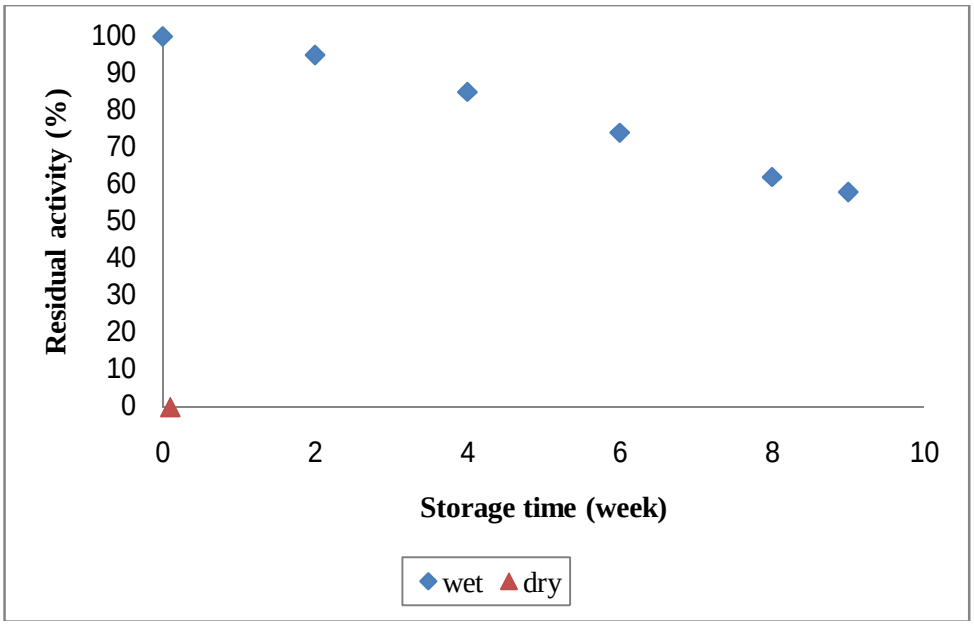


Figure 4.12 Residual activity of immobilized invertase with respect to storage time

In general, enzyme leakage from the surface and denaturation of immobilized enzyme can be the reasons for activity loss with time. Although during storage, leakage of enzyme is not possible because the enzyme is covalently bonded to the films (Taylor 1993; Altınok *et al.* 2008), storage solutions were assayed to determine any enzyme leakage the surface. No leakage was determined from the surface to the storage solutions; therefore the reduction of enzyme activity can be explained by enzyme inactivation due to denaturation during storage.

4.5 Investigation of TiO_2 based photocatalytic immobilized enzyme removal

There are many studies in literature that showed TiO_2 is an efficient photocatalyst for degrading organic compounds and microorganisms (Hoffman *et al.* 1995; Gaya *et al.* 2008; Erdural *et al.* 2008). If TiO_2 is irradiated with light having higher energy than its band gap energy, resulted electron-hole pairs lead to chemical reactions to degrade specific compounds. Even though degradation begins with a partial degradation, the term 'photocatalytic degradation' usually refers to complete photocatalytic oxidation or photomineralisation, essentially to CO_2, H_2O, NO_3^-, PO_4^{3-} and halide ions (Carp *et al.* 2004)

$$\text{Organic contaminant} \xrightarrow[h\nu \geq E_g]{TiO_2} \text{Intermediates} \longrightarrow CO_2 + H_2O + \text{mineral acids}$$

Only the molecules that are in direct contact with the catalyst surface can undergo photocatalytic reactions. These photocatalytic reactions result in products that leave the TiO_2 surface (Hamers *et al.* 2009). Grimes *et al.* (2004) used this concept for the recovery of TiO_2-nanotube based hydrogen sensor from poisoning with the photocatalytic oxidation of the organic contaminating agent by ultraviolet irradiation. With the same idea, photocatalytic activity of TiO_2 was used here to remove immobilized enzymes from thin films which can further be developed as a

patterning technique. This technique must be a selective technique and the removal of enzymes should be carried out in a controlled way.

