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Characterization of enzymes immobilized on modified gold surfaces

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CHARACTERIZATION OF ENZYMES IMMOBILIZED ON MODIFIED GOLD SURFACES

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ABSTRACT

CHARACTERIZATION OF ENZYMES IMMOBILIZED ON MODIFIED GOLD SURFACES

by Saharnaz Bigdeli

In this thesis, Glutamate Dehydrogenase (GDH) and myokinase (MK) were selected as two model systems and their behavior upon immobilization on a gold surface was studied. The surface was first modified by employing Self Assembled Monolayers (SAMs), followed by enzyme immobilization. To determine the activity of these enzymes upon immobilization, assays had to be developed. Flexibility and stability of both enzymes along with the attachment strength and binding capacity of GDH in a continuous flow operation were tested.

Two assays, one bioluminometric and one fluorimetric, for myokinase along with one fluorimetric assay for GDH were developed with success. By employing these assays, the activities of free and immobilized forms of both enzymes were determined. The continuous flow operation performed on GDH revealed that the enzyme had remained active for over 24 hours without any detachment from the surface. The binding capacity of GDH on a gold surface was also determined.

This Thesis is dedicated to:

My dear parents,
For setting all the right examples, and for your endless love and support.

My dear husband,
For your never-ending support.

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Table of Contents

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

REFERENCES

List of Figures

List of Tables

CHAPTER ONE
INTRODUCTION

There has been a tremendous progress in biology and nanotechnology during the last two decades and as a result, these two previously unrelated areas have intersected. This connection has lead to formation of a variety of new research topics. In the present work, the main focus is to provide a novel technology that enables the measurement of the concentration of metabolites present in biological fluids.

It is known that the enzymes' specific kinetic properties, such as the ability for conformational changes in the structure due to contact with specific metabolites, lead to production of signals. These metabolites can be electrically detected and analyzed by preparing and employing certain standards. Thereby, the concentrations of the metabolites can be determined.

For stability and reusability purposes, enzymes can be immobilized in a manner, which would provide retention of their native properties and catalytic activity. Enzymes can eventually be immobilized on electrodes to form cost effective and sensitive biosensors or nanosensors. Biosensors are large scale nanosensors, which consist of a bioreceptor and a transducer. One type of bioreceptor consists of an enzyme immobilized on the surface, which recognizes the target analyte. The transducer converts the enzyme response into measurable electronic signals. Biosensors provide great specificity, sensitivity and convenience along with accurate and fast analysis. They are also widely used for clinical and industrial purposes. A good example is the biosensor, which is used for measuring the glucose level in a diabetic patient's blood.

Immobilization of enzymes on nano-scale channels for metabolite detection will enormously reduce manufacturing, packaging, storage, and shipment costs of nanosensors. This is due to employing less material, packaging appliances, storage area, and shipment weight. In addition to these advantages, this technology may provide the possibility for real time analysis of metabolites available in all body fluids using small volumes of samples. This is especially useful when large sample volumes are not available and there are limitations in sample collection. Some of the main components of a nanosensor and preliminary studies related to its successful construction are outlined in the following section.

1.1 Background

1.1.1 Enzymes

The word enzyme is the Latin word for "in yeast," where enzymes were originally detected. A Swedish chemist by the name of Jöns Berzelius was the first scientist to study enzymes in 1835. James B. Sumner, the 1946 Noble-prize winner, obtained the first enzyme in a pure crystalline form in 1926 [1].

Enzymes are biological catalysts of high molecular weight, which accelerate biochemical reactions occurring inside living organisms. They are proteins and therefore are assemblies of amino acids. Enzymes are effective at low concentrations and are not conserved in the reaction they catalyze. Each enzyme normally catalyzes only one specific chemical reaction in which a substrate binds to a specific site on the enzyme, called the active site. A substrate is a small molecule that fits into a certain

region on the much larger molecule [2]. Figure 1 is a representation of the key and lock model, which describes the function of an enzyme.

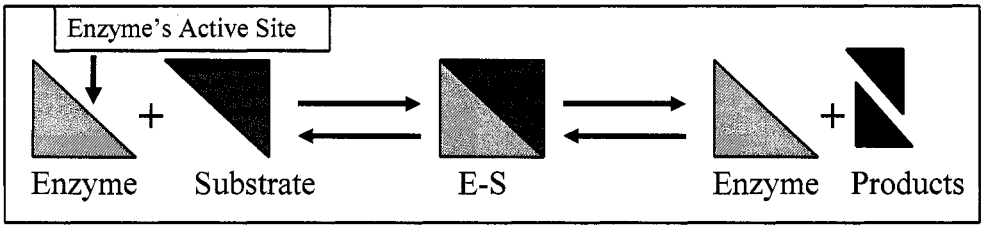


Figure 1. Key and lock model. Modified from original [5].

Enzymes are specific proteins for one particular reaction or a group of related reactions. They will only function if the shape of the substrate matches their active sites. They can be specific even down to reacting with the L- or D- isomer, and this specificity results in fewer unfavorable by-products. As a result of binding with the substrate and during catalysis, conformational changes occur, which may then be detected and quantified [2].

The rate of production [P] depends on the enzyme concentration [E] and substrate concentration [S]. Enzymatic reactions are approximately ten billion times faster than non-enzymatic reactions. Enzymes are widely used in chemical and drug industries, clinical diagnostics and therapeutics. They are also biodegradable and therefore, can return to nature without any human recycling [2].

1.1.2 Immobilization Surface

The surface, which an enzyme is to be immobilized on, must possess specific structural properties. These properties allow the formation of non-covalent interactions such as hydrogen bonding, hydrophobic interactions or chemical associations with the enzyme molecule. Self assembled monolayers (SAMs) provide a suitable surface for the purpose of immobilization with the possibility for retention of flexibility and stability.

1.1.2.1 Self-assembly on Gold

Self-assembly on gold was developed in 1983 by employing alkanethiols. Self assembled monolayers on gold have proven to be extremely suitable for immobilization experiments. Formation of two-dimensional crystals on gold by employing alkanethiols and sulfhydryl groups that covalently bind to the surface, greatly affects the biochemical properties of the substrate. Self assembled monolayers form on the gold surface with a thickness of nanometer scale and are structurally well defined [6].

Thiol (S-H) head groups and gold substrates are commonly used for forming SAMs. The thiol molecules get adsorbed to the gold surface from the solution as shown in Equation 1, creating an intense monolayer with tail groups protruding outwards from the surface. Figure 2 is a schematic of the formation of SAMs on a gold surface. Self assembled monolayers form rapidly on the surface. However, it is necessary to allow the covalent bonding process to continue for 15 hours or more. By

mixing two differently terminated thiols in the preparation solution, mixed SAMs may be constructed [7].

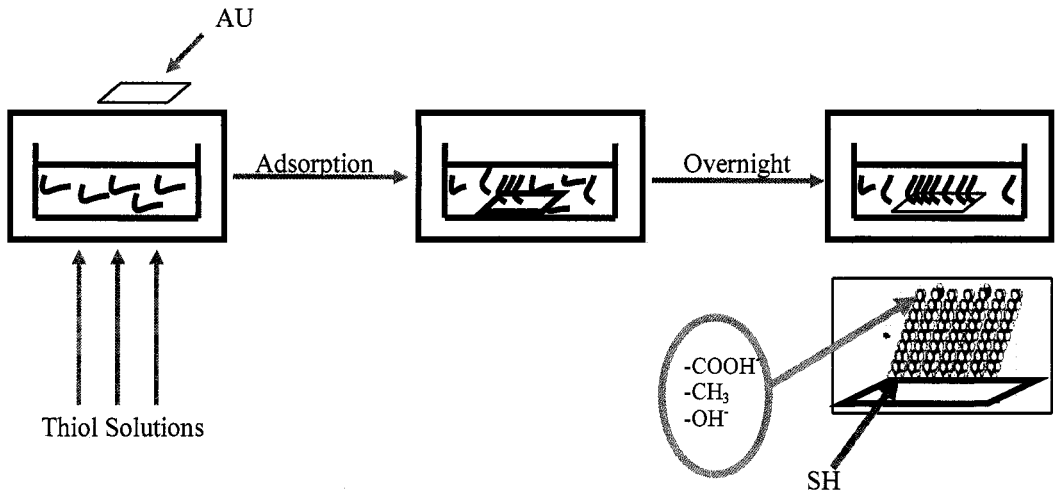


Figure 2. Formation of self assembled monolayers on gold. Modified from original [7].

$$R\text{-}S\text{-}H + AuO_n \rightarrow R\text{-}S\text{-}Au^{+} \cdot AuO_n + \tfrac{1}{2} H_2 \qquad \text{Equation 1}$$

1.1.2.2 Contact Angle

Contact angle (CA) is the angle θ between the tangent line to a liquid droplet resting on a solid surface, and the solid surface itself. The mentioned angle is usually measured to characterize the wetting and adhesion properties of a solid by calculating its solid-vapor surface tension. It is also used to determine the hydrophobicity or hydrophilicity of the surface. Equation 2 presents the relation between surface tensions at different interfaces and the contact angle as shown in Figure 3.

$$\gamma_{SL} + \gamma_{LG} \cos\theta = \gamma_{SG} \qquad \text{Equation 2}$$

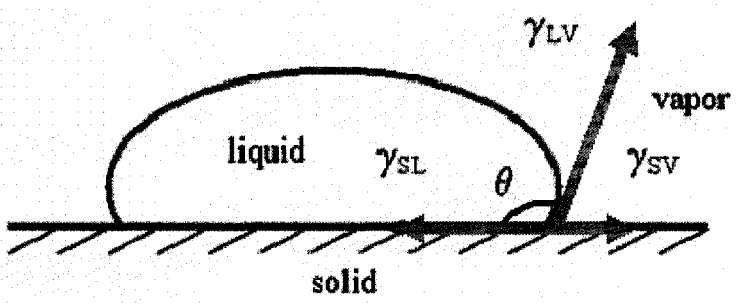


Figure 3. Image of a droplet spreading over a surface. Modified from original [8].

Surface tension is the force generated by surface molecules attracting each other. Cohesive force keeps the liquid molecules united to one another while the adhesive force causes the molecules to adhere to the solid surface. When adhesion forces exceed cohesion forces the liquid wets the surface. On the other hand, greater cohesion force causes the liquid to refuse the surface. In contact angles smaller than 90°, the solid is referred as wettable while in contact angles wider than 90°, the solid is called non-wettable [9].

1.1.3 Immobilization Methods

The idea of immobilization was developed in 1970's, and the Tanaka Seiyako Company in Japan was the first company to use it commercially [1]. Enzyme immobilization refers to making the enzyme stationary by means of adsorption, covalent bonding or gel entrapment. Immobilization stabilizes the enzyme by

bounding it to the surface, and also provides the possibility of multiple usage of the enzyme. It is critical to select a method of immobilization that protects the active sites during attachment.

Covalent immobilization allows the enzyme to be at a fixed position on the surface. The suitability of a carrier material for immobilization is very important. An appropriate carrier material allows the enzyme to stick and stay on the surface with fewer attachments and good retention of native properties. The wide variety of binding reactions, insoluble carriers with functional groups capable of covalent coupling, and surface activation have made this method of immobilization very popular [3].

Physical adsorption of an enzyme onto a solid surface is the simplest and most popular method of enzyme immobilization, which is imposed by hydrophilic and hydrophobic forces. This non-specific physical binding is partly by multiple salt linkages and Van der Waal's forces, and is mainly by hydrogen bonds. In adsorption, no chemical reagents are usually required and therefore, this method is not expensive, can be easily done, and is less damaging to the enzyme in comparison with covalent bonding. Since the formed bonds are weak, desorption of the enzyme is likely to occur by changes in pH, ionic strength, temperature, and even slight presence of substrates [3].

1.1.4 Model Enzymes

Enzyme assay is the determination of the biological properties of an enzyme. Thus, to determine enzymatic activity, an assay must be designed. In the proposed

work, enzymes with high conformational flexibility and stability are desirable as they provide better detection and durability. Myokinase and glutamate dehydrogenase (GDH) are good candidates, as they possess high flexibility and allosteric properties, respectively.

Until the midst of the 20th century, it was not known how the second labile phosphate group in the adenosine triphosphate (ATP) molecule could participate in ATP dependent reactions. Colowick and Kalckar discovered that this problem did not exist in the muscle. They determined that a certain enzyme was catalyzing the reaction:

$$2ADP + H_20 \xleftrightarrow{\text{Myokinase}} AMP + ATP \qquad \text{Equation 3}$$

This enzyme, which could catalyze the conversion of adenosine dephosphate (ADP) to ATP, was then named myokinase (adenylate kinase). Myokinase is a transferase-type enzyme, which transfers phosphate-containing functional groups and is extracted from chicken muscle [3]. Flexibility of myokinase is the reason for its instability and ability to change its conformation, extensively. This high conformational flexibility is the reason for the selection of this enzyme in the present study. These characteristics provide a better possibility for detecting movements electrically. Figure 4 shows the three-dimensional structure of myokinase.

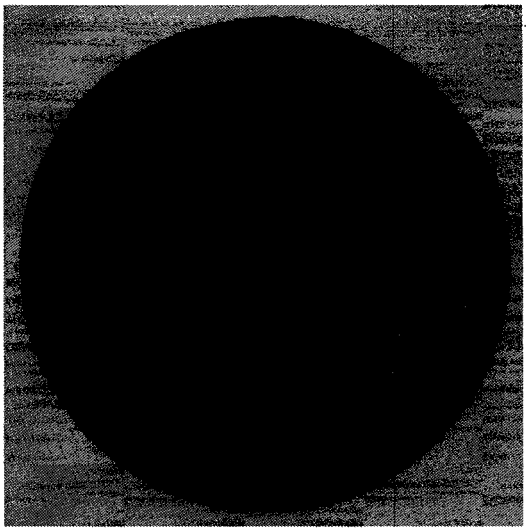


Figure 4. Three-dimensional structure of myokinase.

The catalytic activity of myokinase may also be determined by utilizing a coupled assay based on β-nicotinamide adenine dinucleotide (NADH) determination. Hexokinase catalyzes a reaction, shown in Equation 4, to convert the ATP produced according to Equation 3 to ADP and glucose-6-phosphate. Glucose-6-phosphate can further be catalyzed by glucose-6-phosphate dehydrogenase to produce NADH and 6-phosphoglucolactone as shown in Equation 5.

$$\beta\text{-D-glucose} + ATP \xrightarrow{\text{Hexokinase}} \text{Glucose-6-phosphate} + ADP \qquad \text{Equation 4}$$

$$\beta\text{-D-Glucose-6-phosphate} + NAD^{+} \xrightarrow{\text{G6PDH}} NADH + \text{6-phosphoglucolactone} + H^{+} \qquad \text{Equation 5}$$

Flexibility refers to the movement of atoms, fragments, and residues of a protein/enzyme. This movement causes both hydrophobic and electrostatic

interactions to change. This alteration could either be caused by formation and breakage of salt bridges or by change in pKa of charged residue side chains. The mentioned factors are the main reasons for enzyme stability/instability.

Glutamate dehydrogenase is an allosteric enzyme, which catalyses oxidation/reduction reactions by transferring hydrogen or electrons. The relatively large size and rigidity of GDH are the reasons for the stability of this enzyme. As evident in Equation 6, this enzyme catalyzes oxidation of glutamate to 2-oxyglutarate and ammonia. Here leucine is used as a positive effecter to enhance the catalytic activity of GDH.

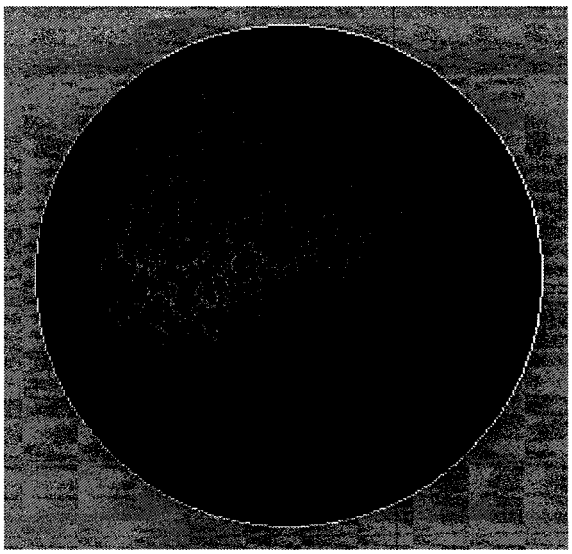
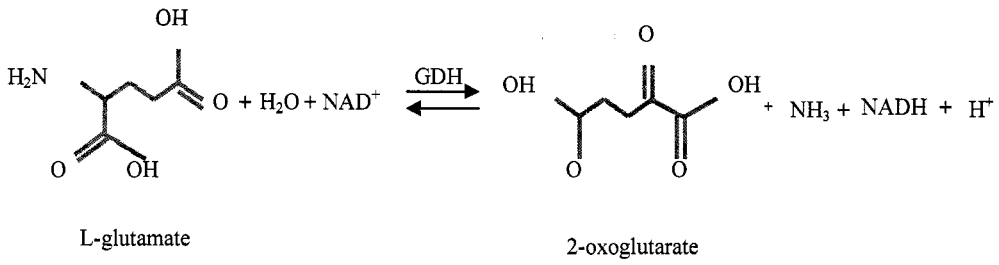


Figure 5. Three-dimensional structure of glutamate dehydrogenase.



L-glutamate

2-oxoglutarate

Equation 6

1.1.5 Enzyme Assays

As a result of employing very low concentrations of enzyme in the testing solution, sensitive assays should be developed to determine the corresponding low rates of product formation. The whole purpose of developing such assays is to perform the immobilization process, while managing to keep the enzyme active. The activity is measured by the amount of product formed from substrate in the presence of the enzyme. If the model enzyme is proven to be active after immobilization, that enzyme can have many applications such as employment in preparation of nanosensors.

1.1.6 Pyrosequencing

Pyrosequencing was first developed and refined by Nyrén in the 1990s [4]. This DNA sequencing method is based on the production of pyrophosphate (PPi) during DNA synthesis. Phosphorylated dNTPs (nucleotides) are incorporated into the DNA template in the presence of four enzymes. Each incorporation, releases inorganic pyrophosphates (PPi), which are proportional to the number of incorporated

nucleotides with a 1:1 ratio. Pyrophosphates trigger ATP sulfarylase and eventually produce ATP. Figure 6 is a visualization of the stated facts.

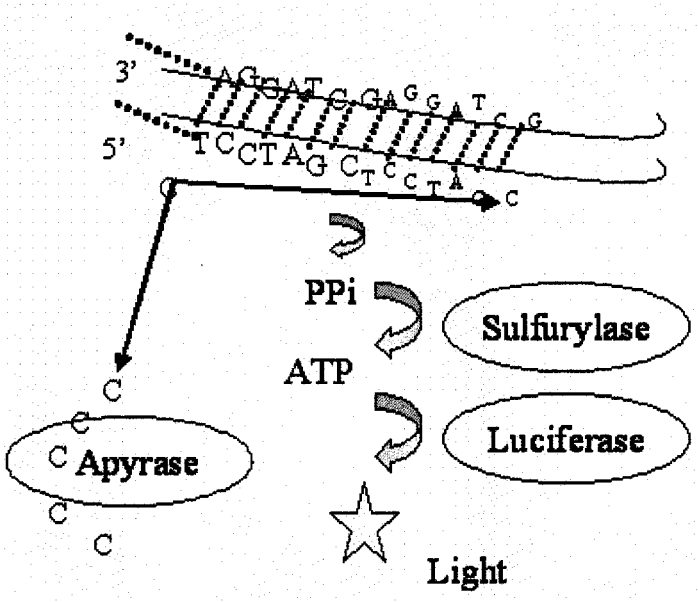


Figure 6. Different steps involved in the Pyrosequencing method. Modified from original [11].

In the present study, the PSQ 96 MA Pyrosequencer is not used for DNA sequencing. Adenosine triphosphate (ATP) itself is inserted into the sampling plates instead of the nucleotides. In other words the sequencing step is eliminated. The assay starts from one step of the Pyrosequencing method, which is for quantifying the amount of the produced ATP.

During a catalytic reaction with myokinase, ADP is converted to ATP and AMP. As shown in Figure 7, adenosine triphosphate oxidizes a substance called luciferin in the presence of luciferase to generate light [12]. Therefore, the amount of ATP produced during the catalytic reaction, is proportional to the intensity of the produced light. The generated light signals are detected by using an extremely

sensitive charged couple device (CCD) camera and the results are reported by employing very accurate software. A comparison between the generated signal from a standard ATP solution of a known concentration and a sample of unknown concentration, determines the unknown.

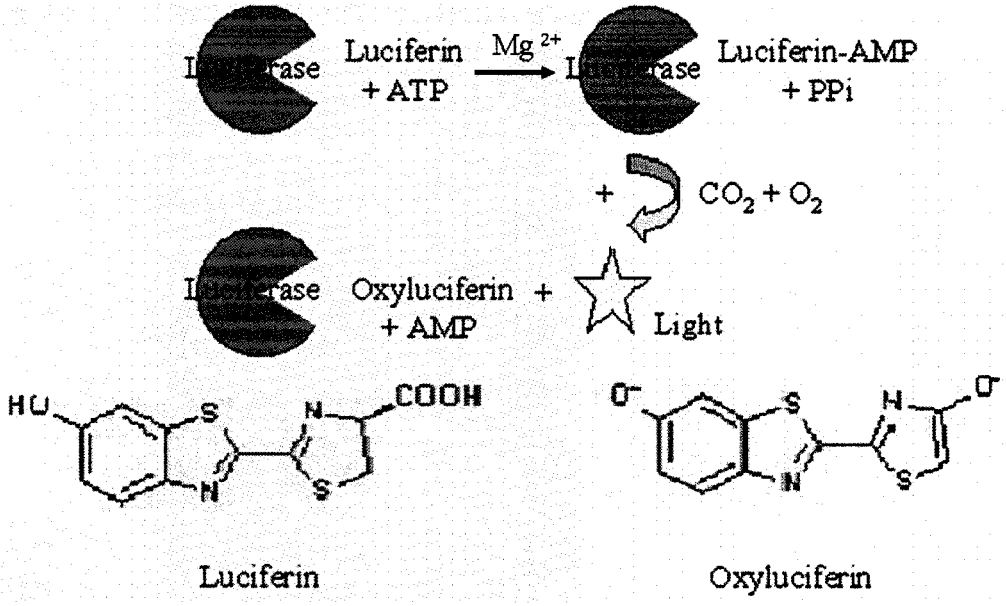


Figure 7. Luciferase bioluminescence mechanism. Modified from original [13].

