Studies of the Preparations and Use of Sol-Gel for Enzyme Immobilization and Analytical Applications

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ABSTRACT

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Much attention has been paid to immobilization of enzymes to improve enzyme stability and permit its reuse. Glucose oxidase entrapment in different kinds of silica sol-gel matrices was investigated. The enzyme showed stable activity for 11 uses in the sol-gel with tetramethyl orthosilicate (TMOS) as precursor, and at least 7 uses in the sol-gel induced electrochemically with tetraethyl orthosilicate (TEOS) as precursor. The sol-gel made with TEOS as precursor and HCl as catalyst showed stability in enzyme activity for 11 uses but the activity decreases on the same sol-gel when modified with silica nanoparticles. Factors such as optimal incubation time of glucose solution, the reproducibility between different sol-gels, and the storage time were investigated. Good linearity and analytical results on real samples were obtained. The detection method was based on a colorimetric method for determining the concentration of hydrogen peroxide produced from the oxidation reaction of glucose.

DEDICATION

I would like to dedicate this thesis to my parents who supported me during my entire journey seeking for education. It would be hard for me to achieve my goals without their understanding and their help.

I would like to thank also my sister and her husband for their support and encouragement while being in United States. A special dedication to my nephew Jason and my niece Cassandra who brought a lot of joy to my life.

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CHAPTER 1

INTRODUCTION

Enzyme-based probes have become popular recently because they can be widely applied in different fields like medicine, food quality control, and chemistry (1).

The development of these sensors is based on immobilization processes that employ a soluble enzyme attached on an insoluble solid support to form a carrier matrix (2). For maximum biostability and activity, the best support is the one that protects the enzyme, preventing it from self-aggregation and microbial attack and at the same time hyphen an aqueous microenvironment similar to the biological media (3).

Sol-gel immobilization method is currently attracting a lot of interest due essentially to the simplicity of preparation and to the high thermal stability of the enzyme (4). The sol-gel technique is also important for a large number of applications, especially in the biological field due to its unique architecture and resistance to microbial attack (5).

Importance of Immobilizing Enzymes

Enzymes play the role of catalysts with unique properties such as sensitivity and specificity in most chemical reactions in the living system (6). Unfortunately, natural enzymes are not always suitable for industrial applications under specific conditions because of their low stability. Many factors can affect the stability of the enzyme such as temperature, pH, and the presence of certain surfactants in the solution. To enhance the stability of the enzyme, three principal procedures were used. The first one is based on isolating the enzyme. The second procedure involves protein engineering where researchers study the effect of mutations in the

structure that have an effect on the stability of the enzyme. The last one is the direct evolution of the enzyme in generating certain diversity capable of producing enzymes with enhanced properties after surviving under harsh environmental conditions (7).

Immobilization of enzyme is a powerful way to enhance the stability of an enzyme as well as its other properties like the conservation of the enzymatic activity by preventing bacterial and chemical degradation, the improvement of specificity, and the reduction of inhibition (3, 6). Furthermore, the immobilization of enzymes makes them more attractive for industrial use due to the cost-effective reusability of the enzyme (3).

Immobilized enzyme has more advantages than free enzyme that suffers from low recovery yield. The use of immobilized enzyme helps in choosing between batch and continuous processes, ending the reactions rapidly, controlling the product formation, removing the enzyme easily from the reaction mixture, and monitoring various engineering designs (8).

Historical Review of Enzyme Immobilization

In 1916, Nelson and Griffin discovered that the invertase enzyme bound on an artificial carrier made of $Al(OH)_3$ was still active (9). In those early days, bio-immobilization techniques were based on the adsorption of proteins on inorganic supports such as glass (10) and alumina (11). The immobilization of enzymes using covalent attachment technique was also known during that time (12).

In the 1950s, physical methods for immobilizing enzyme were basically used, especially the non-specific physical adsorption of enzymes on solid supports like the adsorption of α-

amylase on activated carbon, bentonite, and clay (13). During this period, McLaren worked on immobilizing the enzymes on kaolinite support by adsorption (14). The non-specific adsorption method was improved into specific ionic adsorption like immobilizing chymotrypsin on phosphocellulose (15) and deoxyribonuclease (Dnase) on cellulose (16, 17) and many others. Covalent immobilization of enzymes was also reported but the early-developed carriers used were causing poor retention of enzyme activity (18-25). Around the same time, Dickey proved for the first time that Myoadenylate deaminase (AMP deaminase) entrapped in the sol-gel inorganic matrix made of silicic acid-derived glasses was still active (26).