4.5.1 Effect of irradiation on free enzyme

As it was discussed before, for photocatalytic reactions to take place, photons having higher energy than band gap of TiO_2 must be adsorbed. Acquiring enough energy for band-gap sensitization, the nature or form of the light does not affect the pathway of photocatalytic reactions (Stylidi *et al.* 2004). In order to decide the irradiation source, free enzymes in solution (in the absence of TiO_2) were irradiated with two different light sources; UV-C (peak wavelength at 254 nm.) and blacklight (peak wavelength at 368 nm.) with 9 Watts and 20 Watts respectively as described in Section 3.6.1. The reaction progress curves for UV-C and blacklight irradiated free invertase solutions can be seen in Figure 4.13 and Figure 4.14 respectively and enzymatic activities are tabulated in Table 4.4 including blank as the free invertase solution which was kept in dark.

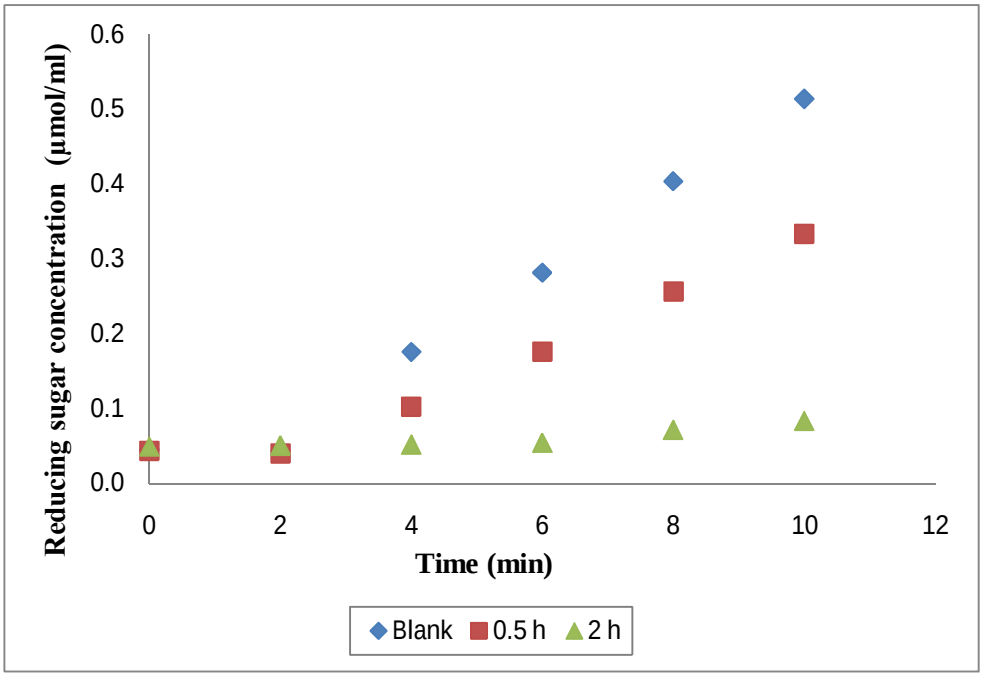


Figure 4.13 Reaction progress curves for UV-C irradiated free invertase (assay conditions at 40°C, pH 5.0, 0.145 M sucrose)