1.1.7 Fluorescence

Fluorescence is based on excitation of a substance by using a light beam of a certain wavelength. This wavelength is referred to as the excitation wavelength. The energy within this beam forces the electrons to go to an orbital with a higher energy level. The high-energy electrons return to their original orbitals after a while. As a result of this return, the surplus energy is emitted at a wavelength above the excitation wavelength. This emission is called fluorescence and Fluorimeters are the

instruments used for its detection. Therefore, in order to use the Fluorimeter for detection of NADH, a fluorescence assay should be developed.

As shown in Equation 3, GDH reduces nicotinamide adenine dinucleotide (NAD^+) to NADH in the presence of glutamate. At 340 nm, NADH can be excited by absorption of light. This absorption enables the NADH electrons to get to a higher level of energy (one orbital closer to the nucleus). Soon after reaching a higher level, the electrons return to their original level while releasing their excess energy as a light emission. This emission starts at 460 nm and will fluoresce under UV light. Nicotinamide adenine dinucleotide (NAD^+) on the other hand, does not excite or fluoresce at the mentioned wavelength.

At 280 nm, NADH exhibits the same excitation property. However, NAD^+ can also be excited at that wavelength making this wavelength unsuitable for NADH detection in the presence of NAD^+. A graphic presentation of the mentioned facts is shown in Figure 8. The property difference between NADH and NAD^+ is the basis for determining the catalytic activity of GDH. By excitation of NADH at 340 nm, the amount of NADH produced during a certain period of time can be monitored.

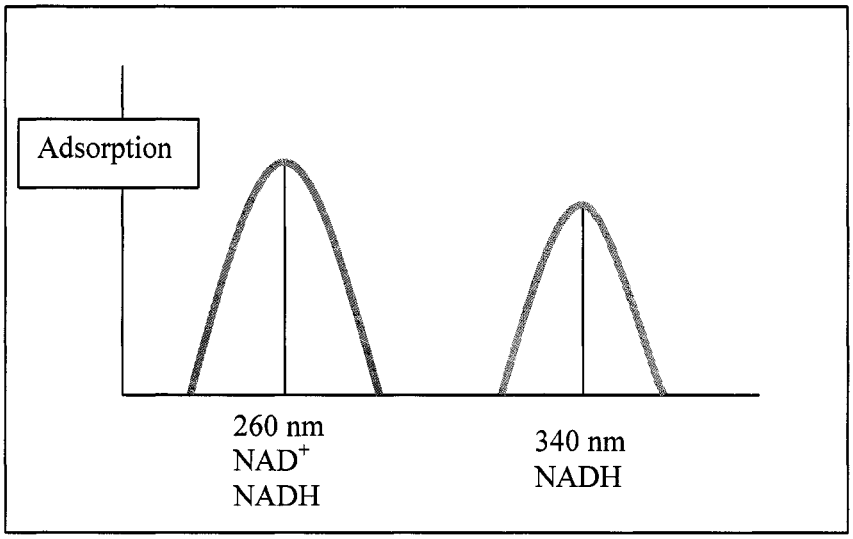


Figure 8. The adsorption spectra for NAD^+ and NADH. Modified from original [14].

Considering the knowledge that metabolite detection can provide, it is important to develop the technology for preparing nanosensors. The nanosensors can provide real time and cost effective detection due to their small size. Therefore, it is necessary to conduct a series of experiments, including characterization of different enzymes, which may lead to the achievement of these desired goals.

CHAPTER TWO
LITERATURE REVIEW

The relevant topics to the present study include, SAMs and immobilization in general, GDH and myokinase immobilization on SAM-modified surfaces, bioluminescence and fluorescence assays, enzyme quantification and some information about nano-scale biosensors (nanosensors). The following section provides a literature review concentrating in the areas mentioned above.

2.1 Self Assembled Monolayers and Enzyme Immobilization

The reason for employing self assembled monolayers is to prepare a suitable surface for immobilization. Various experiments have been conducted during the past 30 years, which have lead to a vast knowledge about the area. However, the initial experiments in the field go as early as 1946. In that year, Zisman investigated the formation of a monolayer on a metal surface by adsorption [1,10].

In 1983, Nuzzo *et al.* published the results of their experiments on preparation and use of self assembled monolayers. Diluted solutions of di-n-alkyl disulfides were adsorbed on the gold surface to form these monolayers. The significance of this study is the use of a crystalline gold surface while avoiding alkyl-trichlorosilanes [6].

In 1996, Ulman published a review paper discussing some of the factors involved in the preparation and formation of self assembled monolayers [1]. In this paper, various types of SAMs were investigated and a thorough description of the special

features of each type was presented. The other topics included the controlling effects of the forces involved in SAM formation and self-assembly on various surfaces.

In 1994, Frey *et al.* showed that the development of an ion pair between lysine ammonium groups and 11-mercapto-undecanoic acid carboxylate provides electrostatic adsorption of a polypeptide poly-l-lysine monolayer into a self assembled monolayer of the employed mixed thiol groups. The significance of this research is the reversibility of the electrostatic adsorption, which can be used in biosensors or fluorescence measurements [15]. In many applications, covalent attachments are preferred over adsorption. This preference is due to the high chance of desorption of enzyme from the surface (with adsorptive attachment) upon exposure to low or high pH, extensive washing, etc [15].

In 1996, in a complementary research to their previous work, Frey *et al.* presented an approach describing covalent bonding of poly-l-lysin on gold surfaces with alkanethiol SAMs. This amide bonding is between n-hydroxy sulfo succinimide ester intermediate and the modified surface. They proved that covalent attachment in this system is irreversible. This method is suitable for covalent attachment of proteins, peptides and oligo-nucleotides [16].

Scientists moved on to another level of research, which was the immobilization of different proteins and enzymes on gold surfaces modified by SAMs. In 2003, Nakano *et al.* investigated the covalent immobilization of glucose oxidase on 11-aminoundecanethiol monolayer-modified gold surfaces. They were the first scientists to perform single molecular imaging simultaneous to covalent immobilization of glucose oxidase on SAM-modified surfaces. They also examined the structure of immobilized glucose oxidase by employing Fourier transform infrared spectroscopy

(FTIR). They later studied an electrode with glucose oxidase immobilized on it for monitoring the electrostatic oxidation of d-glucose [17].

In 2004, Ko *et al.* examined the adsorption of photo-system I (a large protein involved in photosynthesis) on ω-terminated alkanethiol SAMs on gold surfaces [18]. The study was to determine the impact of surface composition on the adsorption process. It was demonstrated that the presence of a Triton X-100 layer on the surface, acts as an inhibitor and reduces the adsorption of photosystem I [18]. Triton X-100 is added to the system for stabilization and will make the surface protein resistant to adsorption on hydrophobic SAMs. The significance of this study is the applicability of this inhibitor to other protein systems [18].

Various experiments on surfaces with different SAMs have been conducted. Different enzymes and proteins have been immobilized and their activities and electrostatic properties have been investigated. A new approach in this field is the inspection of the effects of mixed SAMs and determination of their electrochemical properties. Larsen *et al.* in 2005 tackled these new approaches. They used a blend of mercapto octyl hydroquinone and alkylthiols for this SAM preparation on an electrode. These experiments demonstrated that mixed monolayers overcome the complications associated with intermolecular interactions as they dilute the electrochemical species within the layer. They also minimize head-group-to-head-group interactions while increasing the stability of the SAMs [19]. In addition, mixed SAMs significantly increase the electrochemical response in comparison with pure monolayers.

2.2 Myokinase and GDH Immobilization on SAM-modified Surfaces

Gooding *et al.* in 2000 investigated the influence of the distribution of two enzymes on an electrode. In their work, glucose oxidase and GDH were co-immobilized onto the surface. Self assembled monolayers are formed on the surface of the electrode by using alkanethiol, 3-mercaptopropionic acid thiol groups [20]. Immobilization of the enzymes onto SAMs is to increase the sensitivity of the thin film sensors. The results indicate the existence of a relation between enzyme distribution and sensor responses.

Blasi *et al.* in 2004 tested two different procedures, three-step and four-step procedures for the covalent immobilization of GDH onto silicon supports [21]. They employed atomic force microscopy AFM, FTIR, fluorescence and enzymatic assays to determine the structure, conformational change and activity of the immobilized enzyme. The results demonstrated that coupling through the three-step procedure does not significantly affect the activity, the structure, or the function of the enzyme. This is an indication that this method can enhance the development of well-structured monolayers for use in stable GDH-based planar biosensors [21]. Blasi *et al.* also performed fluorescence measurements and enzymatic assays on immobilized GDH in solid films to study protein conformational changes. In opposition to the three-step procedure, the four-step procedure reduced the surface coverage, due to unfavorable conditions [21].

In another work, Blasi *et al.* in 2005 examined covalent immobilization of GDH onto Si-SiO2 by AFM and enzymatic assays. With a decrease in 3-amino-propyl-triethoxysilane concentration, the specific enzymatic activity decreased while the

surface roughness increased. Therefore, the activity of immobilized GDH depends on surface conditions, and is proportional to the reciprocal of surface roughness [22].

2.3 Bioluminescence and Fluorescence Assays

As mentioned before, myokinase catalyzes the reversible conversion of ADP to ATP. The PSQ 96 MA (Multiple Application) Pyrosequencer can detect the emitted light by a CCD camera. Adenosine triphosphate produces this light as a result of oxidation of luciferin in the presence of luciferase. The detected light is proportional to the amount of ATP produced and therefore, the rate of ATP production in the myokinase enzymatic reaction can be determined. For GDH, determination of the enzymatic rate is done by a Fluorimeter. The detection is based on the fluorescence property of NADH as previously mentioned in the background section.

In 1996, Ronaghi *et al.* took the first steps toward the development of the Pyrosequencing technology. They proposed a new method that could replace gel electrophoresis. They showed that ATP-sulfurylase converts the pyrophosphate produced by DNA-polymerase into ATP. They also demonstrated that the produced ATP could be converted to light by a coupled assay of luciferase and luciferin. In their 1996 article they first brought up the necessity of manufacturing an instrument that would cover larger sequencing experiments while bringing the costs down by performing multiple samples simultaneously [23]. In 1999, Nyren *et al.* published an update on the developments made towards the automation of this DNA sequencing method [24].

In 2001, Ronaghi introduced his novel and accurate technology for DNA sequencing named "Pyrosequencing." In his article he discussed the improvements made to his previous investigations. These improvements included the substitution of dATPαS instead of dATP, and the addition of apyrase to make a four-enzyme system [25].

The Pyrosequencer is commercially available in different models including the PSQ 96 MA. In 2004, Agah *et al.* employed this instrument to model the kinetic properties of the four enzymes involved in Pyrosequencing. These four enzymes are DNA-polymerase, apyrase, luciferase and ATP-sulfurylase. They demonstrated that the results from simulation were compatible with the experimental data. They also provided the readers with the rate-limiting steps and calculated the turnover number and other kinetic properties of each enzyme. Furthermore they repeated their experiments by using mixed combinations of the mentioned enzymes and reported the effects [4].

As mentioned in the Introduction, the difference in the excitation wavelength of NAD^+ and NADH is the basis for determining the activity of immobilized GDH. The excitation wavelength for NADH is 340 nm and its emission wavelength is 460 nm. At the latter wavelength, NADH starts to fluoresce under the UV light. On the other hand, NAD^+ does not fluoresce at the mentioned wavelengths. Various scientists have held studies similar to this type of fluorescence method. Sharma *et al.* in 1994 measured different glucose concentrations by using the fluorescence method. This was done by the reduction of NAD^+ from NADH in an enzymatic reaction between GDH and glucose, and a Fluorimeter was employed to detect the amount of the glucose produced [26].

In another study, the activity of amino acid oxidase was determined by measuring the conversion of NADH to NAD^+ in the presence of GDH. The remaining NADH was destroyed by acid, and the fluorescence of NAD^+ was measured after restoring the pH with imidazole in alkaline condition [27].

In 1993, Kiba *et al.* investigated a flow-injection system with a reactor for the determination of the activity of 3-hydroxy-butyrate dehydrogenase. This enzyme was immobilized on polyvinyl alcohol beads and was packed into a stainless-steel column. The produced NADH was exited at 340 nm and was detected at 465 nm [28].

In 1996, Marcos *et al.* investigated a fluorimetric enzymatic method for determination of ethanol content. The method is based on the fluorimetric alteration caused in alcohol dehydrogenase (ADH) during the enzymatic reaction. This variation is due to the effects of NAD^+ and NADH, which are generated during the enzymatic reaction. This study explains the linear relation between the enzyme response signal and the concentration of ethanol [29].

In 1981, H. C. McDonald *et al.* studied frozen swabs by placing them in a lysis buffer containing an immobilized antibody onto 1, 2 propanediol oxidoreductase [30]. The formed antibody-enzyme composite remained active after adding the substrate. The activity was detected by taking advantage of the fluorescence property of NADH, which luminates under ultraviolet exposure.

2.4 Protein Quantification by Using Fluorescamine

In 2006, Chen *et al.* used the sensitive fluorescence detection method to quantify the total BSA protein available in the human serum. The protein bonds with

fluorescamine to generate a fluorescent derivative. In this study, a Fluorimeter was employed to measure fluorescence at 470 nm. Figure 9 shows the fluorescence spectra with and without BSA. The Figure illustrates that the fluorescamine-BSA complex is excited at 400 nm and starts emission at 470 nm. Also, it demonstrates a great enhancement of fluorescence intensity as a result of an increase in the protein concentration [31].

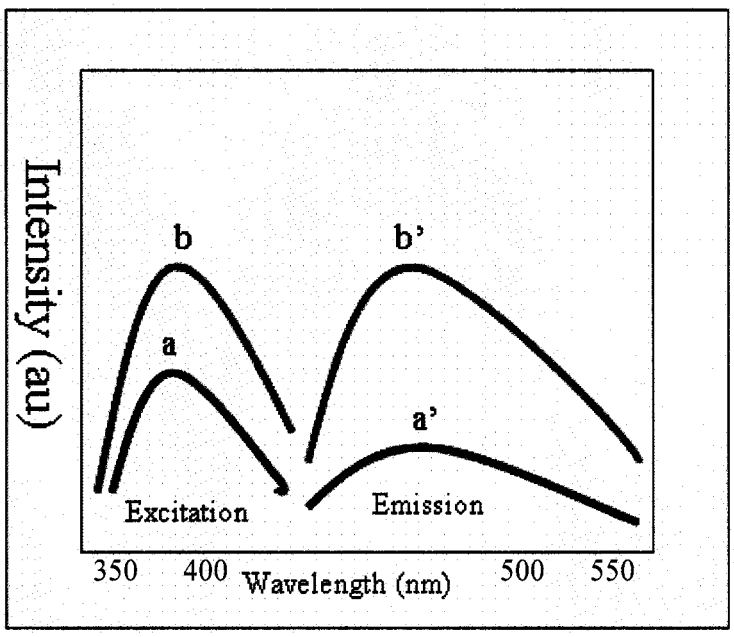


Figure 9. The fluorescence spectra of fluorescamine (a)in presence and (b) in absence of BSA. Modified from original [31].

2.5 Nanosensors

Employing enzyme immobilization as an analytical tool has increased because of the development of enzyme electrodes. The enzyme in a biosensor recognizes the substrate and specifically converts it into a product. If an enzyme in a biosensor is

specific for catalyzing a certain substrate, then the sensor is only useful for detection of the same substrate. The enzyme will not catalyze another substance even if its structure is very similar to that substrate. This fact makes biosensors very specific.

In 2002, Delvaux *et al.* prepared SAMs on gold electrodes. They then converted it to a biosensor by immobilizing glucose oxidase on the nano-electrodes inside nano-tube arrays by covalent carbodiimide attachment. The results generated from the mentioned biosensor indicated high sensitivity [32].

In another study in 2003, Delvaux *et al.* introduced a new method for immobilization of glucose oxidase inside metallic nano-tube arrays. Arrays of nanoscopic gold tubes were prepared by electronless deposition of the metal through the pores of polycarbonate particle track etched membranes (PTM) [32]. Trau *et al.* conducted encapsulation of glucose oxidase microparticles inside a nano-scale layer by layer film, in 2003 [33].

Retention of enzyme stability is extremely crucial in biosensors. In 1999, Von Woedtke *et al.* studied the stability of immobilized enzymes. Long term stability could be reached by using human serum albumin (HSA), rhodalbumin and glutaraldehyde with glucose oxidase (GOD). A rapid decrease in sensitivity was observed after sensor preparation and testing. The same immobilization procedure on the surface of electrochemical sensors has been used very successfully in the case of lactate oxidase (LOD) [34].

2.6 Summary

In the presented section the work of many scientists has been reviewed. Much work has been done on preparation of SAMs and enzyme immobilization on modified gold surfaces. The behavior of immobilized GDH has been studied to some extent, while no work has ever been done on adenylate kinase (myokinase). In the present study, immobilization of myokinase and GDH on different SAM-modified gold surfaces will be investigated and the enzymatic activities will be determined by means of different detection methods.

The two employed detection methods are bioluminescence and fluorescence assays. Fluorescence is a much older method of detection and much work has been performed using this procedure. Determination of GDH activity has been done under different circumstances but similar to the present study. On the other hand, Pyrosequencing is a newly developed technology and limited work has been performed in that area. Determination of the ATP production rate by Pyrosequencing has never been done for myokinase and therefore, is being investigated in the present study.

Immobilization of enzymes on nano-electrodes for preparing a nanosensor is the next step of the presented thesis, and knowledge about the field and previous studies can provide a better outlook for conducting the experiments. Therefore, the literature available on the subject has been briefly reviewed.

CHAPTER THREE
RESEARCH OBJECTIVES

3.1 Research Objectives

The main objective of this study was to show the principle that Pyrosequencing and Fluorimetery can be employed to investigate the effect of four different surface chemistries on immobilized enzyme activities. Myokinase was the enzyme studied with Pyrosequencing since it produced ATP during its catalytic activity. The enzymes studied with Fluorimetery were GDH and myokinase since they both produce NADH during their catalytic activities. In order to complete these objectives the following subtasks were completed:

1. Comparison of immobilized enzymatic activities by using four different thiol group combinations for surface chemistry (100% $COOH^-$, 10% $COOH^-$+ 90% OH^-, 100% CH_3 and 100% OH^-).
2. Determination and comparison of the activities in free and bound forms.
3. Determination of enzyme stability.
4. Determination of the GDH loaded on each chip surface in order to determine specific activity.
5. Determination of GDH release as a result of continuous flow of substrates over a twenty-four hour period.
6. Determination of the effect of allosteric effectors on GDH (with allosteric sites) for both covalent and adsorptive attachments.

Enzyme activity has a linear relationship with product formation and as a result, higher production rate corresponds to higher enzymatic activity. Modifying a gold surface by different thiol groups (different surface chemistries) for immobilization provides a better possibility for determining an environment in which, the enzyme is able to retain its native properties. The properties of four surface chemistries were quantitatively compared with one another to obtain the most efficient one among all. The most efficient method is the one that provides the highest activity, with the strongest association and conformational flexibility.

The activity of an enzyme in immobilized form may be significantly less than its corresponding activity in the free form. This may be due to the fact that some active sites are blocked when they attach to the surface and are not able to undergo the catalytic process. Mass transfer and diffusion limitations may be other contributing factors. The activities of the enzyme were compared in free and bound forms in order to determine the effect of immobilization on enzymatic activity.

Different enzymes have different structures. As a result, their stability and activity upon immobilization is different. The stability of the immobilized enzyme was tested by determination of enzymatic activity several days after immobilization.

Enzymes with allosteric sites, such as GDH, respond differently to different allosteric effectors. In some cases, as a result of immobilization, enzymes with allosteric properties do not respond to allosteric effectors. Covalent or adsorptive methods of attachment exhibit different responses to these effectors. The effect of allosteric effectors was studied on free and bound forms of the enzyme and also on both covalent and adsorptive attachment methods.

CHAPTER FOUR
EXPERIMENTAL DESIGN AND APPARATUS

In the present work, many experiments were conducted. The experiments mainly focused on the development of assays and determination of myokinase and GDH activities using surfaces modified with different monolayers. Figures 10a and 10b present a visual overview of the thesis.

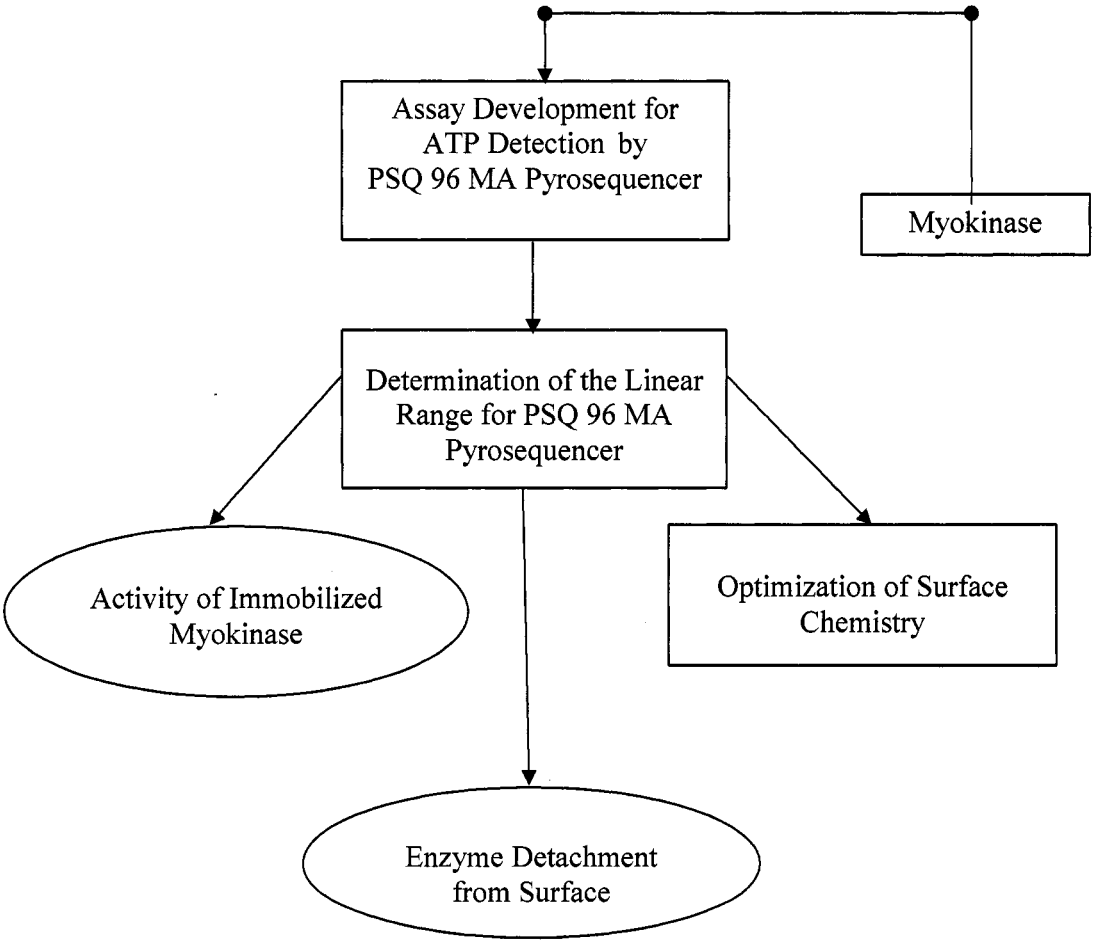


Figure 10a. Experimental overview for myokinase by using bioluminescence assay.