In the 1960s, different techniques of immobilization of enzymes were enhanced by covalent methods (27) and by non-covalent methods such as entrapment (28-30) and adsorption (31). The encapsulation of enzymes was also improved by using synthetic and natural polymers for the sol-gel process (28, 32-34). The cross-linking method was also reported: it was based on the use of the bifunctional cross-linker glutaraldehyde (35, 36). In the late 1960s, research was concentrating on enhancing the solid carriers. The researchers introduced the hydrophilic insoluble supports with defined geometric properties like the cross-linked cellulose, dextran, and agarose supports (27). Important studies concerning cross-linking were known during this time. Richards immobilized carboxypeptidase A crystals (37), Habeeb worked on cross-linking trypsin (38), the studies of Ogata (39) and Jansen, (40) were concerning subtilisin novo and papain, respectively. During this time, the first enzyme electrode also appeared (41). Furthermore, studies on the effect of adding functions to the enzymes for a better control of the immobilization process were sponsored by Glazer et al. (42). Towards the end of the 1960s, the first industrial application of the immobilized enzyme was developed by a

Japanese company and it was based on binding ionically L-amino-acid acylase for the resolution of synthetic racemic amino acids (31).

In the 1970s, new methods of immobilization appeared such as coordination binding and affinity binding (43). In order to improve the properties of enzymes, chemically modified enzymes were immobilized using appropriate immobilization methods like adsorption on cationic exchanger (44). The potential of the technique of enzyme immobilization has been largely improved, consequently new commercial processes using immobilized enzyme appeared, like the immobilization of penicillin G acylase for production of 6-aminopenicillanic acid (6-APA), a product intermediate for semi-synthetic penicillins (44) or the immobilization of glucose isomerase to obtain the fructose syrup from glucose (45).

In the 1980s, many efforts were carried out to create a stable immobilized enzyme especially in organic solvents (46-48).

From 1990 on, researchers focused on the rational design of immobilized enzymes. More sophisticated techniques for immobilizing enzymes, and new strategies to improve the enzyme activity and stability were developed (27). Cross-linked enzyme crystals (CLEC) used for biotransformations in nonaqueous media or in organic-water mixture were developed (49-51). During this period, the sol-gel technique was known as one of the interesting techniques for immobilization of enzyme after 40 years of their first use (27).

In the last 15 years many important dates were known in the history of the immobilization methods. Important researches on physical adsorption were done in 2000 by Liu et al. (52) and in 2004 by Niculescu et al. (53). Doretti et al. in 1996 (54) and Li et al. (55) made important studies about covalent attachment methods. Cross-linking method had also

been studied in 2004 by Chiu et al. (56) and Yang et al. (57). Entrapment of enzymes was studied by Peter et al. in 1996 (58), Schmidt et al. in 1996 (59), Yang et al. in 1997 (60), Tag et al. in 2000 (61), and Kwan et al. in 2004 (62).

The supports used for enzyme immobilization were made of inorganic materials (63-66), organic polymer (67-69), and biomaterials (70-75).

Braun et al. (76) proved in 1990 that the enzymes immobilized in silica supports retained their biological activity. Braun published another article in 1992 (77) about the encapsulation of glucose oxidase with peroxidase and a chromogen in a tetramethyloxysilane sol-gel matrix to determine glucose concentration. Yamanaka et al. (78) studied in 1992 the activity of glucose oxidase immobilized in a sol-gel matrix by entrapment. In this article, the photometric detection method was based on measuring the amount of produced peroxide. After this work, a great variety of immobilization techniques were developed to construct a glucose sensor that had better properties of reusability, stability, recovery, and self life. In 1998, Sosnitza et al. (79) published an article about immobilizing glucose oxidase by binding the enzyme to a chelating agent sepharose with the help of different metal ions and the lectin cocanavalin A that is covalently attached to sepharose. Other works using different immobilization techniques were also significant in the history of glucose determination (80, 81). In 2003, Babu et al. (82) immobilized glucose oxidase on glass beads modified by silanization.