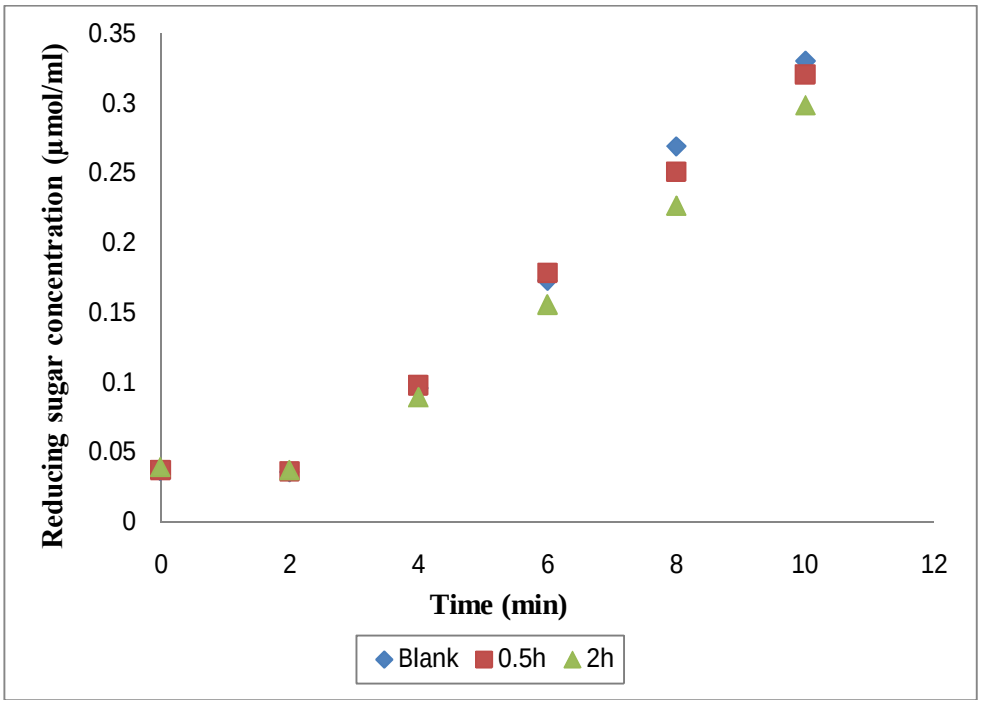


Figure 4.14 Reaction progress curves for blacklight irradiated free invertase (assay conditions at 40°C, pH 5.0, 0.145 M sucrose)

Table 4.2 Residual enzymatic activities of free invertae solution after irradiation

	Blacklight	**UV-C**
Blank	0.63 EU/ml±0.01	0.73 EU/ml±0.01
0.5 h exposure	0.60 EU/ml±0.01	0.46 EU/ml±0.01
2 h exposure	0.58 EU/ml±0.01	0.09 EU/ml±0.01

No significant inactivation was observed on free enzyme after 30 minutes of irradiation with blacklight and almost the same activity was sustained after 2 hours of irradiation. However, irradiation with UV-C caused a dramatic decrease in free enzyme activity after 2 hours of irradiation. Due to those results, blacklight irradiation is more suitable for the studies of photocatalytic enzyme removal from the TiO_2-SiO_2-Au thin films.

4.5.2 Blacklight induced photocatalytic effects on immobilized enzyme

Blacklight (20 W, peak wavelenght 368 nm.) was utilized for the investigation of photo-induced controlled enzyme removal from TiO_2-SiO_2-Au thin films. Invertase immobilized thin films with known activity were subjected to blacklight for 15, 30 and 120 minutes. The results were compared with the experimental results of blank sample which represents invertase immobilized thin films that were kept in dark. In Figure 4.15 reaction progress curves can be seen and residual immobilized enzyme activities after photocatalytic treatment are tabulated in Table 4.3.

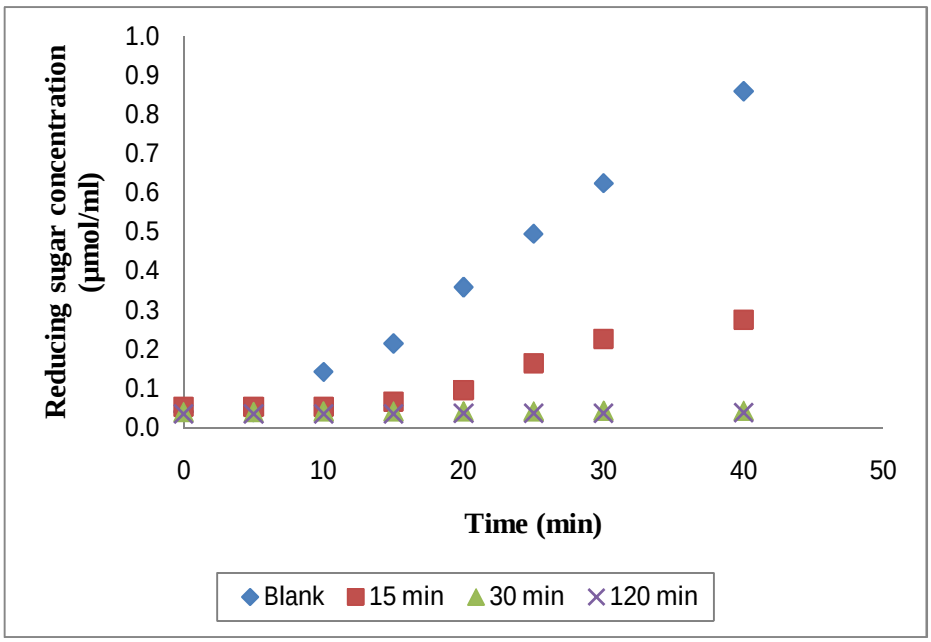


Figure 4.15 Reaction progress curves of blacklight irradiated immobilized invertase on TiO_2-SiO_2-Au thin films (assay conditions at 40^oC, pH 5.0; 0.145 M sucrose)