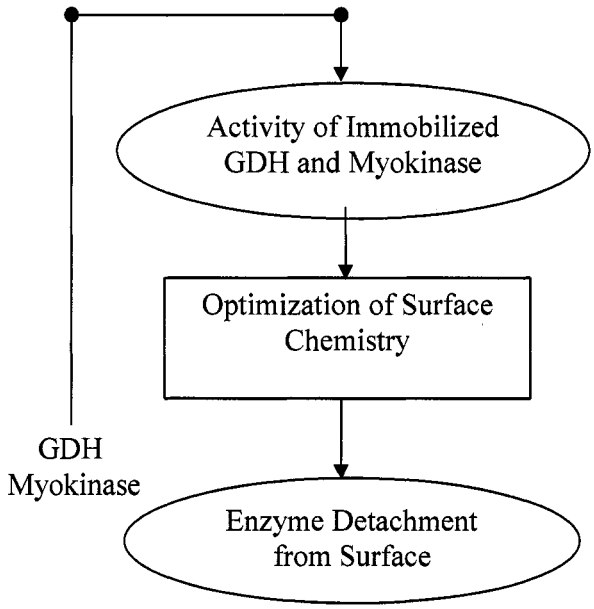


Figure 10b. Experimental overview for myokinase and GDH using fluorescence assays.

4.1 Assay Development for ATP Detection with the PSQ 96 MA Pyrosequencer

The PSQ 96 MA Pyrosequencer was used to detect the level of ATP produced during the enzymatic activity. Since this apparatus was originally designed for DNA sequencing, a new assay had to be developed for ATP detection.

For this assay determination, the total volume of 55 μL and the ATP concentration of 0.002 mM were chosen as fixed parameters. One micro-liter of luciferin and 1 μL of luciferase with five different concentrations of each were used in combination with other varying factors as shown in Table 1 (page 44). The total number of experiments was 130. The condition, which gave the highest signal level with a saw tooth peak, was chosen. A saw tooth peak is an indication of instant saturation of luciferase in reaction with luciferin.

4.2 Determination of the Linear Range of the PSQ 96 MA Pyrosequencer for ATP Detection

The PSQ 96 MA Pyrosequencer was not optimized for the kind of ATP detection used in this work. If the concentration of ATP would exceed a certain level, the plot of signal versus ATP concentration would no longer remain linear. Therefore, the linear range of the signal levels versus ATP was determined by performing the experiments stated in Table 2 (page 45). The total number of experiments was 30.

4.3 Determination of Immobilized Myokinase Enzymatic Activity; Bioluminescence Assay

After determining the best assay for ATP detection by PSQ 96 MA and defining its linear range, the experiments with immobilized myokinase were performed. As shown in Tables 3a and 3b (on pages 45 and 46), different surface chemistries with covalent or adsorptive attachment methods were prepared and tested. The total number of experiments for ATP was 736.

4.4 Determination of Immobilized Myokinase and GDH Enzymatic Activities; Fluorescence Assays

After determining the best assay for NADH detection for both enzymes by the Fluorimeter and defining the linear range, the experiments with immobilized myokinase and GDH were performed. As shown in Tables 3a and 3b (on pages 45

and 46), different surface chemistries with covalent or adsorptive attachment methods were prepared and tested. The total number of experiments for each enzyme was 736.

4.5 Equipment

The myokinase activity determination experiment is based on detecting the level of the product (ATP or NADH) formed during the enzymatic reaction at equal time intervals. For bioluminescence enzymatic assays, the PSQ 96 MA Pyrosequencer was employed; whereas for the fluorescence enzymatic assays, the Perkin-Elmer Victor counter was utilized. The front view of the PSQ 96 MA Pyrosequencer and the Fluorimeter are presented in Figures 11 and 12, respectively.

4.5.1 The PSQ 96 MA Pyrosequencer

In this study, the PSQ 96 MA Pyrosequencer shown in Figure 11 is used to detect the level of ATP produced during catalytic activity. During a coupled assay of luciferin and luciferase with ATP, light is generated and the CCD camera inside the instrument is used to detect the produced light. Based on the intensity of the produced light, the instrument exhibits different signals, which correspond to the level of ATP production. The following are some special features of the PSQ 96 MA Pyrosequencer [35]:

1. Generates actual sequence information.
2. Provides quality control in every run for each sample by means of an automatic quality assessment done by the software.

3. Its pyrograms (the resulting plots) are quantitative and are proportional to the nucleotide base sequence.
4. Is ideal for genetics and clinical research.
5. Provides prompt access to sequencing information.
6. Performs sequencing of 50 bases in less than an hour.
7. Its 96 well microtiter plates enables up to 96 sequences in parallel.

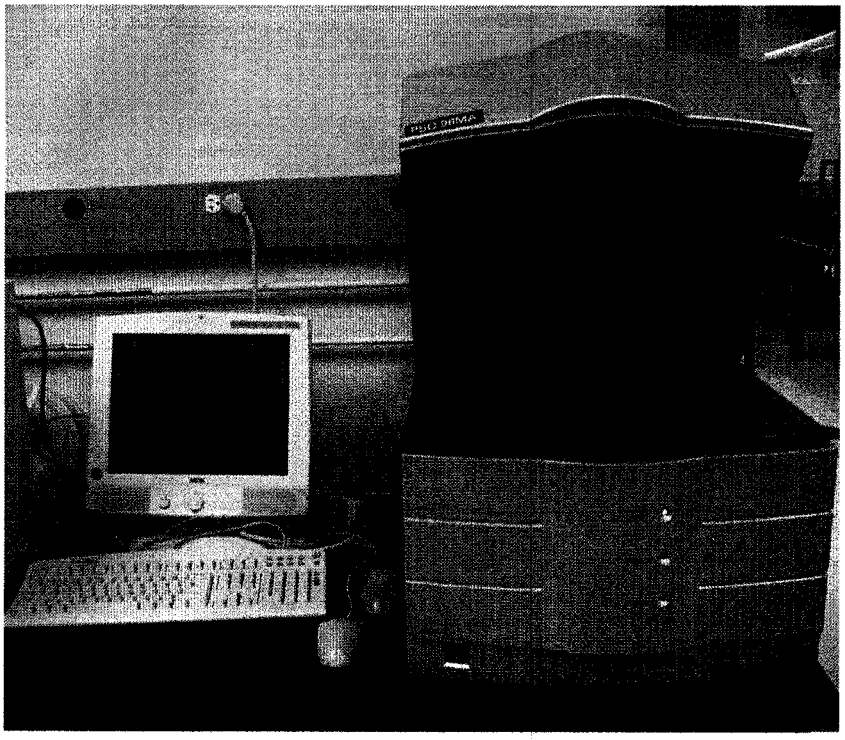


Figure 11. The front view of the PSQ 96 MA Pyrosequencer.

4.5.2 The Fluorimeter

The Perkin-elmer Victor light shown in Figure 12 is a bioluminescence instrument for micro-plate applications such as cell-based assays and toxicology

screens. The system can be upgraded by means of available accessories in case of specific experimental requirements. This small unit can operate as a stand-alone unit or as a robotic system [36]. The filters installed in this instrument excite NADH at 355 nm and start reading at 460 nm. The excitation wavelength is a bit off from the absorption peak but that should not significantly affect the results.

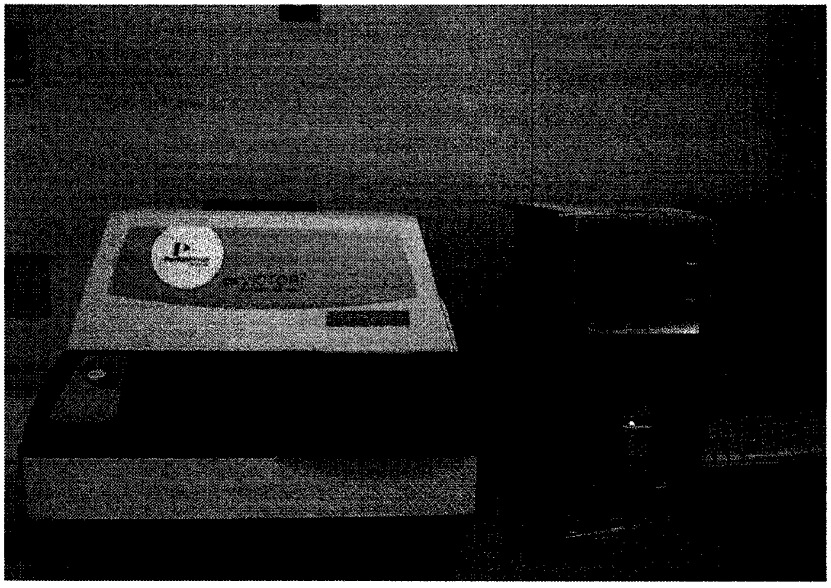


Figure 12. The front view of the Victor light Fluorimeter.

4.5.3 Continuous Flow Measurement Setup

The continuous flow measurement hand made setup shown in Figure 13a was designed and prepared especially for the experiments conducted in this study. The gold chip with immobilized enzyme was placed inside the minireactor shown in Figure 13b and a continuous flow of substrate was allowed to pass over the chip over a period of time.

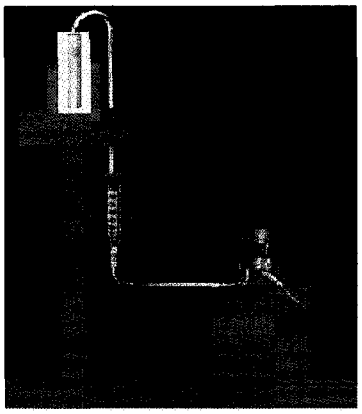


Figure 13a. Continuous flow measurement setup.

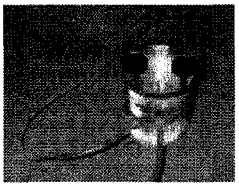


Figure 13b. Hand made mini reactor used in the continuous flow operation.

4.6 Procedure Steps

4.6.1 Preparation of ATP-free ADP

The prepared ADP solution contained a high level of ATP. The coupled assay of luciferase-luciferin with ATP required the ADP solution to be free of ATP. Therefore, all the ATP available in the solution had to be converted to ADP. During an enzymatic reaction, hexokinase catalyzes the conversion of ATP to ADP in the presence of β-D-glucose (see Equation 4, page 9). This conversion is the result of ATP binding to the enzyme in a complex with Mg^{++}. It should be noted that since this conversion is not 100 percent, therefore the solution cannot be completely ATP free.

Since ATP is produced during the coupled assay with myokinase, hexokinase had to be filtered out of the system. Otherwise, it would convert all the produced ATP back to ADP. In order to filter hexokinase, a centrifuge and a 10,000 Molecular Weight Cut Off (MWCO) Viva-science filter were required.

In order to prepare the ADP solution, 74 mg of dry ADP was weighted out and 50 mL of Tris-$MgCl_2$ buffer (pH=7.9) was added to it. To make the conversion of ATP to ADP possible, 2 mg of lyophilized hexokinase along with 90 mg of β-D-glucose was added to the ADP solution, and the mixture was left on a shaker for 10 minutes at room temperature. Then the mixture was poured on top of the filters and was placed inside the centrifuge to spin at 3000 rpm for 15 minutes. The filtered samples were tested for ATP level and were stored in the freezer to avoid conversion of ATP back to ADP.

4.6.2 Chip Cleaning with RCA (Developed in Radio Corporate of America)

The 0.7 cm × 0.7 cm chips, which were prepared by sputtering 99.999% pure gold on top of a layer of chromium attached to a silicon body, were placed inside toluene and were allowed to shake on a shaker for 1.5 hours. After being washed first with ethanol and then by DI water, they were cleaned by the RCA procedure. After RCA cleaning, the chips were washed with DI water. Following this, the chips were washed with ethanol and dried with argon. Each chip was placed inside a 5 mL eppendorf tube.

4.6.3 Thiol Groups and Self Assembled Monolayer Preparation

The following are the four chosen thiol solutions used for preparation of SAMs:

1. 100% ($COOH^-$)-C_{11}, (FMW=218.36)
2. 100% (CH_3)-C_{16}, (FMW=258.1)
3. 100% (OH^-)-C_{11}, (FMW=204.38)
4. 10% ($COOH^-$)-C_{11} + 90% (OH^-)-C_{11}

Based on the chosen surface chemistry, a 1 mM thiol solution in 99.6% purified ethanol was prepared. For both pure thiol groups and combination chemistries, 900 μL of the prepared thiol solution was placed on top of each chip inside the tubes. The tubes were kept in a dark place over night in order for the monolayers to start forming on the surface of the chips.

4.6.4 Enzyme Immobilization: Adsorptive and Covalent Attachments

4.6.4.1 Adsorptive Attachment

The chips from step 4.6.3 were washed with ethanol, dried under a stream of argon and were placed inside new 10 mL BD tubes. An enzyme solution was prepared and 900 μL of it was pipetted on top of each chip inside the new tube. The tubes were allowed to shake on the shaker for four hours so the enzyme would attach to the surface. It should be noted that for myokinase, 1000 units of lyophilized myokinase (Sigma, 0.47 mg solid) was dissolved in 10 mL of 3-(n-morph olino propane sulfonic acid (MOPS) buffer (pH=8.3). On the other hand, GDH was

provided in solution (Sigma, 43 mg/mL), therefore 5 μL of it (10 units, 0.215 mg) was added to 1 mL of the MOPS buffer (pH=8.3).

4.6.4.2 Covalent Attachment (a): Surface Activation

In order to make an activating solution, 3.6 mM 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) plus 11 mM n-hydroxy succinimide (NHS) was prepared in 30 mM 2-morpholino ethane sulfonic acid monohydrate (MES) buffer, pH=5.5. The modified chips from step 4.6.3 were washed with ethanol, dried with argon and were later placed inside new 10 mL tubes. Nine hundred μL of the activation solution was placed on top of each chip inside the new tubes and the chips were allowed to shake on a shaker for one hour. This step was only used for $COOH^-$ ending monolayers that could gain the capacity to bond covalently to the enzyme as a result of surface activation.

4.6.4.3 Covalent Attachment (b): Coupling

The chips from Section 4.6.4.2 were washed with di-ionized water (DI) and were placed inside new 10 mL BD tubes. Lyophilized enzyme was dissolved in MOPS buffer (pH=8.3) and 900 μL of this solution was placed on top of each chip inside the new tubes. The tubes were allowed to shake on a shaker for four hours. Once the four-hour coupling period was over, the tubes were placed in the refrigerator.

4.6.5 Sample Preparation for Myokinase Bioluminescence Assay

Five hundred μL of 1mM ADP solution (ATP free substrate solution) was placed inside a vial 30 minutes prior to testing a chip. This was done to allow the ADP solution to reach room temperature in order to avoid variation in the activity of the enzyme caused by temperature difference. Each chip had to be tested separately in a 40-minute experiment. Therefore, the chip was placed in a new tube containing 1 mL Tris-$MgCl_2$ buffer (pH=7.9) and then it was left on shaker for one minute. This washing step was repeated three times in order to wash away any unbound enzyme present on the surface of the chip.

4.6.6 Myokinase Bioluminescence Enzyme Assay

For the myokinase (Sigma, 1000 unit, 0.47 mg solid) bioluminescence assay, an assay mix had to be prepared first. To prepare the assay mix, an assay buffer was prepared by adding 1 μL of 0.2 mM dithiothreitol (DTT) to 99 μL of 1 mM coenzyme A in Tris acetic (TA) buffer. Also, a luciferase buffer had to be made for preparation of luciferase solution. This buffer was prepared by addition of 0.003 g of Tris succinate and 0.023 g of DTT to 50 mL distilled water. Finally, the assay mix was prepared by adding 48 μL of the prepared assay buffer to 1 μL of the 5 fold diluted luciferase (14.9 mg/mL from Promega) and 1 μL of the sample. The luciferase was diluted by 50% glycerol, which was made by adding 1 volume of luciferase buffer to 1 volume of glycerol.

For the assay of the immobilized myokinase, the washed chip from Section 4.6.5 was placed in the substrate solution vial also prepared in Section 4.6.5, and the vial was immediately placed on the shaker. One-microliter samples were taken every two minutes for 24 minutes while the vial was shaking on the shaker. The 1 μL samples were directly pipetted into their designated wells on the 96-well plate. It should be noted that the designated wells were filled with 49 μL of the assay mix right before adding the sample.

Addition of luciferin was done at the very end by injection from the Pyrosequencer's cartridge. To prepare the luciferin solution, 5 mL of luciferin buffer (50 mM magnesium acetate buffer, pH=7.6, plus 0.2 g polyvinyl pyrrolidone PVP) was added to 5 mg of D-luciferin from Sigma (4, 5-dihydro-2- [6-hydroxy-2-benzothiazolyl]-4-thiazole carboxylicacid, sodium salt, $C_{11}H_7N_2O_3S_2Na$). Seventy-five microliters of this solution was placed inside the Pyrosequencing cartridge. Five microliters of the solution inside the cartridge was injected to the sample plate by the PSQ MA instrument to obtain real-time results. The cartridge had to contain at least 50 μL of luciferin at all times, for accurate injection.

4.6.7 Stabilization of Myokinase

In order to stabilize the immobilized myokinase, a 50% glycerol + 45 mM imidazole buffer pH=7 was prepared. The first step was to prepare a 0.2 mM imidazole buffer by adding 1.36 g imidazole to 100 mL distilled water. The second step was to adjust the buffer's pH to 7. By adding 45 mL of the prepared buffer to 55 mL glycerol, the desired stabilizing buffer was prepared.

For free enzyme stabilization, 100 μL of 2000 unit/mL myokinase dissolved in MOPS buffer (pH=8.3) was added to 900 μL of 0.1 mM imidazole (pH=7). After preparing this enzyme solution, 750 μL of it was added to 750 μL glycerol to make the stabilizing buffer for free myokinase, which is 50% glycerol plus 45 mM imidazole (pH=7) plus 3.5 mM MOPS buffer (pH=8.3).

4.6.8 Sample Preparation for Myokinase Fluorescence Assay

Five hundred microliters of the assay mix, ATP free ADP plus NAD^+-glucose plus hexokinase/glucose-6-phosphate-dehydrogenase (HK/G6PD) was placed inside a vial, thirty minutes prior to testing a chip. This was done to allow the substrate solution to reach room temperature to avoid variation in the activity of the enzyme.

Each chip had to be tested separately in a five-minute experiment. Therefore, the chip was placed in a new tube containing 1 mL Tris-$MgCl_2$ buffer (pH=7.9) and then it was left on the shaker for one minute. This washing step was repeated three times in order to wash away any unbound enzyme present on the surface of the chip. For the free enzyme test, the myokinase solution was prepared by addition of 1 mL of the MOPS buffer (pH=8.3) to 1000 units of myokinase.

4.6.9 Myokinase Fluorescence Enzyme Assay

For the myokinase fluorescence assay, an assay mix had to be prepared first. NAD^+-glucose solution was prepared by the addition of 20 mg NAD^+ and 25 mg glucose to 500 μL of Tris $MgCl_2$ buffer (pH=7.9). Due to the unstable nature of

NAD^+, it should be prepared in the same day as the experiment day. Since the testing conditions for free and immobilized forms were slightly different, the assay mix for each form was prepared separately.

The assay mix for immobilized myokinase was prepared by adding 20 μL of the prepared NAD^+-glucose solution to 477 μL of ADP (ATP free) plus 3 μL of HK/G6PD. The washed chip from Section 4.6.8 was placed inside the assay mix vial (also prepared in Section 4.6.8) and was immediately placed on a shaker. Fifteen-microliter samples were taken every minute for five minutes. The samples were directly pipetted to the designated wells on the 384-well plate belonging to the Fluorimeter.

The assay mix for free myokinase was prepared by adding 1 μL of the NAD^+-glucose solution to 17 μL of ADP solution (ATP free) plus 1 μL of HK/G6PD. Finally, 0.2 μL of the myokinase solution prepared in Section 4.6.7 was added to 19 μL of the assay mix inside a vial, and the vial was immediately placed on the shaker. Samples with 19.2 μL volumes were taken every minute for five minutes. The samples were directly pipetted to the designated wells on the 384-well plate belonging to the Fluorimeter.

4.6.10 Sample Preparation for GDH Fluorescence Assay

Five hundred microliters of the assay mix, potassium phosphate (pH=7.7) plus EDTA plus L-glutamic acid plus NAD^+, was placed inside a vial 30 minutes prior to testing a chip. This was done to allow the substrate solution to reach room temperature to avoid variation in the activity of the enzyme.