Many electrochemical deposition procedures involving glucose oxidase based on entrapping the enzyme within polypyrrole polymer were published in the 1980s (83-85). In 1988, other publications about the electrodeposition of glucose oxidase using polyalanine (86) and polyindole polymers (87) appeared. In recent years, glucose oxidase was widely

immobilized onto different kinds of modified electrodes such as electrodeposition using polymerized o-aminophenol (88) and the polymer formed from dopamine oxidation at an Au electrode (89).

Furthermore, many studies for immobilizing glucose oxidase within nanomaterials on different types of electrodes were conducted. Zhao and coworkers were able to immobilize glucose oxidase/colloidal gold nanoparticles on a carbon electrode by the Nafion film intermediate (90). Reviews on this topic had also been published like the immobilization of glucose oxidase on electrodeposited nickel oxide nanoparticles (91).

Immobilization Procedures for Enzymes

Enzyme immobilization is the imprisonment of the enzyme in a phase separated from the solution phase where inhibitor molecules exist. Although the enzyme is separated from the bulk phase, there is possibility of access of specific molecules to the enzyme phase. The enzyme phase is usually insoluble in water and is made of a polymer with high molecular weight (92). Different techniques can be used for the immobilization of enzymes. They can be classified as irreversible and reversible techniques (93).

Irreversible Techniques

In these techniques, the enzyme cannot be detached from the support without destroying the support or altering the enzyme (93). They include covalent bonding and entrapment.

Covalent Binding. It is the most used method. The enzyme is covalently bound to its polymeric support. The enzymes are attached to the support by using the functional groups on

the enzyme molecule that are not related to the catalytic activity of the enzymes. A simple procedure to improve the activity yield is to carry out the reaction in the presence of its substrate or a competitive inhibitor to protect the active site (94). Some of the most frequent investigated functional groups are the amino, carboxyl and the phenolic group of tyrosine (93), amino group of lysine or arginine, thiol group of cystein, carboxylic group of aspartic or glutamic acids, and the hydroxyl group of serine or threonine (95). Studies have shown that the selection of the support is important because the level of activity of the immobilized enzyme depends on the hydrophilicity of the polymer matrix. For that reason polysaccharide polymers such as cellulose were extensively used for immobilization due to the presence of hydroxyl groups in the sugar residues of the polymer (92, 95). The problem with these polymers is their susceptibility to microbial attack. Other supports investigated were the porous glass and the porous silica. They are strong and durable but less hydrophilic (95). The advantages of covalent bonding technique are the stability of the enzyme-support complex and the low possibility of enzyme leakage. On the other hand, it is a complicated and time-consuming method where the possibility of activity loss is high because the immobilization can sometimes involves some functional groups essential for the biological activity of the enzyme (1).

Multipoint Covalent Attachment. This technique involves many functional groups existing on the surface of the enzyme. It is used to provide more rigidity to the enzyme structure. The choice of the support is critical in this case. The support should have a geometry compatible with the enzyme surface and it should have adequate number of reactive groups so an intense multipoint covalent attachment can be obtained. The stability of the reactive groups on the support and on the enzyme surface is also important to ensure a long-term interaction.

The glutaraldehyde chemistry is one of the most used techniques to have a multipoint covalent attachment (96).

Entrapment. Entrapping the enzyme molecules can happen by using three different means. The first method is based on the inclusion of the enzyme within the pores of a highly cross-linked polymer, the second method is the microencapsulation of the enzyme molecule which leads to its separation from the bulk phase. The last procedure of entrapment is done by dissolving the enzyme in a distinct non-aqueous phase (92). The advantage of the first two methods is that the enzyme molecules are free in solution and their movement is only limited by the porosity of the lattice structure of the gel, consequently there are no steric problems that can be due to covalent or electrostatic binding (92, 95). In the third way, the enzyme is dissolved in a solution containing the reagents essential for the formation of the enzyme phase and then the solution is treated so that a chemically distinct phase appears. The polyacrylamide gel is the most used matrix as a cross-linked polymer made from monomeric precursors. In the case of microencapsulation, semi-permeable microcapsules of nylon formed around the enzyme molecules had been the most investigated (92).