Table 4.3 Residual immobilized enzyme activities after irradiation

	Residual Activity (EU/plate)
Blank	1.20±0.07
15 min exposure	0.40±0.01
30 min exposure	0
120 min exposure	0

These results indicate that invertase immobilized over thin films can be completely inactivated by using 30 minutes of blacklight irradiation. To see if this inactivation is reversible or not, partially inactivated thin film sample (sample denoted as 15 min) were assayed after one day storage for a second time and results can be seen below.

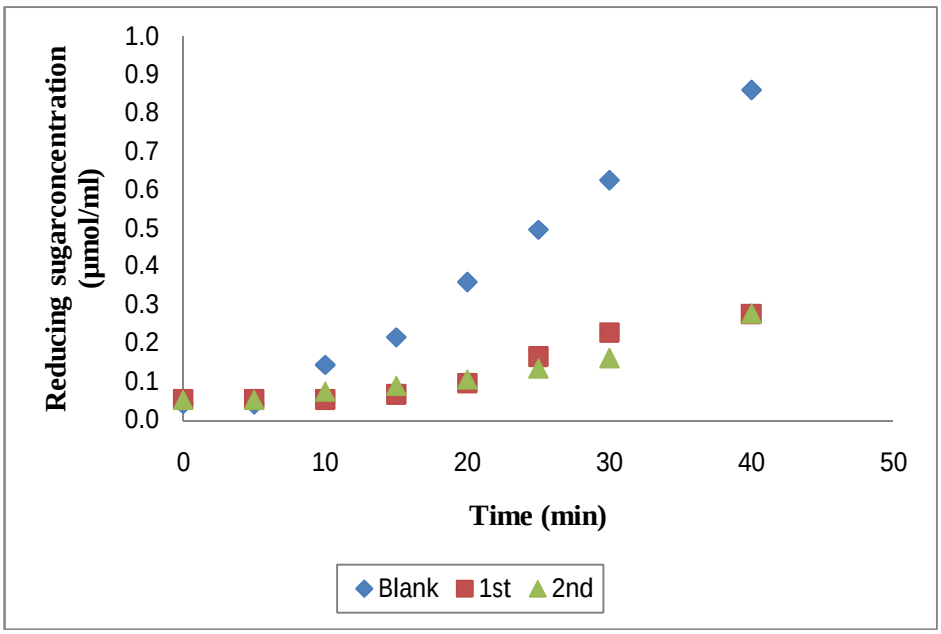


Figure 4.16 Reaction progress curves of irradiated immobilized invertase after storage (assay conditions at 40°C and pH 5.0, 0.145 M sucrose)

Immobilized invertase activities for the first and second assays were 0.40 EU/plate and 0.42 EU/plate respectively. This verifies that the photocatalytic enzyme inactivation is not a reversible process and can be applied for partial or complete removal of enzyme from the surface.

4.5.3 Enzyme re-immobilization on irradiated thin films

Immobilized invertase over the thin films was successfully inactivated by the photocatalytic property of TiO_2. However, to be used as a patterning technique, immobilized enzymes must be removed from the surface and surface sites which are responsible from immobilization should be preserved to achieve re-immobilization. The enzyme re-immobilization to the irradiated surfaces will be promising technique in order to immobilize another enzyme by the successive application of immobilization-patterning cycles. For this purpose, the re-immobilization of invertase to the irradiated samples was examined. Figure 4.17

shows the re-immobilized invertase activity with respect to irradiation time. The data point which was shown at '0' irradiation time represents the immobilized invertase activity before the irradiation process.