Each chip had to be tested separately in a five-minute experiment. Therefore, the chip was placed in a new tube containing 1 mL of 0.1 M potassium phosphate plus 0.2 mM EDTA (pH=7.7) and then it was left on shaker for one minute. This washing step was repeated three times in order to wash away any unbound enzyme present on the surface of the chip. For the free enzyme test, the enzyme solution was prepared by addition of 5 μL (10 units, 0.215 mg) of the GDH solution provided by the manufacturer (Sigma, 43 mg protein/mL) to 1 mL of the MOPS buffer (pH=8.3).

4.6.11 Fluorescence Assay for GDH

For the GDH fluorescence assay, an assay mix had to be prepared first. The prepared assay mix was 0.1 mM EDTA (BioRad) plus 30 mM L-glutamic acid (Sigma) plus 0.1 M potassium phosphate (JT Baker) (pH=7.7) plus 0.2 mM NAD^+. Due to the unstable nature of NAD^+, 0.0013 g of it was added to 10 mL of the assay mix right before the experiment.

For the immobilized GDH, the washed chip from Section 4.6.10 was placed inside the assay mix vial (also prepared in Section 4.6.10) and was immediately placed on a shaker. Fifteen-microliter samples were taken every minute for five minutes. The samples were directly pipetted to the designated wells on the 384-well plate belonging to the Fluorimeter.

For free GDH, 5 μL of the enzyme solution (prepared in 4.6.10) was added to 200 μL of the assay mix inside a vial and the vial was immediately placed on the shaker. Samples with 19.2 μL volumes were taken every minute for five minutes.

The samples were directly pipetted to the designated wells on the 384-well plate belonging to the Fluorimeter.

4.6.12 Addition of Allosteric Effectors to GDH

As mentioned before, GDH is an enzyme with allosteric properties. It is important that this property would be retained upon immobilization to ensure retention of GDH flexibility. Therefore, both free and immobilized forms of GDH were tested for there response to allosteric effectors.

For the free enzyme test, five GDH solutions were prepared (see 4.6.10) in separate vials. Allosteric effectors were added to vials 2-5 to make 2 mM NAD^+, 0.2 mM NAD^+, 7.5 mg/mL L-leucine, and 0.2 mM ADP solutions, respectively. The activities of all enzyme solutions were measured after 20 minutes using the fluorescence assay for free enzyme.

For the immobilized GDH test, four surface chemistries were chosen. For each of the four chemistries, five chips were prepared. Chip number one of each chemistry, was tested with normal testing conditions (see 4.6.11) while the other chips were stored for 20 minutes in the same effecter solutions used for free enzyme. The chips were all tested using the fluorescence assay for immobilized enzymes. The tested chemistries were:

1. 100% ($COOH^-$)
2. 100% (CH_3)
3. 100% (OH^-)
4. 10% ($COOH^-$) + 90% (OH^-)

4.7 Experimental Design

4.7.1 Experimental Design for ATP Detection by the PSQ 96 MA Pyrosequencer

Table 1 provides concentrations and volumes that were used in combination for developing the best assay for ATP detection. In this experiment the total assay volume was 55 μL, and the concentration of the employed ATP was 0.002 mM.

Table 1. Experimental Design for ATP Detection by PSQ 96 MA Pyrosequencer.

Luciferin 1 μL	Luciferase 1 μL	Coenzyme A in TA buffer Mm	CoA mM	DTT in TA buffer mM	DTT in TA μL
2 mg/mL	14.6 mg/mL	6	10	6	90
1 mg/mL	7.3 mg/mL	5	20	5	80
0.5 mg/mL	2.92 mg/mL	2	50	2	50
0.25 mg/mL	1.46 mg/mL	1	90	1	10
0.1 mg/mL	0.73 mg/mL	0.5	99	0.5	1

4.7.2 Experimental Design for Determination of the Linear Range for the PSQ 96 MA Pyrosequencer

Table 2 provides the concentrations of ATP that were used for determining a linear range for the PSQ 96 MA Pyrosequencer. This experiment was repeated 6 times to ensure accuracy of the results.

Table 2. Experimental Design for Determination of the Linear Range for the PSQ 96 MA Pyrosequencer.

ATP Concentration mM	Instrument Reading
0.0002	Desired
0.0005	Desired
0.001	Desired
0.002	Desired
0.005	Desired

4.7.3 Experimental Design for Immobilized Myokinase and GDH Activities

Tables 3a and 3b provide the experimental plan used for testing enzyme immobilization in covalent and adsorptive forms, respectively.

Table 3a. Experiments for Determination of Immobilized Enzyme Activity (Covalent).

Action	Thiol Group	Number of Chips	Enzyme	Activity mmole/min
Normal Condition (Within 8 Hours after Immobilization)			MK, GDH	
	100% $COOH^-$	10		Desired
	10% $COOH^-$ + 90% OH^-	10		Desired
Stability after one Week of Storage at 4° C			MK, GDH	
	100% $COOH^-$	2		Desired
	10% $COOH^-$ + 90% OH^-	2		Desired
Stability after one Month of Storage at 4° C			MK, GDH	
	100% $COOH^-$	10		Desired
	10% $COOH^-$ + 90% OH^-	10		Desired

Table 3b. Experiments for Determination of Immobilized Enzyme Activity (Adsorptive).

Action	Thiol Group	Chips	Enzyme	mmole/min
Normal Condition (Within 8 Hours after Immobilization)			MK, GDH	
	100% OH^-	10		Desired
	100% CH_3	10		Desired
Stability after one Week of Storage at 4° C			MK, GDH	
	100% OH^-	2		Desired
	100% CH_3	2		Desired
Stability after One Month of Storage at 4° C			MK, GDH	
	100% OH^-	10		Desired
	100% CH_3	10		Desired

4.7.4 Experimental Design for Free Myokinase and GDH Activities

Table 4 provides the general plan and concentration of the enzymes used in determining the activity of free enzyme.

Table 4. Experiments for Determination of Free Enzyme Activity.

Action	Enzyme Concentration unit/ml	Enzyme	mmole/min
Normal Condition (Within 8 Hours)	10	MK, GDH	Desired
Stability after one Week	10	MK, GDH	Desired
Stability after over one Month	10	MK, GDH	Desired

4.7.5 Experimental Design for Stabilization of Myokinase

Table 5a and 5b include the experiments used for stabilization of free and immobilized myokinase, respectively. The storage temperatures were –20°C and 4°C and each solution was stored for 2 weeks. For the free enzyme stabilization (Table 5a), the concentration of the enzyme used for the experiments was 2.56 unit/mL. For the immobilized enzyme test (Table 5b), surfaces modified with 100% $COOH^-$, 100% OH^-, 10% $COOH^-$ plus 90% OH^-, and 100% CH_3 were employed.

Table 5a. Experiments for Stabilization of Free Myokinase.

Storage Conditions
Condition A: Myokinase in 50% Glycerol + 45 mM Imidazole + 3.5 mM MOPS buffer pH=8.3
Condition B: Myokinase in 90 mM Imidazole pH=7 + 7 mM MOPS Buffer pH=8.3
Condition C: Myokinase in 70 mM MOPS Buffer pH=8.3
Condition D: Condition A + 10 mg/mL BSA

Table 5b. Experiments for Stabilization of Immobilized Myokinase.

Storage Conditions
Condition A: Myokinase in 50% Glycerol + 45 mM Imidazole + 3.5 mM MOPS pH=8.3
Condition B: Myokinase in 90 mM Imidazole pH=7 + 7 mM MOPS Buffer pH=8.3
Condition C: Myokinase in 70 mM MOPS Buffer pH=8.3
Condition D: Condition A + 10 mg/mL BSA

4.7.6 Determination of the Fluorescamine-GDH Linear Range

Table 6 provides the values that were used to determine a linear range for working with fluorescamine in the enzyme quantification experiments. In this experiment, the concentration of the employed fluorescamine solution in acetone was 50 mg/mL. The GDH concentration in MOPS buffer was 0.215 mg/mL and the volume taken from the prepared GDH solution for each experiment was 100 μL.

Table 6. Experiments for Determination of the Fluorescamine-GDH Linear Range.

Fluorescamine Concentration In GDH Solution (mg/mL)	Fluorimeter Reading
0.10	Desired
0.25	Desired
0.50	Desired
0.98	Desired
1.46	Desired
2.38	Desired
4.55	Desired

4.7.7 Determination of the Amount of GDH Attached to the Surface by Using Fluorescamine-GDH Assay

Table 7 provides the plan used for determination of the amount of enzyme attached to surface upon immobilization for 4 different surface chemistries. Each experiment was repeated 5 times and the employed surface chemistries were:

1. 100% ($COOH^-$)
2. 100% (CH_3)
3. 100% (OH^-)
4. 10% ($COOH^-$) + 90% (OH^-)

Table 7. Experiments for Determination of the Amount of GDH Attached to the Surface.

Activity of Free GDH before Immobilization (mmole NADH/min)	Activity of Free GDH after Immobilization (mmole NADH/min)	Surface Chemistry	Immobilized GDH Activity (mmole NADH/min)
Desired	Desired	100% $COOH^-$	Desired

4.7.8 Activity of GDH in a Continuous Flow Operation

Table 8 includes the experimental plan used for determination of GDH activity and its binding strength to the surface. This experiment was repeated many times with the same surface chemistry to ensure the accuracy of the results. The 100% $COOH^-$ surface chemistry was the chemistry employed for this experiment.

Table 8. Experiments for Determination of GDH Activity in a Continuous Flow Operation.

Time (min)	NADH Production mmole/min
0	Desired
30	Desired
60	Desired
90	Desired
120	Desired
150	Desired
180	Desired
1440	Desired
1470	Desired
1500	Desired

4.8 Data Analysis

4.8.1 Graphing Product Signal Level versus Time for Myokinase Bioluminescence Assay

As mentioned before, samples were taken every two minutes for a period of 24 minutes and were later tested by the PSQ 96 MA Pyrosequencer. The instrument provided 12 data points by generating a signal proportional to the concentration of the formed product. Although, the signal level versus time (min) is theoretically a straight line some of the generated points had minor deviation from a line. It was assumed that experimental errors caused these minor deviations. The regression line equation was determined to give us the catalytic activity (production rate) and the value of R^2. R squared represents the closeness of the regression line to a straight line. To determine the mmole ATP produced per minute, further calculations were done with consideration of the internal standard. Table 9 is an example of data inferred from the graphs generated by the PSQ 96 MA Pyrosequencer and Figure 14 is an example of the plot of signal versus time.

Table 9. Signal Levels at Different Times (ATP Production).

Time (min)	Signal	ATP Level	ATP produced in 10 min	385
0	280	0	Signal/min	38.5
2	350	35	ATP 0.0002 mM	115
4	410	95	mmole/min ATP	0.00067
6	500	185	Background Average	315
8	572	257		
12	800	485		
14	900	585		
20	1050	735		

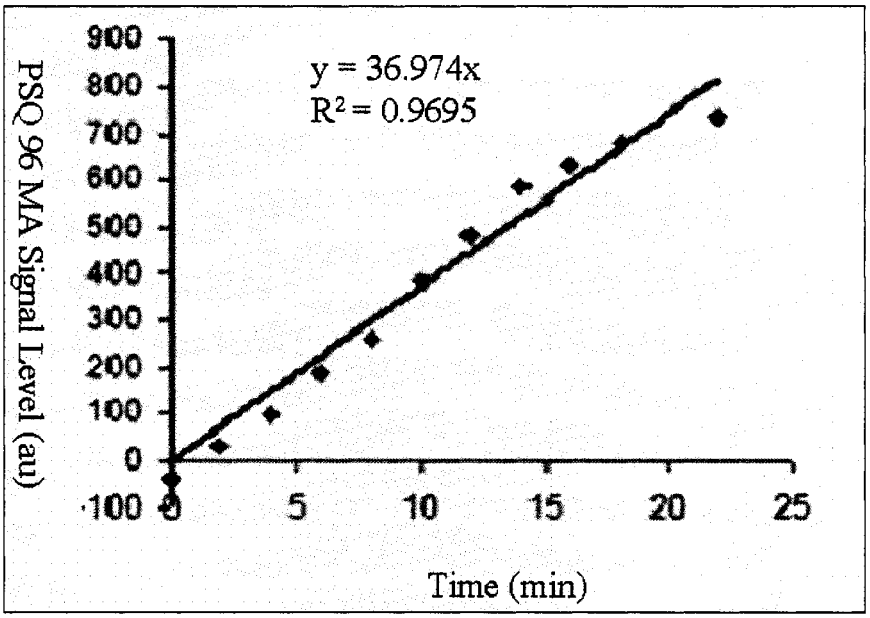


Figure 14. Product signal versus time (ATP production).

In each run, three samples were prepared solely with a 0.002 mmole ATP solution as an internal standard and the average of the signal levels were used as the reference signal. This standard was defined in order to reduce the possible errors that might have occurred from run to run. As shown in Equations 7 and 8, the amount of signal produced in 10 minutes was divided by 10 to obtain the signal/min value. The amount of mmole/min (amount of product) was further calculated by multiplying the signal/min value by 0.002 mM, followed by dividing the result by the value of the signal corresponding to that concentration (0.002 mM).

$$\text{signal/min} = \text{signal level produced in 10 minutes}/10 \qquad \text{Equation 7}$$

$$\text{mmole/min} = (\text{signal/min}) \times 0.002 \text{ mmole/standard ATP signal} \qquad \text{Equation 8}$$

4.8.2 Graphing Product Reading versus Time for Myokinase and GDH Fluorescence Assay

For myokinase and GDH fluorescence assays, samples were taken every other minute for five minutes and the samples were later tested by the Fluorimeter. The instrument provided five data points by generating a reading proportional to the concentration of the formed product. An internal standard was necessary for the same reason as mentioned in Section 4.8.1. To determine the mmole NADH produced per minute, further calculations were done with consideration of the internal standard. Table 10 is an example of data generated by the Fluorimeter and Figure 15 is an example of the plot of reading versus time.

Table 10. Signal Levels at Different Times (NADH Production).

Time (min)	Fluorimeter Reading
0	18376
2	19636
4	20515
6	20877
8	21721

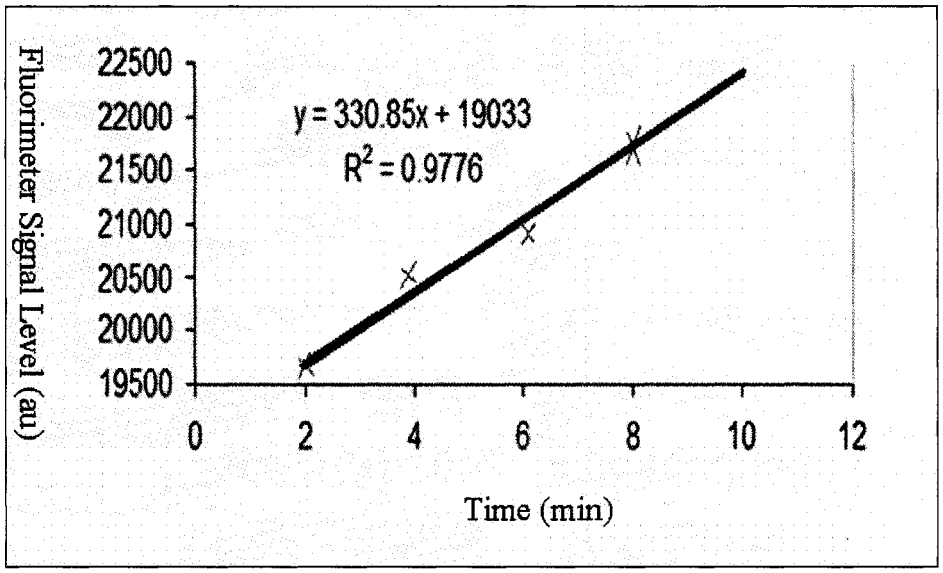


Figure 15. Product signal versus time (NADH production).

In each run, three samples were prepared solely with a 0.086 mM NADH solution as an internal standard and the average of the readings were used as the reference NADH reading. The standard fluorescence reading by the Fluorimeter was 4,5862.5. The amount of signal produced in four minutes was calculated from the regression line equation and was divided by four to obtain the reading/min value as shown in Equations 9 and 10.

$$\text{reading/min} = \text{NADH reading in 4 minutes}/4 \qquad \text{Equation 9}$$

$$\text{mmole/min} = (\text{reading/min}) \times 0.086 \text{ mmole/standard NADH reading} \qquad \text{Equation 10}$$

4.8.3 Statistical Analysis

Six chips from each of the immobilization batches were tested in one day to avoid variation in the results due to enzyme instability. A statistical analysis was performed on the data. The values that were 20 percent off from the majority of the values were eliminated before taking the average. For the statistical analysis, the average or mean value of the data ($\overline{X}$), the standard deviation (SD) and the standard error (SE) were calculated by employing Equations 11-13, respectively.

$$\overline{X} = \Sigma \text{ Activity (i)} / \text{n} \qquad \text{Equation 11}$$

$$\text{SD} = \sqrt{\frac{(X1-\overline{X})\text{^}2+(X2-\overline{X})\text{^}2+(X3-\overline{X})\text{^}2+.....}{n-1}} \qquad \text{Equation 12}$$

n is the number of the conducted experiments and $\overline{X}$ is the average activity of the n runs.

$$\text{SE} = \text{SD} / \sqrt{n} \qquad \text{Equation 13}$$

$$\text{Activity} = \overline{X} \pm \text{SE} \qquad \text{Equation 14}$$

4.8.4 Determination of the Amount of Enzyme Bound to the Surface

The amount of enzyme available in the enzyme solution, before and after immobilization, was measured by the fluorescamine-GDH assay. The difference between these values would be equal to the amount of enzyme attached to the chip. Therefore, the readings from the Fluorimeter were converted to their corresponding amount of enzyme (mg). As a result, the amount of enzyme loaded on the chip was determined.

CHAPTER FIVE
RESULTS AND DISCUSSION

In this chapter, the results of the experiments conducted in the present study are presented and discussed. The results include the developed assays for determination of the activities of myokinase and GDH, the linear range of product detection for each assay, the activities obtained for each enzyme and, tests on stability and flexibility of the two enzymes. Possible sources of error in each case have been pointed out and the approaches taken for their reduction or elimination have been discussed.

5.1 Bioluminescence Assay Developed for Myokinase

In this experiment, developing a bioluminescence assay for determination of myokinase activity was desired. As shown in Equation 3 on page 8, myokinase catalyzes the conversion of ADP to ATP. In a coupled assay consisting of luciferin and luciferase, the produced ATP is converted to light as shown in Equation 15. As discussed in Section 1.1.6 on page 11, the CCD camera inside the PSQ 96 MA Pyrosequencer is used for the detection of light. The produced light is proportional to the amount of ATP available in the sample as shown in Equation 15.

$$\text{Luciferin} + \text{ATP} + O_2 \xrightarrow{\text{Luciferase}} \text{AMP} + \text{PPi} + \text{Oxyluciferin} + CO_2 + h\nu \qquad \text{Equation 15}$$

The PSQ 96 MA Pyrosequencer was originally designed for DNA sequencing where the ATP would be produced in a series of enzymatic reactions. In this study,

samples were taken and tested during an enzymatic reaction where more ATP would be produced as the reaction proceeded. Because of the proportionality between the ATP concentration and the generated light, and also the relation between the generated signals and the intensity of the produced light, concentrations of ATP at different times during the reaction were determined. This was done by analyzing the signals generated by the PSQ 96 MA Pyrosequencer.

Since this instrument was not designed specifically for these types of experiments, and as shown in Figure 16a, the signals generated by the Pyrosequencer exhibited a curve shape rather than a clear peak. The clear peak would correspond to a definite value as the signal level. This problem was caused because the luciferase in the system would get saturated gradually rather than instantly. This was traced to the fact that the amounts of the substances in the assay affected the signal. Therefore, it was necessary to determine acceptable assay proportions in a standard way to get reproducible levels of signal. As described in the following paragraph, using the right concentrations of materials in the assay along with employing some additives in the assay, could contribute to the modification of the signals in a way that an instant saturation of luciferase would occur.

Normally, as luciferin is injected into a sample containing luciferase, light is immediately generated. The generated light reaches its peak at 0.3-0.5 seconds and then it starts to decay instantly [37]. The decay is caused as a result of luciferase inhibition by the production of the oxyluciferin by-product as shown in Equation 15. Oxyluciferin inhibits luciferase activity and therefore, to overcome this rapid extinction, coenzyme A (CoA), a thiol containing biomolecule, was added to the system. Addition of CoA was carried out to make the assay more sensitive by

providing a constant light emission rather than a rapid flash [37]. Coenzyme A makes the assay more sensitive as it displaces the inhibiting oxyluciferin substrate from luciferase to facilitate its turnover [37].

In the luciferase assay, it is very likely that CoA molecules form disulfide bonds and therefore, addition of another chemical is necessary to protect the active sulfhydryl (SH) site of the coenzyme A. Dithiothreitol (DTT), $C_4H_{10}O_2S_2$, is a thiol reducing agent that protects thiol containing proteins and molecules such as CoA from forming disulfide bonds [38]. Dithiothreitol was added to the system to protect CoA while CoA was added to make the assay more sensitive. Therefore, both of these additives, directly and indirectly contribute to reaching a more sensitive assay for luciferase. The luciferin buffer was chosen as the medium for dissolving luciferin powder as it contained polyvinyl pyrrolidone (PVP). Polyvinyl pyrrolidone has a stabilizing effect on the luciferase enzyme and this fact is useful because while using the PSQ 96 MA Pyrosequencer, the luciferin gets injected in a sample containing luciferase. The presence of PVP insures the stability of luciferase especially during long testing periods [23]. The luciferin buffer was chosen also because it was utilized in the commonly used reporter assay kits for any standard experiment employing firefly luciferase as the luciferin medium [37,38].