The advantages of the entrapment technique in general are the mild procedures used to immobilize the enzyme and the universal use of this technique for any enzyme (1). But the major disadvantage of the entrapment is the diffusion limitation especially when large molecules like starch and proteins are used, which can have an adverse effect on the enzyme kinetics (94, 95).

Cross-Linking. The chemical cross-linking process is based on using homo or hetero bifunctional cross-linking agents such as glutaraldehyde, bis-isocyanate derivatives or bis-

diazobenzidine (1, 94). Immobilization of enzymes can happen by linking only enzyme molecules to each other. However, it is usually done by using the linkage of an inert protein like gelatin, albumin, and collagen (94). Two procedures can help to get a solid matrix. The first one is to induce the agent to polymerize and the second is to precipitate the enzyme. The major disadvantage of this technique is that the cross-linking agent is able to attack the active site of the enzyme. For this reason this technique results in relatively low enzyme activity (1, 92).

Reversible Techniques

Under gentle conditions, the enzyme can be detached from the support making it possible to be reused and to immobilize fresh enzyme. These are the reversible techniques of immobilization (93).

Adsorption. The nonspecific adsorption is the oldest and simplest method of enzyme immobilization that involves a dynamic equilibrium between the adsorbed enzyme and the support. The binding forces could be hydrogen bond, hydrophobic, ionic bonding, or Van der Waals interactions (92). These forces are weak but can produce a reasonable strong interaction (95). One of the major advantages of this technique is that it does not cause any damage or chemical modification to the enzyme or to the support. However, leakage of enzyme from the support can be seen because of the reversible nature of the forces, and it is dependent on factors like the pH, the nature of solvent, and the temperature (1, 95).

In the case of ionic binding, the interactions are similar to the interactions used in the ion-exchange resins. It is a simple method but the difficulty is to keep the enzyme strongly bound and fully active (93).

Other examples of adsorption can be used to link the enzyme to the support like the affinity binding, the metal binding, and the disulfide bonds. The interaction in the case of affinity binding is selective and requires the presence of an attached ligand to the support (93). The transition metal salts used for chelation or metal binding are attached to the nucleophilic groups on the matrix. Some of the sites on the metals remain free to coordinate with the enzymes. This method is simple but not as reproducible because of the lack of uniformity between the occupied sites (93). The disulfide bonds can also be used to adsorb the enzyme on the support. In this example, a stable covalent bond is formed between the thiol groups on the matrix and the enzyme. To get a high activity, an appropriate thiol-reactive adsorbent with high specificity should be used (93).

Choice of Immobilization Method

Different requirements should be taken into consideration in order to choose the most appropriate immobilization method. First, the conditions of the immobilization technique should not alter the stability of the enzyme. Second, if cross-linking procedure should be used, then the cross-linking reaction should be prevented from penetrating the active site of the enzyme. Sometimes, protecting the active site is necessary. This can be done in the case of sulphydryl enzymes by adding to the enzyme cystine first to protect the group then reactivating the enzyme after immobilization. Third, the nature of the reaction that the enzyme will catalyze is an important factor to consider in choosing the immobilization technique. For example, choosing to immobilize the enzyme by physical entrapment in sol-gel is not appropriate if the enzyme will be used as a catalyst in the decomposition reaction of a high

molecular weight polymer like polysaccharide. Finally, the immobilization technique should be compatible with the future application of the immobilized enzyme. For this reason, the mechanical properties of the support are important considerations. For example, if the final design of the immobilized enzyme should be a membranous sheet, then covalent bonding on cellulose polymers is the best choice because these polymers can be easily transformed into sheets (92).

Choice of the Support

The nature of the support can have a big effect on the activity of the immobilized enzyme. Different characteristics should be taken into consideration when choosing a good support. An ideal support should have good physical strength and is resistant to compression. It is preferred that the support have a high degree of porosity with a large available surface area. It should be hydrophilic (capable of binding water molecules), inert towards enzymes, stable during the storage, capable of maintaining the enzyme activity, resistant to any microbial attack or change in pH and temperature, biocompatible, and available at low cost (95).