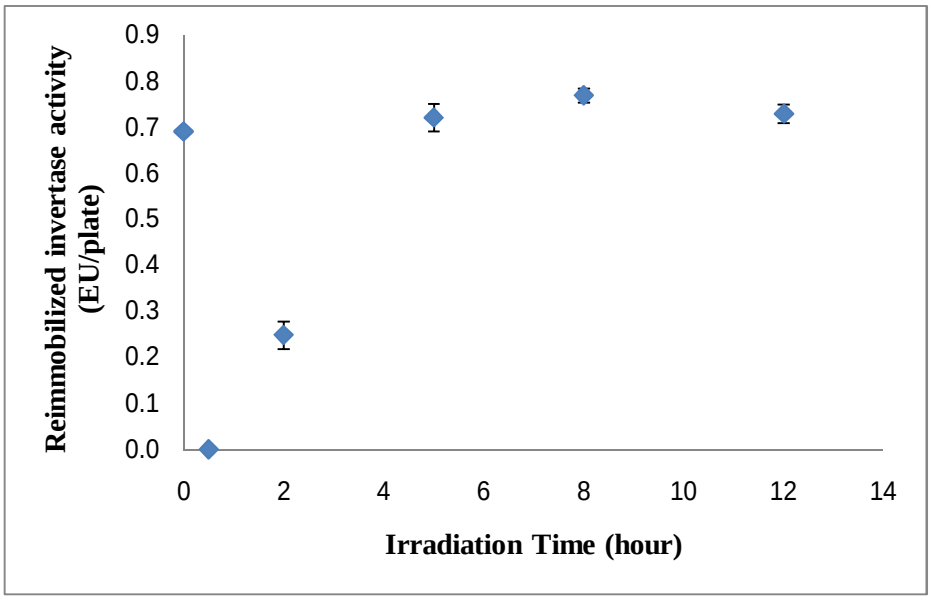


Figure 4.17 Re-immobilized invertase activity on irradiated surfaces with corresponding irradiation time

As it is seen, although immobilized invertase was completely inactivated in 30 minutes, re-immobilization of invertase on those films could not be achieved. This might be due to the presence of partial degradation of invertase. The surface residues were stable and not able to be removed from the surface by washing with buffer solution inhibiting the re-immobilization. As exposure time increased, the complete removal was achieved after 5 hours of irradiation and re-immobilization succeeded.

The fundamental conclusion according to these results is the relationship between the inactivation of immobilized invertase and its photocatalytic degradation. Immobilized invertase might not be completely degraded and removed from the

surface although being inactivated. Despite the possibility of re-immobilizing invertase onto the residues which occupied the surface after irradiation, it is very plausible that immobilized invertase was photocatalytically degraded and removed from the surface after sufficient exposure which made the re-immobilization of invertase to the irradiated thin films possible. With this concept, new application area of photocatalytic activity of TiO_2 in biosensor technology can be introduced. By choosing appropriate irradiation power and time, the immobilized enzyme over the surface can be removed with controlled way and another enzyme might be immobilized. Thus, the production of enzyme arrays is viable with this technique.

Generally, TiO_2 thin films and powders are used in biosensor applications because of their non-toxicity, high biocompatibility and good retention of biological activity for protein binding. Also their high surface area, optical transparency and conductivity are preferred properties for bioanalytical applications (Topoglidis *et al.* 1998). In literature, the photocatalytic activitiy of TiO_2 in the presence of enzymes was used to improve the response of the biosensor (Ganadu *et al.* 2002; Zu *et al.* 2009). It was reported that the sensitivity of detection increased when the enzyme immobilized TiO_2 surfaces were UV irradiated due to the additional capture of electrons from the photoexcited TiO_2. However, inactivation of enzymes in the presence of photoexcited TiO_2 is perceived as a drawback (Ganadu *et al.* 2002; Zu *et al.* 2009).

Use of TiO_2 in the photopatterning of biomolecules for biosensor applications is a promising technique. Very recently, TiO_2 was utilized for the photo-patterning of biomolecules with the UV initiated photochemical grafting of functional groups by the exclusion of water and O atoms (Li *et al.* 2009; Franking *et al.* 2009). However, our study showed the possibility of photocatalytic removal of immobilized enzymes from the thin films without destructing the immobilization capability which can be utilized for the preparation of enzyme site arrays for biosensor applications. The representative sketch in Figure 4.18 was drawn to give an idea about how the photocatalytic enzyme removal and re-immobilization can be utilized to have enzyme patterns. As discussed in Section 2.7, photochemical

patterning techniques are quite advantageous when compared with the other methods, and it is certain that the use of photocatalytic activity of TiO_2 will contribute to the development of enhanced photochemical patterning techniques.