In a five-month period of experimentation, the suitable amounts of enzymes and substrates for usage in the assay were determined. Employing acceptable amounts of assay components led to generation of signals shown in Figure 16b, which resulted in an instant saturation of luciferase. It was found that the best combination of concentrations used for developing an optimized bioluminescence assay for myokinase was obtained by first pipetting out 48 μL of a solution consisting of 1 mM

coenzyme A and 0.2 mM DTT (1 μL of DTT + 99 μL of CoA). This volume was pipetted inside a designated well of the Pyrosequencer's testing plate. Then, 1 μL of 2.98 mg/mL luciferase prepared in 50% glycerol was added to the well followed by addition of 1 μL of the sample ATP. Finally, 5 μL of 1 mg/mL luciferin prepared in luciferin buffer (50 mM magnesium acetate buffer pH=7.6 plus 0.2 g PVP) was injected inside the same well by using the Pyrosequencer's cartridge. The 50% glycerol solution was prepared by addition of one volume of luciferase buffer to one volume of glycerol. Also, the 2.98 mg/mL luciferase solution was prepared by the addition of 80 μL of 50% glycerol to 20 μL of luciferase stock solution (14.9 mg/mL enzyme solution from Promega). Extended detail is provided in Section 4.6.6 (page 38). Figures 16a and 16b exhibit the shape of the curves before and after optimization, respectively.

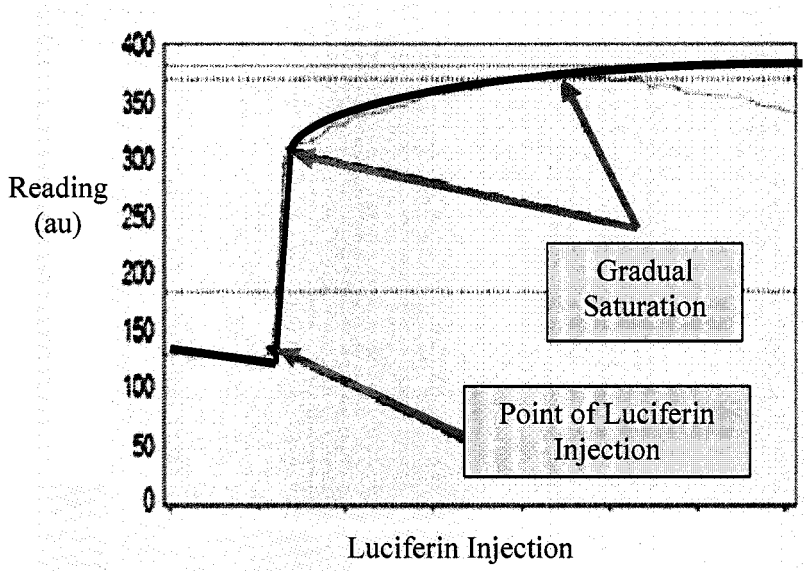


Figure 16a. Signals from PSQ 96 MA Pyrosequencer before optimization.

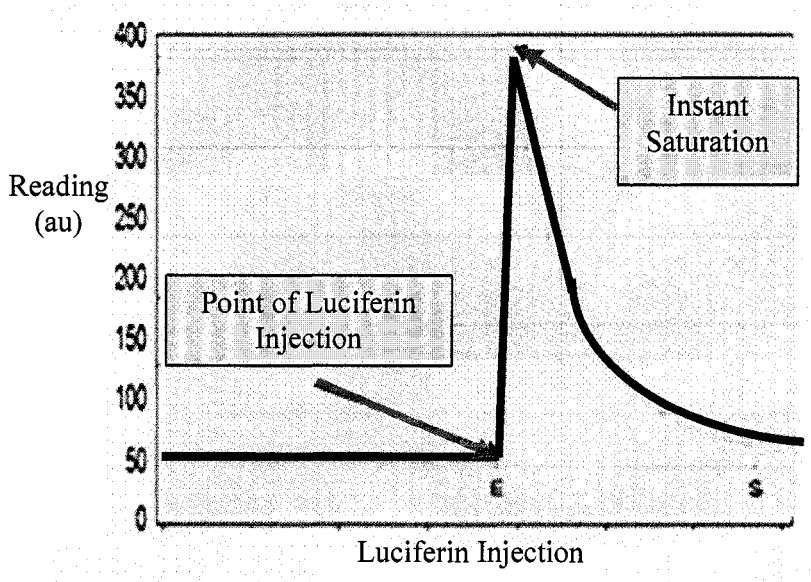


Figure 16b. Signals from PSQ 96 MA Pyrosequencer after optimization.

As evident in Figure 16a, for a chosen ATP concentration, the light signal level reaches 400 units in a gradual manner. This trend is an indication of a gradual saturation of the luciferase enzyme. In summary, the reason for getting such signals was the fact that in this thesis, the PSQ 96 MA Pyrosequencer was used in a different type of experimentation procedure than it had originally been designed for. This issue was solved by using the right amounts of chemicals in the assay, which led to instant reaching of the signal level to 400 units as a result of instant saturation of luciferase (see Figure 16b). Achieving the desirable type of signal by employing acceptable concentrations of enzymes and substrates was necessary for the next steps of the thesis. This optimization was especially necessary before proceeding to activity determination experiments.

5.1.1 Linear Range for Detection of ATP by the PSQ 96 MA Pyrosequencer

In this experiment, determination of a linear range for detection of ATP by the PSQ 96 MA Pyrosequencer was desired. One problem associated with using the PSQ 96 MA Pyrosequencer was its limitation for detection of light when light intensity exceeded a certain level. The instrument was only linear up to a particular concentration of ATP. Upon exceeding that concentration, the instrument was no longer sensitive since the signal became saturated. This occurred when 4,000 units of the instrument's signal level was reached. Therefore, it was necessary to determine the linear range for ATP detection by testing different concentrations of ATP and preparing a plot of ATP concentration versus signal level. Figure 17 is the plot prepared based on the values obtained from testing different concentrations of ATP by the instrument.

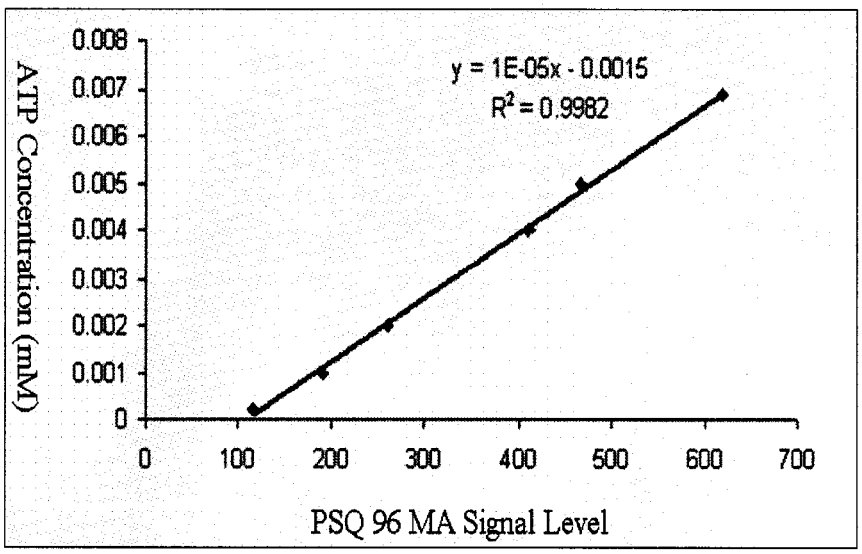


Figure 17. Standard curve for determination of ATP concentration from the produced signals.

As evident in Figure 17, the value of R^2 obtained from the regression line of the acquired data was 0.998. R squared is the relative predictive power of a model, which is a descriptive measure between 0 and 1. The closer it is to unity, the greater its ability is to predict and thus the better the model. Therefore, in this experiment the closeness of the obtained R^2 value (0.998) to one, indicated that the prepared plot was a predictable model with a straight line in which, the data fit well. Also, the linear range for detection was determined to be between 0.0002 mM and 0.0069 mM of ATP concentration. Therefore, for the proceeding activity determination experiments, ATP concentrations higher than 0.0069 mM were avoided. By staying within this range, one major source of error and variation in activity values was greatly reduced.

The equation obtained from the regression line of the prepared plot, was used as the standard equation for finding unknown ATP concentration values that were within the specified range (see Equation 16). The unknown ATP concentrations were determined by plugging in the value of the generated signal level into the equation as x and solving the equation for y.

$$y = 1 \quad 10^{-5} x - 0.0015 \qquad \text{Equation 16}$$

Initially, when testing the ATP samples in triplicate (for preparing the standard curve), some variation was observed in the generated signals. The source of this problem could either be normal pipetting errors, well to well variation, or change in the concentration of the ATP solution as a result of being left out at room temperature. Therefore, it was necessary to make sure that the observed variation was not caused by the latter case. In other words, it was necessary to check for stability of the ATP

solution over a long period of time. This experiment was performed by means of preparing samples with a certain concentration of ATP over a 40-minute period of time, followed by quantifying the generated signals and then comparing the obtained values. The samples were taken every other minute for 40 minutes. The results are presented in Figure 18.

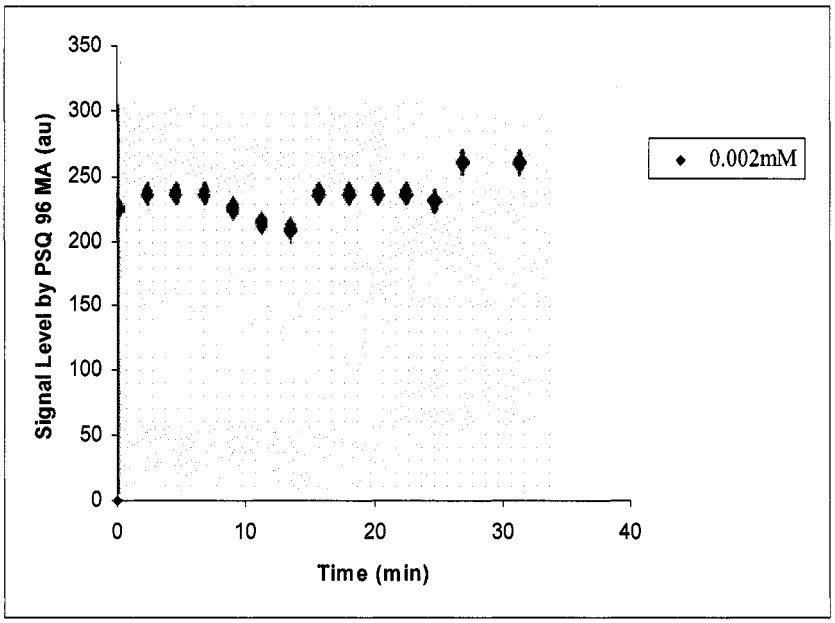


Figure 18. Stability of ATP during 40 minutes.

As shown in Figure 18, variation in the generated signals was evident but it was not significant. Therefore, it became clear that the concentration did not change considerably over time and the minor variation was caused only by normal pipetting or similar, errors.

The variation issue was further minimized to negligible well to well variation, by using more accurate pipettes and extreme attentiveness while pipetting. Also, to eliminate another possible source of error, the temperature of the samples was kept constant by allowing the samples to rest at room temperature 30 minutes prior to testing. Once acceptable conditions for using the PSQ 96 MA Pyrosequencer for the myokinase assay were determined, the experiments for determining myokinase activity were performed.

5.1.2 Activity of Myokinase Obtained from the Bioluminescence Assay (Covalent and Adsorptive)

In this experiment, determination of the activity of immobilized myokinase by means of the developed bioluminescence assay (explained in Section 5.1 on page 57) was desired. The enzyme was immobilized on the surfaces modified with four different monolayers while employing both covalent and adsorptive attachments. For each of the four surface chemistries, six chips were prepared and the immobilized enzyme activities were determined by testing all the prepared chips. Multiple tests were performed to ensure the accuracy of the obtained results and according to Section 4.6.6 (page 38). The samples were taken every two minutes, for the total time of 24 minutes, by employing some hand pipettes. A shaker was used for shaking the solution so that the taken samples would be homogenous. The collected samples were later tested by the PSQ 96 MA Pyrosequencer in order to determine their ATP level.

At the beginning of the thesis, the immobilization process was performed simultaneously for all chemistries (24 chips were prepared). Therefore, after four

hours of immobilization, there was insufficient time for testing all the prepared chips. Consequently, on the average, only four of the prepared chips could be tested on the chip preparation day and the remaining chips were stored in the fridge between 24 hours to one week before being tested. As a result of storing the immobilized chips, the activity obtained for these immobilized enzymes showed small reproducibility from chip to chip. At times, there were two orders of magnitude difference in the obtained results, which was an indication of either a problem in the testing procedure, the sensitivity of the instrument, the instability of the enzyme, or a combination of all the above.

The values obtained for immobilized myokinase activity using the initial approach, mentioned in the previous paragraph, showed great variation from one chip to another. As the values in some cases were apart by two orders of magnitude, the obtained values were not reliable and therefore, are not presented here. This variation issue was so substantial that its source was greater than could be attributed to normal pipetting and similar errors. Also, as mentioned in the previous section (Section 5.1.1), pipetting errors were minimized before starting this round of experiments and could not have been the cause of such large variation.

Some factors causing the observed variation in the results could include the instability of the enzyme, deficiency of certain chemicals in the assay, employment of wrong amounts of chemicals in the assay, unequal formation of monolayers on the gold surface, low sensitivity of the testing instrument and, technical issues of the testing instrument. Since the injection unit (for injecting luciferin into the sample plate) of the PSQ 96 MA Pyrosequencer was not functioning properly, it was decided

that this source of error would be eliminated first. If the variations persisted after fixing the unit, other factors should be identified and tackled.

Several months were spent in identifying and fixing the technical difficulties of the PSQ 96 MA Pyrosequencer. Simultaneously, the literature was explored for possible sources of error in the activity determination process. After fixing the injection unit of the instrument, the variation issue was reduced but still persisted. The difference between the generated results (not presented due to inaccuracy) was reduced to one order of magnitude and needed to be further reduced. Therefore, various experiments and troubleshooting strategies were performed to verify or rule out other relevant sources of error. Finally, one issue stood out after the easier to fix problems were eliminated.

The results showing the variation between the obtained values are not presented as they were not reproducible or reliable. All the performed tasks up to this point were basically troubleshooting steps taken to optimize the testing conditions to obtain reproducible data. The obtained reproducible data are presented and discussed later during this section. While performing the troubleshooting experiments, it was found that the variation was especially high when experiments were performed on different days. As mentioned in Section 1.1.4 on page 7, myokinase is an enzyme with high ability to change conformation. This characteristic is especially useful for electrically detecting the signal produced as a result of the enzyme's conformational change as it provides a more detectable signal [12]. On one hand, the greater the change in the enzyme conformation is, the higher the ratio of the detected signals to the system noise gets, and the higher the quality of the obtained signals becomes [12]. On the other hand, this ability for conformational change seemed to be the reason for the

experimental difficulties involved in working with myokinase since the enzyme was found to be unstable. The issue seemed to be the instability of myokinase upon storage and immobilization.

While doing the initial activity determination experiments (results not presented), it became evident that most of the variations were caused when activity determinations were performed in separate days. It also came to attention while working with the immobilized chips that after less than 48 hours, no ATP production could be observed. This activity loss could either be due to enzyme detachment from the immobilization surface, or the enzyme's significant or complete loss of activity. Therefore, it was necessary to determine and eliminate the reason behind the activity loss.

To determine the reason for the observed loss of enzymatic activity, 24 hours after immobilization, it was first necessary to clarify whether the drop in ATP production was caused by enzyme detachment from the surface or by the enzyme's instability. In other words, proof of existence of a strong association with the surface was mandatory before moving on to the enzyme instability issues. The attachment strength in immobilization is one of the main purposes of this thesis. Therefore, to test the attachment strength between myokinase and the modified gold surface, the activities of the enzymes immobilized on three chips were first measured and then extensive washing of the chips were performed using different time interval washing steps (washing each chip 3 times for 30 minutes or longer). The activities were measured again after the wash while the same experiment was repeated twice for assurance of the accuracy. The two obtained values, before and after the wash, were within 5% of each other. The obtained results have not been presented here as they

were only performed to ensure that the enzyme would not detach from the surface as a result of washing. Also, this experiment was a side experiment to reach the suitable experimental conditions for the activity determination experiment. In addition to testing the immobilized pieces before and after the washing steps, the washoffs from the immobilized enzyme were tested and no activity could be detected (no ATP production could be observed). This showed that the enzyme had not detached from the surface to enter the washing solution, otherwise some ATP production would have been observed. The results of this experiment have not been presented either, as they were not the main target of this experiment and were only performed as part of several months of experimentation for eliminating sources of error. Therefore, it was concluded that enzyme detachment from the surface was not the issue here because the obtained activity values, before and after the washing step, remained close in value (within 5%). This variation could be considered insignificant as the inevitable pipetting, operator, calculation and operation errors existed. At this point, one of the other possible sources of error, which was the instability of myokinase upon storage needed to be investigated.

Once it was verified that enzyme detachment from the surface was not the cause of the observed variations, it was decided that the immobilization and activity determination processes be performed all in one day to avoid the enzyme's loss of activity and day to day variations. Also, to check the reproducibility of the immobilized enzyme activities, six immobilized chips were tested in one day according to Section 4.6.6 (on page 38). Performing a statistical analysis and calculating the standard error could certify whether or not testing all the chips in one day could undertake the enzyme's instability problem and therefore, the analysis was

adopted (see Section 4.8.3 on page 55). One surface chemistry with only covalent attachment (monolayer formed by 100% $COOH^-$ terminating thiol group) along with two chemistries with only adsorptive attachments (monolayers formed by 100% OH^- and 100% CH_3 terminating thiol groups) were chosen for activity determination. A fourth chemistry with combination of both adsorptive and covalent attachments was also employed (monolayers formed by a mixture of 90% OH^- and 10% $COOH^-$ terminating thiol groups). In total, four different surface chemistries were chosen to investigate the effect of covalent and adsorptive types of attachments on the resulting activity. As mentioned in Section 4.6.6 (page 38), 50 units/mL (0.0235 mg/mL) of myokinase was used for immobilization of the enzyme on each chip. Testing conditions were exactly the same for all surface chemistries. It was decided that the experiments would start with testing the 100% $COOH^-$ surface chemistry and if any reproducibility was observed, the other chemistries would be tested as well. The activities obtained for all the chemistries are presented in Table 11.

Table 11. Activities of Immobilized Myokinase Using Different Surface Chemistries.

Surface Chemistry	mmole ATP produced/min
100% $COOH^-$	0.000412 ± 0.000064
10% $COOH^-$ + 90% OH^-	0.000720 ± 0.00011
100% OH^-	0.000408 ± 0.000078
100% CH_3	0.000269 ± 0.000066

The experiments started with testing the 100% $COOH^-$ surface chemistry (first row on Table 11). Reproducible data with a small standard error that was within 15% of the average value was obtained and therefore, the other chemistries were tested.

As evident in the results, the rest of the obtained activities exhibited high reproducibility as well. The calculated standard error values (calculated by equations on page 55) for all four surface chemistries, were between 15-24% of their corresponding average values obtained as the myokinase activity. This error was considered as insignificant and normal for our testing conditions as there were several factors causing this error. In every experiment, there were inevitable experimental errors such as pipetting, operator, operation and instrument errors. In addition to these errors, the low number of the conducted experiments contributed to the generated error. Generally, it is better to have 10 to 15 data points for determining the standard deviation and standard error. This was not the case here as only six chips were tested for each of the four chemistries. The reason for choosing limited number of experiments was the fact that both the immobilization and the chip testing should have been performed in one day for enzyme stability reasons. Therefore, there would not be enough time left to test more than 6 chips in one day. Also, due to the relatively high cost of both the enzymes and the chips, repeating the experiment in separate batches was impossible. So the 15-20% error was considered as acceptable. Other sources contributing to the present error were the minor errors generated by the instrument due to its low sensitivity, and NADH concentration exceeding its linear detection range (mentioned in Section 5.1.1 and Figure 17 on page 62) at some point during the activity determination process.

The reproducible results with an acceptable error range indicated that the cause of activity variation from chip to chip was the enzyme instability upon storage over night. This was concluded as the problem was solved when storage was avoided. Therefore, one remedy for eliminating this type of variation in future experiments seemed to be performing activity determination experiments all in one day. The other remedy would be to stabilize myokinase so it would retain its activity upon storage. Great effort was put into stabilization of myokinase and the results are presented in Section 5.2.3 on page 77.

The main reason for choosing four different surface chemistries for activity measurements was to determine the chemistry that generated the highest activity among all four, by comparing the obtained results. As shown in Table 12, the highest activity belongs to the chemistry with both covalent and adsorptive attachments (10% $COOH^-$ plus 90% OH^-). Also, it is shown that hydrophilic chemistries ($COOH^-$, OH^-) have higher activities than the hydrophobic chemistry (CH_3). A higher production of ATP, which is caused by the change in enzyme conformation, corresponds to a higher activity value [12]. Therefore, for myokinase it was concluded that a hydrophilic surface containing both covalent and adsorptive types of attachment, provided the highest ability for conformational change and activity. Activity determination for immobilized myokinase on the four mentioned monolayers by employing the bioluminescence assay has never been performed by anyone before. In other words, the obtained results are unique and no similar results are available in the literature for comparison.

From the beginning of the thesis, it was intended that an alternative approach for determining the activity of myokinase would be developed. Therefore, it was decided

that the stabilization of myokinase would be performed after developing the alternative method, which was the fluorescence assay of myokinase. The development of the fluorescence assay, the enzyme stabilization process, and the results and discussions are presented in the following section (Section 5.2) and its subsections. It was also intended that a fluorescence assay would be developed for GDH. The results of this assay development are also provided in Sections 5.2.6 and 5.2.7 on pages 92 and 93, respectively.

5.2 Fluorescence Assays for Myokinase and GDH

In this experiment, developing fluorescence assays for determination of the enzymatic activity of myokinase and GDH was desired. As mentioned in Section 1.1.7 on page 13, fluorescence assays can determine the enzymatic activity of enzymes that catalyze reactions that produce or utilize NADH. Glutamate dehydrogenase can catalyze such reactions and therefore, a fluorescence assay was needed to be developed to determine its catalytic activity.

As an alternative to the bioluminescence assay for myokinase, a fluorescence assay was developed to determine the activity of the free and immobilized forms of the enzyme while investigating the impact of stabilization methods on the activities. These testes were performed through the use of a coupled assay involving hexokinase and glucose-6-phosphate dehydrogenase as shown in Section 1.1.4 and Equations 3-5 (pages 8,9).