The supports are usually classified into organic and inorganic supports. The organic supports could be natural or synthetic. The natural polymers include polysaccharides (cellulose, agar, chitin, and alginate), proteins (collagen, albumin), and carbon. The synthetic polymers are the polystyrene, polyacrylamide, vinyl, and allyl polymers. The inorganic supports are classified into two categories, the natural minerals such as bentonite or silica and the processed materials such as glass, metals, and controlled pore metal oxide (93).

The Enzyme Structure during Catalysis

It is worth examining the conformational changes of the enzyme during the catalysis process in order to understand the influence of the immobilization on the structural properties of the enzyme. The monomeric enzymes usually have two distinct structures. The first is an open structure relative to the enzyme without the substrate. The second one is a closed conformation indicating the presence of bound substrates and the formation of the enzyme-substrate complex. The closed conformation is essential to orient the catalytic groups in a suitable position around the substrate and to prevent the escape of the intermediates during the catalysis phase.

According to different kinetic models, the free enzyme molecule or the enzyme-substrate complex can undergo conformational changes due to atomic displacements during catalysis. According to the induced-fit mechanism, the catalytic groups on the enzyme should have a precise orientation so the enzyme can bind to specific substrates. The resulting conformational transition (allosteric transition) will have a big impact on the enzyme response to the ligand concentration (97).

Effect of Immobilization on Enzyme Activity

The Microenvironment

Two different effects can arise in the microenvironment surrounding the enzyme due to the existence of the polymer matrix. The first effect is the partitioning effect. This effect is due to the capacity of the polymer to attract or repulse different molecules such as substrate, product, inhibitor, or protons. The distribution of the molecules around the enzyme and in the

bulk will not be homogeneous, and unexpected results can occur when applying the Michaelis-Menten type kinetics (92). The partitioning effect is obvious in the case of using an ionic polymer.

The second effect is the diffusion limitation effect resulting from the role of the polymer as a barrier that inhibits the free diffusion of the molecules from the bulk to the enzyme and vice-versa (92). The diffusion limitation is largely depending on the physical size of the pores of the polymer. The diffusion effect will usually result from the difference in concentration between the microenvironment and the solution phase. When the immobilized enzyme and the solute are mixed, a difference in concentration between the enzyme phase and the solution causing a concentration gradient to arise until a steady state is reached when the rate of diffusion of the substrate at certain point is equal to the rate of desorption. If the point of interaction between the enzyme and the substrate is hard to reach, the rate of diffusion of the substrate will decrease and the profile of the substrate gradient concentration will lose its linearity (92).

Conformational Changes

If the enzyme is immobilized to the support by covalent bonding, the ability of the enzyme conformation to adopt its correct position and to change during the catalysis process will be affected. Because the catalytic activity is depending on the conformational changes, the values of the kinetic parameters will be perturbed especially in the case of multipoint covalent attachment. The inhibition of some conformational changes that are considered important during catalysis may force the enzyme to lose its allosteric properties (92).

Stability

If the immobilization is properly done, the stability of the enzyme will be improved. Immobilization of enzyme in a porous structure will protect the enzyme from interacting with other enzymes molecules preventing any aggregation, autolysis, or proteolysis (in the case of proteases). The enzyme molecules can also be protected from air bubbles that can cause the deactivation of the enzymes. The air bubbles result from supplied gases or vigorous stirring needed to control the pH. It is also important to stabilize the enzyme when working in organic solvent phase because the free enzymes can get deactivated or denatured in organic solvent (94, 96). However, not all immobilization techniques can enhance enzyme stability. For example in the case of immobilizing the enzymes on non-porous nanoparticles, they will be in direct contact with external reagents. In such situation, other procedures should be used to improve the stability of the enzyme (96).

Inactivation

When the immobilization techniques require extreme reaction conditions (high pH or presence of oxidizing agent), some of the immobilized enzyme molecules can get deactivated. Furthermore, the enzyme can get its active site attached in such a way that the substrate will not have access to the active site that is blocked by the polymeric support. Thus, the activity of the immobilized enzyme is usually less than the free enzyme (92).