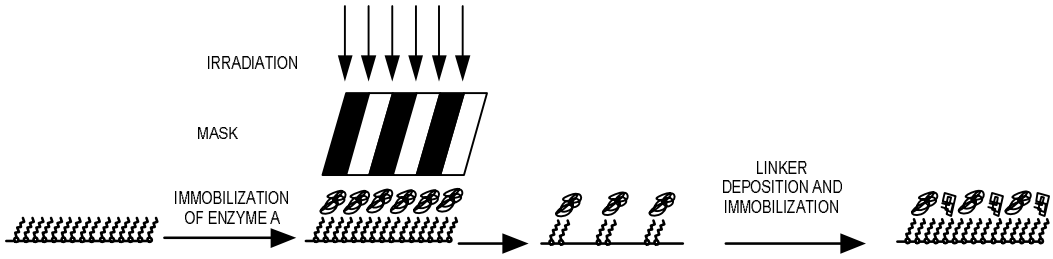


Figure 4.18 Possible steps for patterning with the use of photocatalysis

CHAPTER 5

CONCLUSIONS

Within the scope of the present study, model enzyme invertase was successfully immobilized on TiO_2-SiO_2-Au thin films. TiO_2 based photocatalytic removal of invertase by blacklight irradiation was achieved and invertase was re-immobilized successfully to the irradiated thin films.

Well adhered and uniform TiO_2-SiO_2-Au thin film coatings were obtained on glass substrates. TiO_2-SiO_2-Au thin films were functionalized with amine groups by cysteamine linker and the immobilization of model enzyme invertase was achieved. The immobilized invertase activity was found independent from the immobilization temperature within the range tested. The optimum enzyme concentration and period for immobilization was determined as 10μg/ml and 12 hours respectively. With the optimized conditions, invertase immobilized thin films exhibited high storage stability retaining more than 50% of their initial activity after 9 weeks.

The immobilized invertase over thin films was completely and irreversibly inactivated with 30 minutes of blacklight (368 nm.) irradiation by the photocatalytic activity of TiO_2-SiO_2-Au thin films. Invertase was re-immobilized to the irradiated thin films and initial immobilized invertase activity (before the irradiation) was attained when the invertase was re-immobilized to thin films that were irradiated for 5 hours. Photocatalysis-reimmobilization cycles can pave the way to new studies on enzyme patterning with the use of photocatalytic activity of TiO_2.

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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

DNSA METHOD FOR REDUCING SUGAR DETERMINATION

3,5 -Dinitrosalicylic acid (DNSA) is an aromatic compound which reacts with reducing sugars, hence it provides a method for quantitative determination of reducing sugar concentrations in a sample. In a typical reaction, equal volumes of the DNSA reagent and the sample, which will be analysed, are mixed. The mixture is heated in boiling water for 5 minutes, cooled for 5 minutes in ice bath and allowed to warm up to room temperature. Colorimetric changes are detected by following the change in optical density at 540 nm. Chemicals used to prepare DNSA reagent are given in Table B.1. DNSA reagent was prepared by dissolving 3,5 -dinitrosalicylic acid and Na-K tartarate in distilled water separately before mixing all the chemicals.

Table B.1 Chemicals and relative amounts for DNSA reagent

Chemicals	Amount % (w/v)
3,5 -dinitrosalicylic acid	1
NaOH	1
Sodium sulphide	0.05
Phenol	0.2
Na-K tartarate	36.25

APPENDIX C

GLUCOSE STANDART CURVE

Reducing sugar concentrations in the reaction mixtures were determined by using glucose standart curves for the activity calculations of the free invertase and immobilized invertase. Different glucose concentrations were prepared from the 1mg/ml stock solution of glucose (Table C.1)

Table C.1 Concentrations of the glucose solutions for glucose standart curve

Glucose concentrations (mg/ml)	**0**	**0.05**	**0.07**	**0.09**	**0.12**	**0.15**
Sodium acetate buffer (ml)	10	9.5	9.3	9.1	8.8	8.5
Glucose stock solution (ml)	-	0.5	0.7	0.9	1.2	1.5

Glucose concentrations were correlated with optical densities by DNSA method. A representative glucose standart curve can be seen in Figure C.1 and the slope of the linear part was used in activity calculations.