To develop a fluorescence assay, it was first necessary to determine the linear range of the Victor Fluorimeter for NADH detection. It was also useful to prepare a

standard curve of the NADH concentration versus the Fluorimeter reading within the determined linear range. By having such curve, any sample containing an unknown concentration of NADH could be tested by the instrument. If the reading would be in the specified liner range, the NADH concentration could be determined. The standard curve was prepared according to the procedure explained in the following section. Also, the developed assay for each enzyme is explained in separate sections (Sections 5.2.2 and 5.2.6).

5.2.1 Preparation of a Standard Curve for NADH

In this section, preparation of a standard curve for NADH detection by the Fluorimeter was desired as it would enable conversion of fluorescence readings to NADH. Although, the Victor fluorescent instrument provided accurate readings for a very wide range, it was necessary to determine the upper and lower ranges of the instrument's linearity.

Solutions of NADH with different concentrations were prepared in Tris buffer (pH=8.6) and five samples from each concentration were tested by the Fluorimeter. At the beginning of the experiment, an approximate range of linearity was not known and therefore, the chosen concentrations were random and widespread. By plotting those random values, the points that exhibited deviation from a straight line were eliminated. The results of the mentioned experiments have not been presented as they were only performed to find the final range. Additional points (concentrations) within the narrowed down range were chosen and again five samples of each were taken and tested. Table 12 contains the points chosen for preparing the standard curve, while

Figure 19 presents the prepared standard curve. The final value for each reading is the average value of five readings from five different samples.

Table 12. The Points Used for Preparing a Standard Curve for NADH.

NADH mM	NADH Concentration
0.000868	750.8 ± 4.3
0.00868	6121.2 ± 97.2
0.04	21660.8 ± 96.5
0.05	26848.8 ± 187.2
0.06	32032.4 ± 94.4
0.07	37216.4 ± 236.8
0.0868	45682.4 ± 53.5

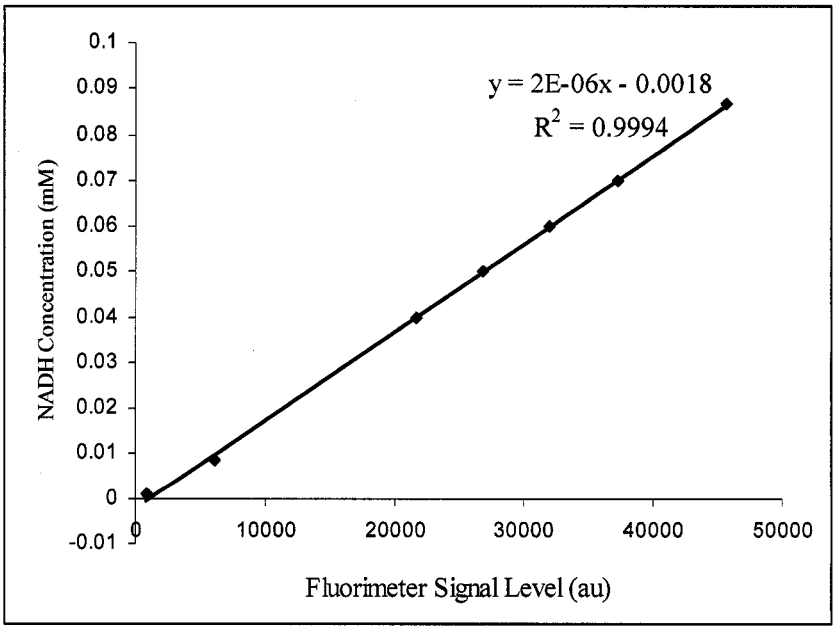


Figure 19. Standard curve for NADH.

As shown in Figure 19, the value of R^2 obtained from the regression line of the acquired data was 0.9994. The closeness of this value to unity indicated that the prepared plot was in fact a straight line and the data fit the line well. The linear range of the Fluorimeter reading versus NADH concentration was between 0-50,000. Therefore, the standard curve for NADH detection by the Victor Fluorimeter was set for readings below 50,000 units of Fluorimeter reading, which corresponds to concentrations of NADH below 0.08 mM. This experiment verified that concentrations higher than 0.08 mM, generated readings that deviated from the straight line making the measurements less accurate and undesirable. Equation 17 is the equation used for extrapolating the unknown concentrations of NADH by placing the obtained reading from the Fluorimeter as x and solving for y.

$$y = 2 \quad 10^{-6} x - 0.0018 \qquad \text{Equation 17}$$

5.2.2 Fluorescence Assay Developed for Myokinase

In this experiment, a fluorescence assay for determination of myokinase enzymatic activity was desired. As shown in Equations 3-5 (pages 8 and 9), myokinase can generate NADH in a series of reactions. To develop a fluorescence assay for myokinase, finding the volumes and concentrations of enzymes, substrates and buffers along with the suitable pH of the medium was necessary similar to what was done in Section 5.1 on page 57. The performed experiments, which took one month in duration, led to the following conditions as the best assay conditions for immobilized and free myokinase, respectively.

For immobilized myokinase, the assay mix was prepared by taking 477 μL of the ADP (ATP free) solution and adding 20 μL of the NAD^+/glucose solution to it followed by addition of 3 μL of the Hexokinase/G6PD solution. The NAD^+/glucose was prepared by adding 20 mg of NAD^+ and 25 mg of glucose to 500 μL of Tris-$MgCl_2$ buffer (pH=7.9). For extended detail see Sections 4.6.8 and 4.6.9 (on page 40). Finally, the assay was performed by placing the immobilized chip inside 500 μL of the assay mix.

For the free myokinase, the assay mix was prepared by taking 17 μL of the ATP free ADP and adding 1 μL of the NAD^+/glucose solution (mentioned in the previous paragraph) followed by addition of 1 μL of hexakinase/G6PD. To start the assay, 0.2 μL of the free myokinase with varying concentrations, was added to the assay mix.

As mentioned in Section 5.2.1, myokinase is not stable after 12 hours of preparation. Therefore, strategies were adopted for its stabilization. The employed methods for stabilization are mentioned in the following section.

5.2.3 Stabilization of Myokinase in Free Form

In this experiment stabilization of myokinase was desired. Therefore, four stabilizing conditions were chosen based on the indications available in the literature [39,40]. In the literature, no work has been performed on stabilization of myokinase and therefore, the chosen stabilization conditions were only similar to some conditions used for stabilization of other enzymes. Four different free enzyme solutions with the same final concentration of 2.56 unit/mL were prepared. The base solutions for preparing the four mentioned enzyme solutions were the stabilization

solutions (storage conditions mentioned in Table 14). After preparation of the four storage conditions, the activity of the free enzyme in each of the four prepared solutions was determined by employing the fluorescence assay.

As mentioned in Section 5.1.2 on page 65, one of the main reasons for being unable to obtain reproducible data at the beginning of the myokinase activity determination experiments, was the enzyme's unstable nature. Therefore, as an alternative to testing all the chips (with immobilized enzyme) in one day, a considerable amount of time was spent on stabilization of myokinase.

For the stabilization experiments, the Victor Fluorimeter and the myokinase fluorescence assay were employed, rather than the PSQ 96 MA Pyrosequencer and the myokinase bioluminescence assay. This was because the fluorescence assay involved less time consuming assay preparation steps, and the cost of reagents were much lower in comparison with the bioluminescence assay.

For stabilization of myokinase, different concentrations of some additives (explained in the following paragraph) in combination with glycerol were used while storing at 4°C and –20°C, respectively. Glycerol is a known chemical for enzyme stabilization and cases of its usage are abundant in the literature [39]. Imidazole on the other hand is not a very common additive for this purpose, but some studies in the literature have reported employment of imidazole as a stabilizing buffer [40].

In total, eight conditions were chosen for the stabilization experiments. Out of the eight chosen conditions, only four have been presented in Table 13. This was because during the initial tests, only these four conditions exhibited a stabilizing effect and the enzymes stored in the rest of the conditions lost their activities, completely. Therefore, the mentioned four were chosen for a complete and thorough

experimentation. The results of the initial tests on the discarded conditions have not been presented here as they were lengthy and redundant. Table 14 presents the obtained activity results for free myokinase stored in the conditions mentioned in Table 13. The concentration of the prepared myokinase used for these experiments was 2.56 units/mL of lyophilized myokinase prepared in the conditions mentioned below (A-D) as their base solution. For each of the four mentioned storage conditions, six identical samples were prepared and tested to determine the reproducibility and reliability of the results.

Table 13. Storage Conditions for Stabilization of Myokinase.

Condition A	Myokinase in 50% Glycerol + 45 mM Imidazole + 3.5 mM MOPS Buffer (pH=8.3)
Condition B	Myokinase in 90 mM Imidazole pH=7 + 7 mM MOPS Buffer (pH=8.3)
Condition C	Myokinase in 70 mM MOPS Buffer (pH=8.3)
Condition D	Condition A + 10 mg/mL BSA

Table 14. Activities of Free Myokinase upon Storage in Various Conditions.

Storage Condition	Tested Days After Preparation	% Activity Change	mmole/min Production
Condition A	Same Day		0.0075 ± 0.0005
–20°C	14	13.3	0.0086 ± 0.0001
4°C	14	37.2	0.0119 ± 0.0013
Condition B	Same Day		0.0047 ± 0.0009
4°C	14	52.4	0.0098 ± 0.0009
–20°C	14	None	0.0046 ± 0.0003
Condition C	Same Day		0.000097 ± 0.000002
–20°C	14	24.9	0.00012
4°C	14		None
Condition D	Same Day		0.0074 ± 0.0001
–20°C	14	35.8	0.015 ± 0.0007
4°C	14	51.2	0.0235 ± 0.0005

By looking at the results presented in Table 14, a relation could be observed between the activities obtained from the examined conditions. Storage at condition C generated the lowest values for myokinase activity while conditions A and D generated the highest values. The activity values generated upon storage in condition B, lay between conditions C, D and A ($C < B < D = A$).

The standard error values calculated (by equations on page 55) for all four storage conditions, were between 1-10% of their corresponding average values obtained as the free myokinase activity. This error is considered as insignificant and

normal for the current testing conditions as there are several factors causing this error. As discussed in Section 5.1.2 on page 65, in every experiment there are inevitable experimental errors such as pipetting, operator and operation errors along with errors generated by the instrument, and the low number of the conducted experiments. As mentioned at the beginning of this section, only six duplicates were tested for each of the four storage conditions. The reason for choosing limited number of duplicates for this experiment was this preference that all tests would be performed in one day to avoid day to day variations. Also, due to the relatively high cost of myokinase, repeating the experiment in separate batches was not economically feasible and the 1-10% error was considered as acceptable. Other sources contributing to the existing errors were the instrument's lack of sensitivity and also possible exceeding of NADH concentration from its linear detection range at some point during the activity determination process (see Section 5.2.1 and Figure 19).

Also, as shown in Table 14, two of the four conditions mentioned in Table 13 provided the highest value of enzymatic activity while helping to stabilize free myokinase to an extent were the enzyme retained its activity for more than two weeks. These two conditions were conditions A and D.

The results shown in Table 14 exhibit that storing the enzyme in condition C (70 mM MOPS buffer, pH=8.3) at 4°C for 2 weeks, resulted in a complete loss of enzymatic activity. Under equal testing conditions and after two weeks of storage, the highest activity was obtained from storage in conditions A and D (condition A + BSA). Since addition of Bovine Serum Albumin (BSA) showed no significant improvement in myokinase stability, condition A (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3) was chosen as the most effective storing condition

among all the tested conditions for stabilization of myokinase. Myokinase had retained its activity upon storage for up to two weeks in all of the conditions mentioned in Table 14. This retention of activity was especially evident when stored at 4°C. Therefore, since storage at this temperature was more convenient than storage at –20°C, 4°C was chosen as the most effective storage temperature. Overall, the most effective condition for stabilization and storage of myokinase was chosen to be condition A (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3 stored at 4°C).

As a storage condition (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3 at 4°C) was developed and chosen for stabilization of myokinase, it was time to determine the activity of immobilized enzyme upon storage as well. Therefore, the activity of immobilized myokinase was first determined immediately after preparation without being exposed to the storage condition prior to being tested. The chips with immobilized enzyme were then stored in the mentioned condition and were tested for activity after a while. The results are shown and discussed in the following section.

5.2.4 Activity of Immobilized Myokinase Obtained from the Fluorescence Assay (Covalent and Adsorptive)

In this experiment, determination of the activity of both free and immobilized forms of myokinase by means of the fluorescence assay developed in Section 5.2.2 (page 76) was desired. In addition to the activity determination experiments, the effect of storing the immobilized myokinase in the storage conditions developed in

Section 5.2.3 (the previous section) was investigated. The enzyme was immobilized on surfaces modified with the same four surface chemistries used in Section 5.1.2 on page 65. The employed surface chemistries are also mentioned in Table 15.

The myokinase concentration employed for immobilization on the gold surface was 0.047 mg/mL (100 units/mL) of the lyophilized enzyme prepared in MOPS buffer pH=8.3. Similar to the bioluminescence assay for myokinase, four surface chemistries were employed for formation of self assembled monolayers in order to modify the gold surface. For each of the four surface chemistries, immobilization of myokinase was performed on 9 chips and the fluorescence assay was preformed according to Section 5.2.2 on page 76. From the nine prepared chips for each of the four surface chemistries, only six were used for immobilized activity determination. The remaining three chips were stored in the storage condition developed in Section 5.2.3 (condition A: 50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3 stored at 4°C) to be tested in a few weeks after preparation. For activity determination, samples were taken every other minute for total of 10 minutes. The four employed surface chemistries along with the obtained results for the immobilized myokinase activities are presented in Table 15.

Table 15. Activities of Immobilized Myokinase by Fluorescence Assay.

Thiol Group Terminated in	mmole/min NADH Production
100% $COOH^-$	0.00111 ± 0.00008
10% $COOH^-$ + 90% OH^-	0.00121 ± 0.00006
100% OH^-	0.0012 ± 0.0002
100% CH_3	0.00100 ± 0.00009

As evident in Table 15, the surface chemistry with both covalent and adsorptive types of attachments showed a slightly higher activity than all other surface chemistries. This result was in accordance with the results obtained from the bioluminescence assay of myokinase presented in Section 5.1.2. The immobilized myokinase activities obtained by the fluorescence assay, for all four surface chemistries, were the same in contrast with the activities obtained from the bioluminescence assay in which, the surface modified with 100% CH_3 had the lowest activity (see Table 11 on page 70). This result was due to the higher sensitivity of the Fluorimeter and the fluorescence assay. The important issue in this thesis was to determine whether the enzyme had remained active or not. Therefore, as long as there was retention of activity, the results were considered as desirable results.

The standard error values calculated (by equations on page 55) for all four storage conditions, were between 5-16% of their corresponding average values obtained as the immobilized myokinase activity. This error is considered as insignificant and normal for the current testing conditions as there are several factors causing this error. As discussed in Section 5.1.2 on page 65, in every experiment

there are inevitable experimental errors such as pipetting, operator, calculation and operation errors along with errors generated by the instrument and the low number of conducted experiments. As mentioned at the beginning of this section, only six gold chips (with immobilized myokinase) were tested for each of the four surface chemistries. The reason for choosing limited number of chips for this experiment was the preference that all the tests would be performed in one day to avoid day to day variations and loss of myokinase activity. Also, due to the relatively high cost of myokinase, repeating the experiment in separate batches was not economically feasible and the 5-16% error was considered as acceptable. Other sources contributing to the existing errors were the instrument's low sensitivity and also possible exceeding of NADH concentration from the instrument's linear detection range at some point during the activity determination process (see Section 5.2.1 and Figure 19). In addition, the filters on the Victor Fluorimeter did not exactly match the excitation and emission spectra of NADH. As mentioned in Section 1.1.7 on page 13, the excitation and emission spectra of NADH were 340 nm and 460 nm, respectively. The filters available on the Victor Fluorimeter were 355 nm for excitation and 460 nm for emission (see Section 4.6.3 on page 36).

After determination of the immobilized myokinase activity for each of the four surface chemistries, the three remaining chips stored in condition A (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3) at 4°C, were tested. In this experiment, similar to the activity determination procedure (see Section 5.1.2), the fluorescence assay was employed for determining the stored myokinase activity by taking samples every two minutes for total of 10 minutes. The obtained results are presented in Tables 16 and 17, respectively.

Table 16. Activities of Immobilized Myokinase upon Storage in Condition A at 4°C (Covalent).

Thiol Group Terminated in:	mmole/min NADH Production Immediately after Immobilization	mmole/min NADH Production, Stored in Condition A @ 4°C for 1 Week	Change in Activity %	mmole/min NADH Production, Stored in Condition A @ 4°C for 4 Weeks
100% $COOH^-$	0.00111 ± 0.00006	0.00129	None	0.0024 ± 0.0002
10% $COOH^-$ + 90% COOH-	0.00121 ± 0.00005	0.00053 ± 0.00007	-56.2	0.00060 ± 0.00006

Table 17. Activities of Immobilized Myokinase upon Storage in Condition A at 4°C (Adsorptive).

Thiol Group Terminated in	mmole/min NADH Production Immediately after Immobilization	mmole/min NADH Production, Stored in Condition A @ 4°C for 4 Weeks	Change in Activity %
100% OH^-	0.00130 ± 0.00015	0.00059 ± 0.00008	-54.6
100% CH_3	0.00177 ± 0.00005	0.00089 ± 0.00007	-52.5

Before using condition A as the storage buffer, myokinase would lose its activity in less than 12 hours. As shown in Tables 16 and 17, employing the stabilization buffer has enhanced myokinase stability to more than one month. As mentioned before, the main concern of this thesis was to keep the enzyme active and therefore, partial loss of activity was not very important although it was not desirable either.

The other factor contributing to the drop in the activity values obtained for the enzyme, beside loss of activity (caused by limitation of enzyme conformation) could be the enzyme detachment from the surface. As evident in the results (Tables 16 and 17), all chemistries except 100% $COOH^-$ had lost 50% of their activities within one

month after immobilization. This might be because the 100% $COOH^-$ surface chemistry was the only chemistry among all four that purely formed covalent bonds with the enzyme's lysine groups. The 100% CH_3 and 100% OH^- surface chemistries, attached to myokinase entirely by natural adsorption, while the fourth surface chemistry (10% $COOH^-$ + 90% OH^-) attached to myokinase, partially with covalent bonds and partially by adsorption. Therefore, as covalent bonds provide a stronger association of myokinase to the surface, it is logical to think that myokinase detachment from the surface would be less in the covalent attachment, in comparison with the other chemistries. The results presented in Tables 16 and 17 are also an indication of the stated fact. For all the other chemistries besides the 100% $COOH^-$ chemistry, a 50% decrease in the activity was observed upon storage in the stabilization buffer. This decrease might have been caused as adsorptive immobilization provides a weaker association with the enzyme.

In addition to determining the immobilized myokinase activity, the activity of the free form of the enzyme was examined. This experiment was similar to the experiment performed in Section 5.2.3 but with one difference. The myokinase solutions made in Section 5.2.3, were prepared in the four stabilization solutions (mentioned in Table 13 on page 80), whereas in this experiment the myokinase solution was originally prepared in MOPS buffer, pH=8.5. The activity of free myokinase was tested and determined using this original solution and the remaining solution was then stored in storage condition A (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3) at 4°C with a 1:1 ratio of storage solution to original myokinase solution (prepared in MOPS) for future tests. After testing the free myokinase activity (prepared in MOPS buffer) several times immediately after

preparation, the enzyme stored in condition A (50% glycerol + 45 mM imidazole + 3.5 mM MOPS buffer, pH=8.3) at 4°C was tested one week after storage. The results are presented in Table 18.

Table 18. Free Myokinase Activities before and after Storage in Condition A at 4°C.

Myokinase Concentration 0.00049 mg/mL	Myokinase in MOPS Buffer (pH=8.3) after Preparation	Myokinase Stored in Condition A for 1 Week
NADH Production mmole/min	0.0129 ± 0.0008	0.195 ± 0.015

As shown in Table 18, the free enzyme has retained its activity upon storage in the stabilization buffer. It was once again observed that the stabilization buffer had successfully helped myokinase maintain its activity.

Up to this point, the activities of free and immobilized myokinase were determined by using the bioluminescence and fluorescence assays also developed during this thesis. Stabilization strategies for myokinase were also adopted and the enzyme activities were determined upon immobilization. As the final experiment performed on myokinase, the following section focuses on investigating the comparability of two values. One of the two values was the difference between the activities of free myokinase in the enzyme solution before being used as the immobilization solution and after use for immobilization. The other value was the activity obtained from the chip that was placed in the same enzyme solution for myokinase to get immobilized on. The details and the results are provided in the following section.

5.2.5 Comparison of the Activities of Immobilized Myokinase with the Activities of Free Enzyme Solution before and after Immobilization

In this experiment, determination and comparison of the activities obtained from the free myokinase solution (used as the immobilization medium) before and after the immobilization procedure was desired. In addition to the mentioned experiment, the activity of the immobilized enzyme prepared in the mentioned immobilization medium was needed. The purpose of this experiment was to determine the relation between the two mentioned values.

An enzyme solution was prepared and its activity was determined once before using it as the immobilization solution and once immediately after. The experiment was performed on two different surface chemistries (see Tables 19 and 20) while repeating each experiment three times to ensure reliability of the results. The concentration of the free enzyme solution used for the experiment was 0.00049 mg/mL (1.0416 unit/mL of lyophilized myokinase in MOPS buffer pH=8.3). The activity results are presented in Tables 19 and 20.

Table 19. Activities of Free Myokinase before and after Immobilization (100% OH^-).

	A	B	A-B	C
	Activity of Free Myokinase before Immobilization	Activity of Free Myokinase after Immobilization		Activity of Myokinase Immobilized on 100% OH^-
NADH Production mmole/min	0.0129 ± 0.0008	0.0105 ± 0.00036	0.0024	0.00130 ± 0.00015

Table 20. Activities of Free Myokinase before and after Immobilization (100% CH_3).