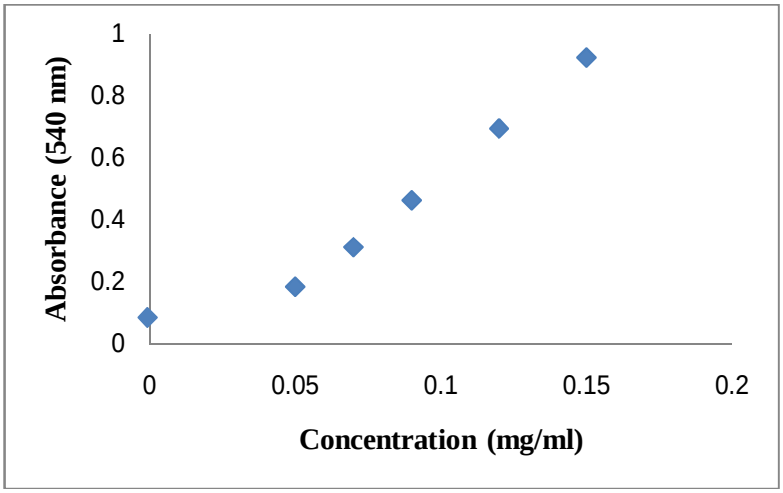

Figure C.1 Representative glucose standart curve

APPENDIX D

EDX ANALYSIS OF TiO_2-SiO_2-Au THIN FILMS

EDX analysis of thin film coated glass substrates were performed by Quanta 200 scanning electron microscope in Gazi University.

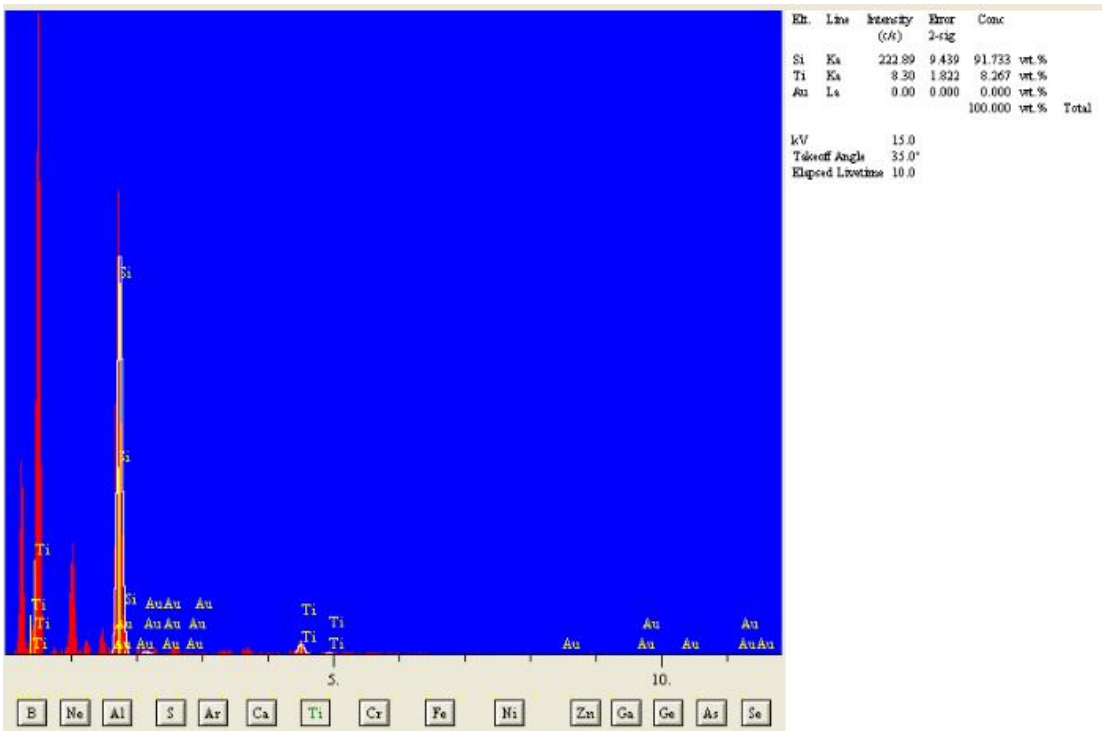

Figure D.1 EDX analysis of TiO_2-SiO_2-Au thin films