	A	B	A-B	C
	Activity of Free Myokinase before Immobilization	Activity of Free Myokinase after Immobilization		Activity of Myokinase Immobilized on 100% CH_3
NADH Production mmole/min	0.0129 ± 0.0008	0.010373	0.0026	0.00177 ± 0.00005

When performing enzyme immobilization, some of the enzymes available in the solution attach to the surface while others remain in the solution. Logically, the amount of enzyme exiting the enzyme solution during immobilization should be equal to the amount of enzyme that attaches to the gold surface. The same concept does not apply to the two activity values, because enzyme immobilization partially limits the enzyme's natural change in conformation and activity [3]. This restriction in conformational change is caused as some of the enzyme's active sites become ineffective upon attachment to the surface [3]. It should be noted that the amount of enzyme/protein is different from the activity of the enzyme. The activity of an enzyme can naturally alter because of denaturing and stability issues, or limitation of the active sites and conformational change [3,12]. On the other hand, the concentration or amount of enzyme can be altered by diluting/concentrating the enzyme solution.

In this experiment, the difference between the activities of the free enzyme solution used for immobilization before and after the procedure was determined so it could be compared with the activity of the enzyme immobilized on the chip. This was done to see how immobilization would affect the activity. By looking at the results shown in Tables 19 and 20, it became evident that the activity difference was higher

than the actual immobilized enzyme activity by about a factor of 1.6. This result indicates that the same number of enzymes in free and immobilized forms did not generate the same amount of activity. This result gives a strong indication that immobilization of myokinase limits its conformational change and activity. This experiment has not been performed on myokinase by any other researcher and therefore, there are no results available in the literature for comparison.

The percent error generated in this experiment is 11% and 3% for the two tested chemistries, respectively. This error range is acceptable considering the inevitable sources of error. The sources include pipetting and operator errors, operation and calculation errors, errors caused by the testing procedure, and inadequate sensitivity of the instrument. Also, in this case only three chips were tested and the low number of experiments contributes to the generated error. In addition, the filters on the Victor Fluorimeter did not exactly match the excitation and emission spectra of NADH. As mentioned in Section 1.1.7 on page 13, the excitation and emission spectra of NADH were 340 nm and 460 nm, respectively. The filters available on the Victor Fluorimeter were 355 nm for excitation and 460 nm for emission (see Section 4.5.2 on page 32).

After performing the activity determination experiments on myokinase, studies on the behavior of GDH were started. The following section explains the development of an assay for GDH activity determination.

5.2.6 Fluorescence Assay Developed for GDH

In this experiment, developing an assay for determination of the enzymatic activity of GDH was desired. As shown in Equation 6 (page 11), GDH catalyzes a reaction that reduces NAD^+ to generate NADH. To develop an assay for GDH, it was necessary to determine the concentrations of the enzymes, substrates and buffers along with the suitable pH of the medium similar to what was done in Section 5.1 on page 57. Many experiments were performed, which led to finalizing the following combinations and concentrations for both immobilized and free GDH assays.

The enzyme solution used for both free and immobilized tests was prepared by taking 5 μL of the GDH solution provided by the manufacturer (Glutamate dehydrogenase, 43 mg protein/mL). The taken volume corresponded to 0.215 mg/mL of GDH in the solution ([43 mg protein/1000 μL enzyme solution] [5 μL enzyme solution/1 mL MOPS buffer] = 0.215 mg/mL = 215 μg/mL MOPS buffer, [215 μg/mL MOPS buffer] [1/336000] = 6.399 10 $^{-4}$ μmoles/mL).

For immobilized GDH, the assay mix was prepared by making a 0.1 M solution of potassium phosphate, and adding EDTA and L-glutamic acid to it to bring their final concentrations in the potassium phosphate buffer to 0.1 mM and 30 mM, respectively. The pH was then adjusted to 7.7 by means of a pH meter. Preparation of the assay mix was completed by addition of NAD^+ to the prepared buffer. As mentioned in section 4.6.10 (page 41), the addition of the unstable NAD^+ was postponed to the day of the activity determination experiment. Therefore, the prepared buffer was divided into 10 mL aliquots and the aliquots were stored in the freezer till the day of the experiment. For each assay, 0.0013 g of NAD^+ was added to

10 mL of the previously stored buffer to make the final concentration of the NAD^+ in the buffer 0.2 mM.

The buffer used for washing the chips in three steps (see Section 4.6.10 page 41) was 0.1 M potassium phosphate plus 0.2 mM EDTA with pH adjusted to 7.7. While the washing was in progress, 500 μL of the assay mix that had reached room temperature was placed inside a vial. After performing the washing procedure, the immobilized GDH assay was initiated by placing the chip inside the assay mix vial (500 μL).

For the free GDH assay, 200 μL of assay mix that had reached room temperature was placed inside a vial and 5 μL of the prepared enzyme solution was added to it to initiate the activity determination assay.

After developing an assay for GDH, the activities of both free and immobilized forms of the enzyme were determined. The following section presents the values obtained for GDH activity and the discussion of the results.

5.2.7 Activity of GDH Obtained from the Fluorescence Assay

In this experiment, determination of the activity of both free and immobilized (only covalent) GDH by employing the fluorescence assay developed in Section 5.2.6 was desired. The surface chemistry employed for modifying the gold surface was the 100% $COOH^-$ surface chemistry.

Ten units/mL of GDH solution (0.215 mg/mL) was used for immobilization of enzyme on each chip. In total, 10 chips were prepared and four of the chips were stored for future activity measurement experiments such as determining the loss of

activity upon storage. For the chosen surface chemistry (100% $COOH^-$), six chips were tested by employing the Fluorimeter and the fluorescence assay, developed in the previous section. The results each present the average value of six experiments and are presented in Table 21.

Table 21. Activities of Immobilized GDH before and after Storage.

Testing Condition	NADH Production mmole/min	Loss of Activity %
Immediately after Immobilization	0.0015 ± 0.00012	
Tested once Right after Preparation, Stored for 4 Weeks in Phosphate Buffer at 4°C and Tested for the Second Time	0.00062 ± 0.0001	-58.7
Stored for 4 Weeks in Phosphate Buffer at 4°C and then Tested	0.00073 ± 0.00004	-51.3

As shown in Table 21, in contrast with the unstable myokinase, GDH remained active without the help of any additives over a long period of time. It did however; lose approximately half of its activity in a one-month period. As mentioned before, the main concern in this thesis was retention of the enzymatic activity and partial loss of activity does not have a significant effect on the nanosensor experiments.

The calculated standard error value (by using equations on page 55) for the immobilized GDH on the surface modified with 100% $COOH^-$ chemistry was 6% of the average value of the six tested chips. This error is considered as insignificant under the current testing conditions as there are several factors causing this error. As discussed in Section 5.1.2 (on pages 65-73), in every experiment there are inevitable

experimental errors such as pipetting, operator, and operation errors along with errors generated by the instrument and the low number of conducted experiments. As mentioned at the beginning of this section, only six chips were tested for the chosen surface chemistry. The reason for choosing limited number of chips in this experiment was the preference that all the tests would be performed in one day to avoid day to day variations. In contrast with myokinase, the cost of GDH was not an important issue in this case. The main issue was the time constraint. It was also decided that all experiments follow one trend to make them more comparable. Other sources contributing to the existing errors were the instrument's low sensitivity and also possible exceeding of NADH concentration from its linear detection range at some point during the activity determination process (see Section 5.2.1 and Figure 19). In addition, the filters on the Victor Fluorimeter did not exactly match the excitation and emission spectra of NADH. As mentioned in Section 1.1.7 on page 13, the excitation and emission spectra of NADH were 340 nm and 460 nm, respectively. The filters available on the Victor Fluorimeter, which were used for all the experiments were 355 nm for excitation and 460 nm for emission (see Section 4.5.2 on page 32).

After determining the activity of immobilized GDH, the other experiment performed on GDH was determination of the activity of the free enzyme solution used for immobilization, before and after the procedure. This task was performed in order to compare the activity difference between the enzyme solution before and after immobilization, with the activity of the enzyme immobilized on the chip. The activity of the immobilized enzyme (ΔA) was later used in determination of the specific activity. Each experiment was repeated three times to ensure the reliability of the

obtained activities. The concentration of the GDH solution used in this experiment was 0.215 mg/mL. The results are presented in Table 22.

Table 22. Activities of Free GDH before and after Immobilization.

	A	B	A-B	ΔA
	Activity of Free GDH in MOPS Buffer before Immobilization	Activity of Free GDH in MOPS Buffer after Immobilization	Activity Difference	Activity of GDH on 100% $COOH^-$ Immediately after Immobilization
NADH Production mmole/min	0.001288 ± 0.000016	0.00113 ± 0.00004	0.000158	0.0015

The results shown in Table 22 indicated that the activity difference for the free enzyme solution (A-B) was one order of magnitude smaller in value than the activity of the immobilized enzyme (ΔA). These results were the exact opposite to the results previously obtained for myokinase. As mentioned in Section 5.2.5 (page 89), when using an enzyme solution for immobilization, it is logical to assume that the amount of enzyme that has attached to the surface would be equal to the amount of enzyme that leaves the solution. The same assumption can not be made for the activity of the enzyme. This is due to the many factors that are involved in the immobilization procedure resulting in the enzyme's change of activity. Upon immobilization, limitation in the enzyme's movement and conformational change may occur as some of the enzyme's active sites get restricted. This restriction may cause a decrease in the enzymatic activity. For GDH, the stated fact does not seem to be the case. The orientation of the enzyme attaching to the surface is another factor causing a difference between the activities of the immobilized enzyme and the activity of the same units of free enzyme that leave the immobilization solution to attach to the

surface. In the literature, no experiments similar to this experiment were conducted and therefore, there were no sources available for comparison of the results.

The results obtained from myokinase (see Section 5.2.5 on page 89), exhibited a lower value of immobilized enzyme activity than the difference between the activities before and after immobilization. The reason for this opposition laid on the difference between the structure and specific characteristics of the two enzymes. Myokinase has a higher ability for conformational change than GDH and therefore, upon immobilization the conformational change could be restricted to a greater extent than GDH. This restriction upon immobilization could be the cause of a greater drop in the value of the activity.

Another important factor in characterizing the behavior of an enzyme was its flexibility upon immobilization. Glutamate dehydrogenase has allosteric properties and therefore, in the following section, the flexibility of the enzyme is being investigated.

5.3 Determination of GDH Flexibility by Addition of Allosteric Effectors

In this experiment, the flexibility of GDH was investigated by using the enzyme's allosteric properties. In theory, immobilization is known to limit the enzyme's flexibility. This limitation causes a drop in the enzymatic activity [41]. The validity of this statement needed to be investigated. In order to examine the flexibility of GDH upon immobilization, allosteric effectors were employed. The addition of allosteric effectors was done to determine how the free and immobilized forms of the enzyme reacted to this addition.

The surface chemistry used for immobilization was 100% $COOH^-$. L-leucine, ADP and NAD^+ were added to both forms of the enzyme and the activities were measured afterwards. For the immobilized enzyme, 18 chips were prepared and the GDH activity was tested once before the addition of the allosteric effectors and once after (six chips for each effector). Similarly, for the free GDH test, 18 identical enzyme solutions were prepared and their activities were determined before the addition of any allosteric effectors. Each of the three chosen allosteric effectors was then added to the solutions (each effector added to six solutions) and the activities were obtained. Making various samples was done to ensure reproducibility of the obtained data. The experiments were performed by the Fluorimeter and by employing the fluorescence assay developed for GDH. The results are shown in Figure 20.

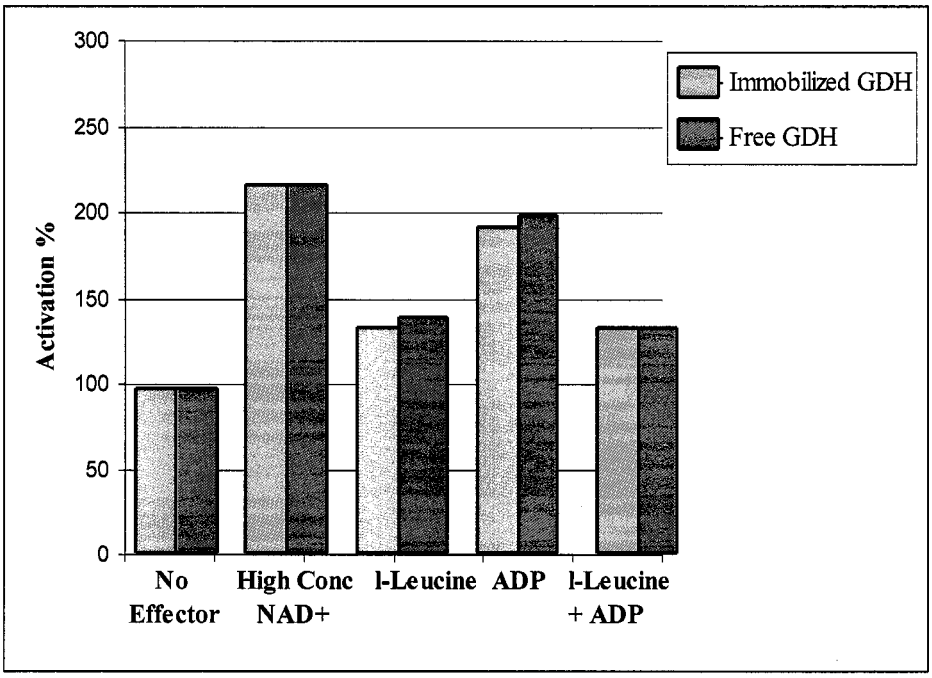


Figure 20. Effect of allosteric effectors on free and immobilized forms of GDH.

As shown in Figure 20, the allosteric effectors activated both free and immobilized forms of the enzyme to the same extent. These results also indicated that immobilization of GDH did not affect the enzyme's flexibility. This piece of information was very useful as it indicated that GDH retained its natural change in conformation upon immobilization. It could be concluded that the orientation of GDH while attaching to the surface, was in a manner that it wouldn't restrict its active sites and conformation. In addition to this result, the results provided in Section 5.2.7 (Table 21) gave the same indication. According to these results, chances were high for good electrical detection of GDH movement upon immobilization, as the conformational change of the enzyme was shown to have retained.

After it was verified that the enzyme had retained its flexibility upon immobilization, it was necessary to determine the binding capacity of the immobilized surface. Determining the amount of enzyme bound to the surface upon immobilization could be used to determine the surface binding capacity. The amount of enzyme attached to the surface upon immobilization could also be used to determine the specific activity of the enzyme in both free and immobilized forms. The significance of determining this value is discussed in the following sections. Also, the steps taken for developing the required assay for determining specific activity, determination of the optimal detection conditions, and preparation of a standard curve for the dye-enzyme complex along with the results of this experiment are presented.

5.4 Fluorescamine-GDH Assay

Up to now, the activities of enzymes in different forms and on various surface chemistries have been measured. In this experiment, the amount of the enzyme attached to the surface upon immobilization was desired. This value was necessary for determination of the specific activity (explained in Section 5.5) and also for determining the binding capacity of the immobilized surface. In order to find the specific activity of the immobilized enzyme, two values were required. The first value was the amount of enzyme immobilized on the surface, and the second value was the activity generated by the same amount of immobilized enzyme. To determine the amount of enzyme attached to the surface, an assay had to be developed. Fluorescamine, a dyeing chemical with fluorescent properties and capability of attachment to GDH, was employed to do the task.

To begin the experiment, the concentration of fluorescamine that generated the optimal Fluorimeter reading, upon binding with GDH, was required. In other words, preparation of a saturation curve for fluorescamine for it's binding to GDH was necessary. The desired data were collected by the Victor Fluorimeter and the actual experiments were started afterwards. The steps taken for determination of the optimal fluorescamine testing conditions are presented in the following section.

5.4.1 Optimal Point for Detection of Fluorescamine-GDH Complex

In this experiment, determination of the optimal fluorescamine-GDH detection condition was desired. In order to obtain the optimal reading for the fluorescamine-

GDH complex by the instrument, various concentrations of fluorescamine were chosen while the concentration of GDH was kept constant. The fluorescamine solution was prepared in acetone, and its concentration was 50 mg/mL. The GDH concentration in MOPS buffer (pH=8.3) was 0.215 mg/mL, which is the same concentration used in all previous experiments. The volume taken from the GDH solution for performing each experiment was 100 μL, which was added to different concentrations of the fluorescamine solution (prepared in acetone) to form the complex. The experiments were performed three times each, and the average values were used for plotting the data. The prepared solutions were tested by the Victor Fluorimeter, and the results are presented in Figure 21.

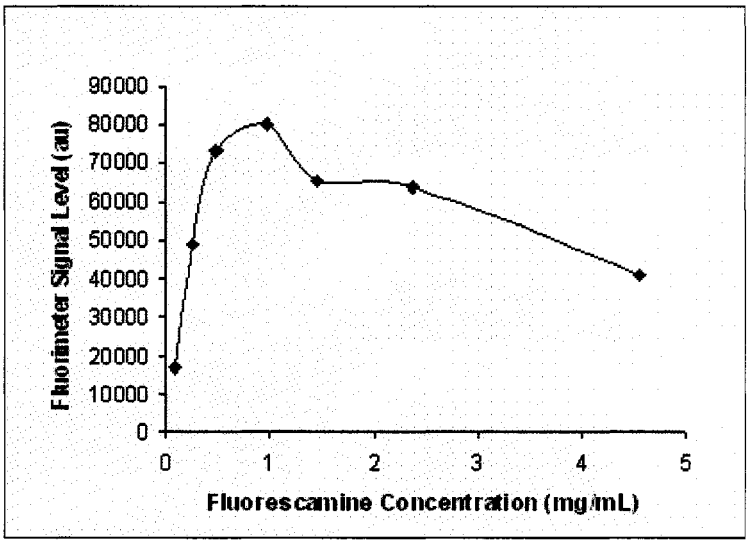


Figure 21. Fluorescamine optimization curve.

As evident in Figure 21, the optimal concentration of fluorescamine that generated the highest reading by the Victor Fluorimeter was 0.98 mg/mL. This complex was prepared by the addition of 100 μL of GDH (0.215 mg/mL) to 2 μL of

the fluorescamine solution. The instrument reading corresponding to this concentration was 81,201.56 units (arbitrary units).

The optimal concentration for fluorescamine-GDH detection was determined and therefore, preparation of a standard curve for determining unknown concentrations of the complex was necessary. The following section explains the procedure in more detail along with the obtained results and discussion.

5.4.2 Standard Curve for Fluorescamine-GDH

In this experiment, preparation of a standard curve for fluorescamine-GDH was desired. To determine the linear range for detection of the fluorescamine-GDH complex by the Victor Fluorimeter, different concentrations of GDH were tested. Hundred microliters of the chosen GDH solutions were added to fluorescamine to make the final concentration of 0.098 mg/mL, which was the optimal fluorescamine concentration developed in the previous section. At the beginning of this experiment, the GDH concentrations were chosen randomly. Triplicates of each concentration were tested by taking three 15-μL samples, and using the Fluorimeter to obtain the reading. When originally plotting the data, the points that showed deviation from the straight line were eliminated. The values that were within a linear range were kept for the next round of experiments and various points between the specified points were chosen for further experiments. Figure 22 exhibits the relation between the tested GDH concentrations and their corresponding readings by the instrument. Many more concentrations than the presented were tested, but the ones exhibiting deviation from

a straight line were eliminated. In other words, only the concentrations within the linear region were accepted.

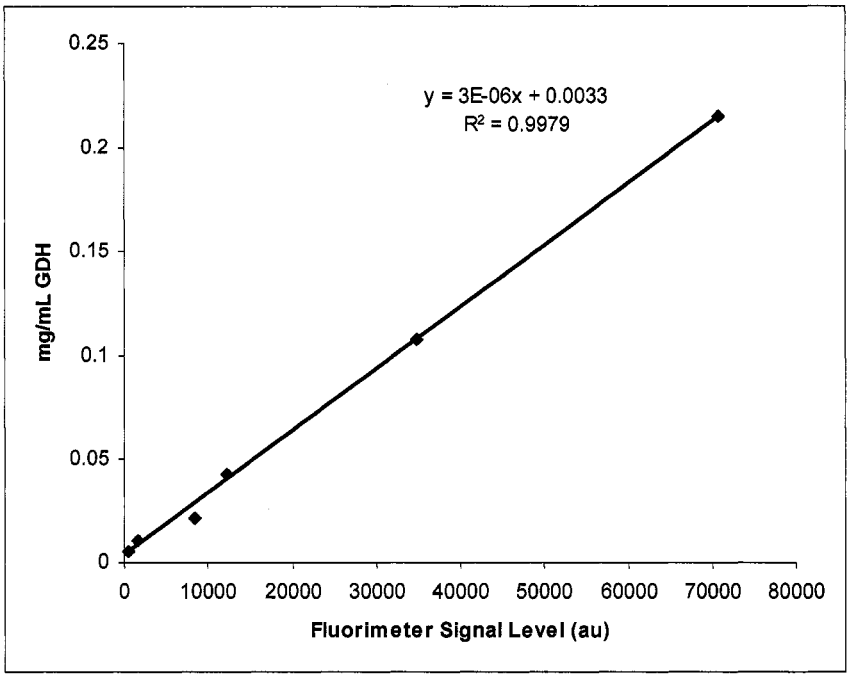


Figure 22. Fluorescamine-GDH standard curve.

The prepared standard curve shown in Figure 22 provides the linear range of operation as the value of R^2 was 0.997. The closeness of this value to unity indicates that the data points (within that range) fit the straight line perfectly. The equation of the regression line was also determined and is presented as Equation 18. This equation can be used to determine unknown concentrations of GDH by simply using the reading obtained from the instrument as the x value, plugging it into the equation, and solving for y.

$$y = 3 \quad 10^{-6} x + 0.0033 \qquad \text{Equation 18}$$

After preparation of the standard curve, the actual amount of enzyme available in the immobilization solution could be determined. The results and discussion of the experiments are presented in the following section.

5.4.3 Amount of GDH Bound to the Surface

In this experiment, determination of the amount of enzyme attached to the surface was desired. This experiment was performed to determine the binding capacity of the immobilization surface and also the specific activity of the immobilized enzyme. After determining the fluorescamine-GDH optimal detection point and preparing the standard curve, the amount of enzyme was determined by testing the enzyme solutions before and after immobilization on the 100% $COOH^-$ chemistry.

In this experiment, it was assumed that the amount of enzyme lost from the solution was the same as the amount of enzyme attached to the surface. However, the accuracy of this assumption should have been investigated first. Therefore, the chip with immobilized enzyme was placed inside 1 mL of MOPS buffer. After half an hour of storage and shaking, the chip was removed from the MOPS buffer and the buffer was tested. The test was to clarify whether or not any GDH would be present in the remaining washing buffer as a result of detachment from the surface. No enzyme could be detected in the remaining buffer either because there was no GDH in the solution (no detachment from the chip to the buffer) or because the amount of enzyme was so low that the instrument was not sensitive enough to detect it. Either way, the enzyme detachment, if any, could be considered as negligible.

The experiments were repeated six times to ensure repeatability. The concentration of the original GDH solution used for immobilization was 0.215 mg/mL. Fluorescamine was added to make the final concentration of 0.98 mg/mL (explained in Section 5.4.1). Six similar solutions were prepared and tested. The background solution was 100 μL of MOPS buffer plus 2 μL of fluorescamine prepared in acetone. The background reading was deducted from the actual readings. The total surface area of the chip, which the enzyme was immobilized on, was 198 mm^2 (7 7 2 for two coated sides) and this val ue was used for determining the binding capacity. The obtained results are presented in Table 23.

Table 23. Amount of GDH Bound to the Surface.

Reading after Immobilization	Reading before Immobilization	mg/mL GDH in Enzyme Solution before Immobilization	Mg/mL GDH in Enzyme Solution after Immobilization	mg/mL (before –after)	ng/mm^2
60719.8 ± 1809.4	70642.7 ± 1245.1	0.215	0.185	0.0295	301

The instrument has generated readings based on the amount of enzyme available in the solutions before and after immobilization. As shown in Table 23, the two values were subtracted to determine the amount of GDH disappeared from the solution by employing Equation 18. By using the value obtained as the amount of enzyme attached to the surface (the difference between the amount of enzyme before and after immobilization) and dividing it by the area of the chip, the binding capacity of the immobilization surface was determined to be 301 ng/mm^2. This value shows that during the two-hour immobilization process of GDH, 301 ng of enzyme had attached to every 1 mm^2 of the immobilization surface modified with the 100%

$COOH^-$ monolayer. A study similar to this experiment has not been performed before and therefore, the obtained value could not be compared with a similar work in the literature.

The standard error values calculated (by equations on page 55) for the Fluorimeter readings, were around 3% of their corresponding average values obtained as the amount of GDH available in the solution before and after immobilization. This error is considered as insignificant and normal for the current testing conditions as there are several factors causing this error. As discussed in Section 5.1.2 on pages 65-73, in every experiment there are inevitable experimental errors such as pipetting, operator, and operation errors along with errors generated by the instrument. The limited number of experiments performed, contributed to the error as well. As mentioned at the beginning of this section, only six GDH solutions were prepared and tested. The reason for choosing limited number of samples was because it was necessary to perform all experiments in one day to avoid day to day variations. Using an instrument not sensitive enough for the task could also be a reason for the existing errors.

In the future experiments (see Chapter 7), an alternative method for determining the binding capacity of the surface will be employed. The instrument used in that method is called the surface plasmon resonance (SPR). Once the binding capacity is determined by the new method, the two values can be compared to ensure the accuracy of the obtained results.

Now that the amount of enzyme in the solution before and after performing immobilization, and also the activity of the enzyme attached to the surface upon immobilization were determined; the value of specific activity could be calculated.

5.5 Specific Activity of GDH

In this experiment, determination of the specific activity of the immobilized enzyme was desired. In order to calculate the specific activity, finding the values of ΔF and ΔA were necessary (will be explained shortly). The difference between the amount of enzyme in the immobilization solution before and after immobilization was calculated to find ΔF. The difference in the reading was 9,419.33 units, which corresponded to 0.0295 mg of GDH. This value was calculated by using Equation 18 and it was marked as the amount of enzyme attached to the surface upon immobilization. The activity of the immobilized enzyme on the 100% $COOH^-$ chemistry determined in section 5.2.7 (page 93), was used as the value of ΔA. Table 24 provides a summery of the values needed for determination of the specific activity.

Table 24. Summary of the Values Necessary for Determining Immobilized GDH Specific Activity.

ΔF (mg/mL) from Equation 18	ΔA (mmole/min.mL)
0.0295	0.0015

Comparing the specific activities of the free and immobilized forms of the enzyme reveals the limitation enforced on the enzymatic activity upon immobilization. If the two values (specific activities of free and immobilized GDH) remain the same, it can be inferred that immobilization has not limited the enzymatic activity of GDH. Retention of specific activity upon immobilization is always considered as favorable as it indicates that the enzyme has retained its enzymatic

properties and flexibility upon immobilization. The specific activity of the immobilized GDH is calculated by using the following equation:

$$\text{Specific Activity} = (\Delta A \text{ or } A) / (\Delta F \text{ or } F) \qquad \text{Equation 19}$$

The specific activity of immobilized GDH was calculated to be 0.0508 mmole NADH/min.mg (specific activity = 0.0015/0.0295 = 0.0508 mmole NADH/min.mg). Here, the specific activity was not determined for free GDH as it required using the exact amount of enzyme as the immobilized enzyme. Experiments for determination of the free enzyme's specific activity will be performed in the future.

The stability, flexibility and specific activity of GDH indicated that this enzyme had good retention of its properties upon immobilization. As a complementary experiment, the behavior of the enzyme when placed in a continuous flow operation was investigated. The continuous flow operation is a large scale model of the nanochannel inside the nanosensor that is being manufactured for metabolite detection. The following section contains the obtained results and the discussion.

5.6 Continuous Flow Over Immobilized GDH

In this experiment, the effect of continuous flow of substrate over immobilized GDH was investigated. In that regards, a continuous flow of the GDH substrate solution, with a constant flow rate of 87 μL/min, was passed over the immobilized GDH by employing the setup shown in Section 4.6.4 on page 36. The passage of flow continued for more than 24 hours. Samples were collected every 30 minutes by

a fraction collector that released the flow after it would pass over the chip. The samples were taken in triplicates, and the average fluorescent reading values were calculated. The average values and their standard errors are presented in Table 25.

Table 25. Results of Continuous Flow over Immobilized GDH for more than 24 Hours.

	Average Fluorescence Reading	Flow Rate (μL/sec)
Day 1 3-hour Duration	2726.9 ± 98.7	0.36
Day 2 3-hour Duration	2691.5 ± 98.7	0.38

By Assuming no enzymes were released from the surface and also no loss of enzymatic activity had occurred, it would be expected that over a three-hour period of taking samples, the same fluorescent readings would be obtained. That was because each sample represented the NADH produced during the first seconds of the enzymatic activity. The results met the expectation since all the readings were approximately the same. Figure 23 is plot of the NADH readings from the samples taken over a 24-hour period.

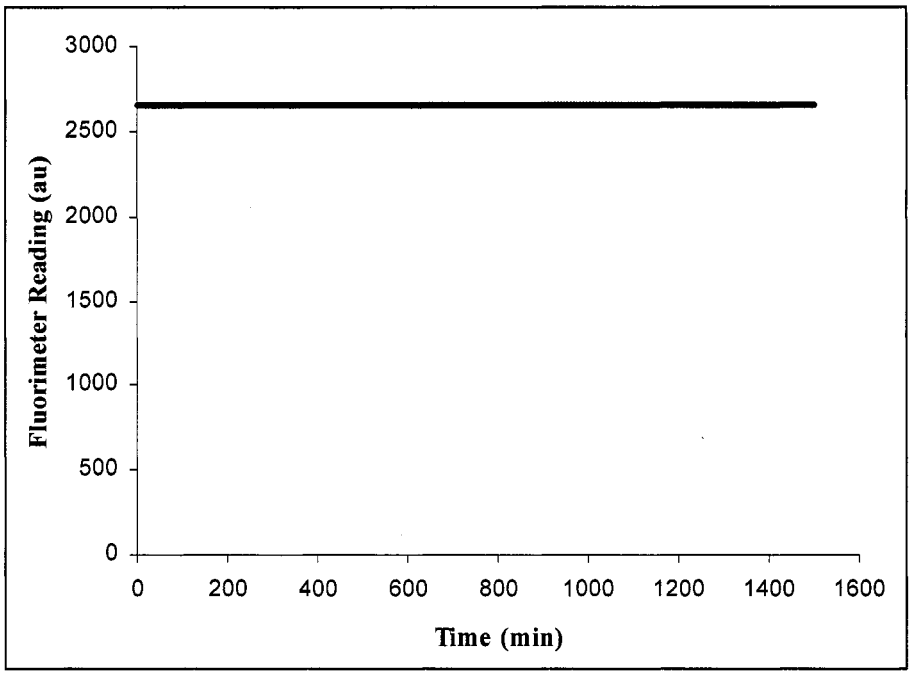


Figure 23. Production of NADH during 24 hours of continuous flow operation.

As shown in Figure 23, the reading value was constant throughout the experiment. Obtaining a constant value of NADH production during 24 hours of operation showed that the enzyme had not detached from the surface as a result of the continuous flow. This finding was a proof of a strong GDH binding strength to the surface. It also revealed that GDH had stayed active for over 24 hours even after being left outside at room temperature overnight. This information was another positive factor for making GDH a suitable candidate for usage in a nanosensor for metabolite detection.

CHAPTER SIX
CONCLUSIONS

The main challenge of the thesis was to establish efficient immobilization strategies that would reduce the loss of enzyme conformational change upon immobilization, to minimum. In order to determine the impact of immobilization on the enzymes' conformational change, investigation of some issues such as enzyme activity and flexibility upon immobilization was necessary. In that regards, two model enzymes were chosen. Myokinase was chosen for its high ability to undergo conformational change and GDH for its stability and allosteric properties.

Self-assembled monolayers were employed to modify the surface by using four different alkanethiol groups. Immobilization of myokinase and GDH was performed on the modified surfaces by both covalent and adsorptive immobilization methods with retention of native properties.

In order to determine the activity of each enzyme upon immobilization, appropriate assays were developed. For determining myokinase activity, two assays, one bioluminescence and one fluorimetric, were developed. The activities were then successfully determined by employing two different instruments. The employed instruments were the PSQ 96 MA Pyrosequencer and the Victor Fluorimeter for the bioluminescence and the fluorescence assays, respectively. For determining GDH activity, a fluorescence assay was developed and the activities were measured using the Victor Fluorimeter. Obtaining reproducible results for myokinase and GDH activities indicated that both enzymes retained their native structure and remained active upon immobilization.

The activity of each enzyme in the free form was also determined. The obtained values were compared with the immobilized form in order to determine the flexibility of the enzymes. The flexibility of GDH was also investigated with the effect of allosteric effectors as GDH possessed allosteric properties. The results indicated that the allosteric effectors had activated both free and immobilized forms of the enzyme to a similar extent. This result revealed that GDH had retained its flexibility upon immobilization.

The stability of the two enzymes in the free form and upon immobilization was investigated. Due to instability of myokinase, different stabilization buffers were prepared and one of the tested conditions exhibited a stabilizing effect on myokinase. In fact, the mentioned condition helped myokinase to remain stable for up to one month.

To determine the specific activity of GDH and the binding capacity of the surface, the amount of enzyme attached to the gold surface during the immobilization procedure was determined. The actual activity of the immobilized GDH on the carboxyl-terminated modified surface (100% $COOH^-$) was also determined. The specific activity was then calculated by using the obtained activity and the amount of enzyme attached to the surface.

Finally, to make a link to immobilization of enzymes in a nanochannel, which works on the basis of substrates passing inside the channel, studies were conducted on the effect of continuous flow on the immobilized GDH. The results exhibited a constant rate of product formation over a 24-hour period of substrate passing over the surface. This result reveled that GDH had remained active over the mentioned period without any detectable detachment from the surface.

CHAPTER SEVEN
FUTURE WORK

The overall goal of this study was to investigate enzyme immobilization patterns in the large scale so the tested enzymes could later on be immobilized in nanochannels to fabricate nanosensors. Therefore, an important future task would be to miniaturize the obtained immobilization patters down to nano scale.

In the present work, the thiol groups employed for surface modification were mainly hydrophilic with carboxylic or hydroxyl terminus. The CH_3 terminating thiol group was the only hydrophobic group used in this work. For future experiments, if other hydrophobic or hydrophilic thiol groups would be desired for modification of surfaces other than gold, contact angle measurements can be conducted to determine the wettability of the surface.

Some experiments conducted on GDH were not performed on myokinase due to the unstable nature of this enzyme. Now that myokinase has been stabilized by use of stabilization buffers, the same type of experiments conducted on GDH should be performed on myokinase. These experiments include the continuous flow test, determination of free enzyme activity, and determination of the enzyme bound to the surface before and after immobilization.

Also to ensure the accuracy of the fluorescamine-GDH assay, another method for determination of the amount of enzyme attached to the surface could become useful. Therefore, preliminary studies in addition to some initial experiments were performed with the SPR instrument, and some useful data were generated. However, this work is in its early stages and a lot more needs to be done in the future.

The specific activity of the free form of GDH was not determined in this work. This was because a kinetic analysis needed to be performed and the amount of enzyme used in free and immobilized forms, needed to be matched to have equal conditions and concentrations. This task is one of the tasks that will be performed in continuance of this thesis in the future. Finally, to achieve a universal immobilization strategy, it is necessary to expand this study to several other enzymes.

REFERENCES

1. Abraham Ulman, "*Formation and structure of Self Assembled monolayers,*" Chemical Reviews, **96** (4), pp.1533-1553 (1996).

2. Michael L. Shuler and Fikret Kargi, Bioprocess engineering basic concepts, second edition. (Dokuz Eylul University, Izmir, Turkey, 2002), pp. 57-104.

3. Martin Chaplin, M.C (2004). Enzyme Technology Web-Book [Online]. Available at www.lsbu.ac.uk/biology (accessed 15 December 2004).

4. Ali Agah, Mariam Aghajan, Foad Mashayekhi, Sasan Amini, Ronald W. Davis, James D. Plummer, Mostafa Ronaghi and Peter B. Griffin, "*A multi-enzyme model for Pyrosequencing,*" Nucleotide acids research, **32** (21), pp.1-15 (2004).

5. Available at http://waynesword.palomar.edu/molecu1.htm (accessed 2001).

6. Ralph G. Nuzzo, David L. Allara, "*Adsorption of bifunctional organic disulfides on gold surfaces,*" Journal of American chemical society, **105** (13), pp. 4481-4483 (1983).

7. *Perkin Elmer online catalog* [Online]. Available at http://las.perkinelmer.com/catalog/productinfopage.htm?productid=1420-060 (accessed 2005). WWW Article.

8. *Contact angle measurements* [Online]. Available at http://www.dmsf.ust.hk/metrology/about_ContactAngleMeasurement.pdf (accessed 2006). Posted pdf file.

9. Giorgio Carboni, *Experiments on surface phenomena and colloids* [Online]. Available at: http://www.funsci.com/fun3_en/exper2/exper2.htm (accessed 2002).

10. Bigelow, W. C., Pickett, D. L. and Zisman, W.A., "*Colloid interface,*" Science, **513** (1), 1946.

11. Enabling next-generation research [Online]. Available at http://www.mscience.com.au/pyro1.htm (accessed 2000).

12. Ronald W. Davis, Research plan proposal, Stanford Genome Technology Center, 2004.

13. Available at http://www.tandur.is/frodleikur/010.html (accessed 2004).

14. F.W.D. Rost, Fluorescence microscopy volume II, New York, NY, Cambridge university press.

15. Claire E. Jordon, Brian L. Frey, Steven Kornguth and Robert M. Corn, "*Characterization of poly-L-lysine adsorption onto alkanethiol-modified gold surfaces with polarization-modulation Fourier transform infrared spectroscopy and surface plasmon resonance measurements*," Langmuir, **10** (10), pp. 3642-3648 (1994).

16. Brian L. Frey and Robert M. Corn, "*Covalent attachment and derivatization of poly (L-lysin) monolayers on gold surfaces as characterized by polarization-modulation FT-IR spectroscopy*," Analytical Chemistry, **68**, pp.3187-3193 (1996).

17. Koji Nakano, Kenji Doi, Kousuke Tamura, Yosuke Katsumi and Masato Tazaki, "*Self-assembling monolayer formation of glucose oxidase covalently attached on 11-aminoundecanethiol monolayers on gold*," Chemical Communication, pp. 1544-1545 (2003).

18. B. Samuel Ko, Brian Babcock, G. Kane Jennings, S. Grey Tilden, Rachel L. Peterson, David Cliffel and Elias Greenbaum, "*Effect of surface composition on the adsorption of photosystem I onto alkanethiolate Self Assembled monolayers on gold*," American chemical society, **20**, pp. 4033-4038 (2004).

19. Allan G. Larsen and Kurt V. Gothelf, "*Electrochemical properties of mixed Self Assembled monolayers on gold electrodes containing mercaptooctyldroquinone and alkylthiols*," American Chemical Society, **21**, pp.1015-1021 (2005).

20. J. Justin Gooding, Lina Pugliano, D. Brynn Hibbert, P. Erokhin, "*Amperometric biosensor with enzyme amplification fabricated using Self Assembled monolayers of alkanethiols: the influence of the spatial distribution of the enzymes*," Electrochemistry Communications, **2**, pp. 217–221 (2000).

21. L. Blasi, L. Longo, P.P. Pompa, L. Manna, G. Ciccarella, G. Vasapollo, R. Cingolani, R. Rinaldia, A. Rizzello, R. Acierno, C. Storelli, M. Maffia, "*Formation and characterization of glutamate dehydrogenase monolayers on silicon supports*," Biosensors and Bioelectronics (2004).

22. L. Blasi, L. Longo, G. Vasapollo, R. Cingolani, R. Rinaldi,T. Rizzello, R. Acierno and M. Maffia ,"*Characterization of glutamate dehydrogenase immobilization on silica surface by atomic force microscopy and kinetic analyses*," Enzyme and Microbial Technology **36**, pp. 818–823 (2005).

23. Mostafa Ronaghi, Samer Karamhamed, Bertil Petterson, Mathias Uhlen and Pal Nyren, "*Real-time DNA sequencing using detection of pyrophosphate release*," Analytical Biochemistry, **242**, pp. 84-89 (1996).

24. Samer Karamhamed and Pal Nyren, "*Real-time detection and quantification of adenosine triphosphate sulfurylase activity by a bioluminometric approach*," Analytical Biochemistry, **271**, pp. 81-85 (1999).

25. Mostafa Ronaghi, *Pyrosequencing sheds light on DNA sequencing* [Online]. Genome Research, Available at www.genome.org (accessed 2001).

26. Ashutosh Sharma and Nigel S.M. Quantrill, "*Measurement of glucose using fluorescence quenching*," Molecular Spectroscopy, **50** (6), pp. 1179-1193 (1994).

27. David J. Holme and David M. Goldberg, "*Development of a fluorimetric assay for amino-acid oxidase activity and its application to the study of human tissues*," Biochemical Medicine, **28** (1), pp. 51-61 (1982).

28. Nobutoshi Kiba, Hidekazu Koemado and Motoshisa Furusawa, "*Flow-injection determination of 3-hdroxybutyrate in serum with an immobilized 3-hydroxybutyrate dehydrogenase reactor and chemiluminescence detection*," Analiytica Chemica Acta, **290** (3), pp. 357-361 (1994).

29. S. de Marcos, J. Galbán, R. Albajez and J. R. Castillo, "*Enzymatic determination of ethanol based on the intrinsic fluorescence of alcohol dehydrogenase*," Analytica Chimica Acta, **343** (1-2), pp 117-123 (1997).

30. H. C. McDonald, P. A. Simon, M. M. Takeguchi, G. Odstrchel, H. H. Weetall and Yehudi M. Felman, "*Detection of neisseria gonorrhoeae by ultraviolet illumination of NADH generated by an immobilized antibody-gonococcal enzyme complex*," Immunology Letters, **3** (6), pp 377-380 (1981).

31. Xuwei Chen and Jianhua Wang, "*A sequential injection fluorometric procedure for rapid determination of total protein in human serum*," Talanta, **69**, pp. 681-685 (2006).

32. Marc Delvaux, Sophie Demoustier-Champagne, "*Immobilization of glucose oxidase within nanotubes arrays for application to enzyme biosensors*," Biosensors and Bioelectronics, **18** (7), pp. 943-951 (2003).

33. Dieter Trau, Reinhard Renneberg, "*Encapsulation of glucose oxidase microparticles within a nanoscale layer-by-layer film: immobilization and biosensor applications*," Biosensors and Bioelectronics, **18** (12), pp. 1491-1499 (2003).

34. Peter Ulrich Abel, Thomas von Woedtke, Birgit Schulz, Theodor Bergann and Alexander Schwock, "*Stability of immobilized enzymes as biosensors for continuous application in vitro and in vivo*," Journal of Molecular Catalysis b: Enzymatic, **7**, pp.93–100 (1999).

35. *Delivering life sciences to the world* [Online]. Available at http://www.hvd.ru/p/p31.html (accessed 2003).

36. *Chayon* [Online]. Available at http://www.chayon.co.kr/shop/mall/pdetail.asp?no=24406 (accessed 2004).

37. *Luciferase Reporter Assay Kit User Manual* [Online]. Available at www.bdbiosciences.com (Published online 11/22/2002).

38. Hualei Li, Dithiothreitol, *Cleland's Reagent* [Online], Available at http://www.medicine.uiowa.edu (accessed 2003).

39. Yong-Hong Liao, Marc B. Brown and Gary P. Martin, "*Investigation of the stabilization of freeze-dried lysozyme and the physical properties of the formulations*," European Journal of Pharmaceutics and Biopharmaceutics, **58** (1), pp. 15-24 (2004).

40. Stephan Huwel, Lutz Haalck, Nikola Conrath and Friedrich Spencer, "*Maltose phosphorylase from lactobacillus brevis: Purification, characterization, and application in a biosensor for ortho-phosphate*," Enzyme and Microbial Technology, **21**, pp. 413-420 (1997)

41. Gulay Bayramoglu, Meltem Yilmaz and Yakup Arica, "*Immobilization of a thermostable α-amilase onto reactive membranes: kinetics characterization and application to continuous starch hydrolysis*," Food Chemistry, **84**, pp. 591-599 (2004)

42. Stephan Hüwel, Lutz Haalck, Nikola Conrath and Friedrich Spener, "*Maltose phosphorylase from lactobacillus brevis: Purification, characterization, and application in a biosensor for ortho-phosphate*," Enzyme and Microbial Technology, **21**(6), pp. 413-420 (1997).