Kinetic Study of Free Enzyme

The reactivity of the enzyme can be represented by the Michaelis-Menten Kinetics. The reaction is represented as follow:

$$E + S \underset{K_{-1}}{\overset{K_1}{\leftrightarrow}} ES \xrightarrow{K_2} E + P \qquad [1.1]$$

E represents the enzyme, S is the substrate, ES is the enzyme-substrate complex, and P is the product. K_m is the Michaelis constant and it is equal to $(K_{-1} + K_2) / K_1$; K_m is almost equal to K_{-1} / K_1 if $K_2 << K_{-1}$ (the formation of product is the rate limiting step). K_m measures the degree of dissociation of ES complex, thus it can evaluate its stability. Another essential parameter is K_{cat} or K_2 which is the turnover number that estimates the rate of formation of the product. The apparent rate constant K_{cat} / K_m can help to evaluate the tendency of the enzyme-substrate complex to dissociate or to form the product (98). V_{max} is also useful to describe the catalytic activity of an enzyme in a dilute solution. Under such conditions, the graph of the reciprocal of V (reaction velocity) plotted against the reciprocal of S (substrate concentration) results in a straight line. The slope of the line is equal to K_m / V_{max}. The intersection of the line with the X axis will give the value of the reciprocal of K_m and the intersection with the X axis will give the $1 / V_{max}$ value (92).

Kinetic Study of Immobilized Enzyme

Because of the presence of new features in the case of immobilized enzyme, it is better to redefine new kinetic constants K_v and V_s instead of K_m and V_{max} where V_s is the highest value for the reaction velocity obtained when all the enzymes are attached to the substrate molecules and k_v is the substrate concentration that can give a reaction velocity of $V_s / 2$ (92).

Many factors can affect the apparent kinetic constants in the case of immobilized enzyme including the partitioning effects and the diffusion limitation.

Partitioning Effects

These effects have a noticeable impact on the measured kinetic parameters in the case of ionic interactions between the polymer and ionic substrates. Taking the example of an enzyme immobilized on a cationic polymer and interacting with an ionic substrate S^-, the concentration of S^- around the immobilized enzyme is greater than its concentration in the bulk phase due to the partitioning effect. The concentration of the solute around the polymer (S_i) is always proportional to the concentration of the solute in the bulk phase (S_0) (92).

If the ionic partition coefficient considered is equal to 2, the Lineweaver-Burk plot for this system shows that the immobilized enzyme is still obeying the Michaelis-Menten Kinetics, the value of V_{max} is the same, and the value of K_m is estimated as lower by a factor of 2. The coefficient p is introduced in the new Michaelis –Menten equation:

$$V = \frac{V_{max} \bullet S_0 \bullet p}{K_m + S_0 \bullet p} \text{ with } K_v = \frac{K_m}{p} \qquad [1.2]$$

Where $P = S_i / S_0$ is the ionic partition coefficient (92).

The value of the apparent K_m depends also on the ionic strength of the solution because the partition effect is inversely proportional to the ionic strength of the solution. If the number of the substrate ions surrounding the matrix increases, then the partition effect of the polyionic matrix on each ion will decrease. In the case of high ionic substrate concentration, the partitioning effect of the matrix is not obvious anymore, the value of K_m is still perturbed showing the existence of other factors affecting the immobilized enzyme kinetics and under these conditions, the Lineweaver–Burk plot is not linear but sigmoidal (92).

Diffusion Limitation

To take into consideration the diffusion factor, a new term h_s must be added to the catalysis equation:

$$S_0 \xrightarrow{h_S} S_i + E \leftrightarrow ES \rightarrow E + P \qquad [1.3]$$

If h_s is << V_{max} / K_m , the reaction will be diffusion controlled and the effective reaction velocity V' = V_{diff} but if h_s >> V_{max} / K_m , the reaction will be kinetically controlled and the rate constant is equal to K_m / V_{max}. In this case, K_v^1 is the value of the concentration at which the reaction velocity is equal to V_{max} / 2 in the case where diffusion controls the rate of the reaction. K_v^2 is the same constant relative to the case where the reaction is kinetically controlled.

Two factors can cause the diffusion limitation of the substrate to the matrix. The first factor is an external factor due to the unstirred layer between the bulk solution and the surface of the polymer or it could be due to the effect of temperature. The other factor is the internal diffusion limitation. In the second case, diffusion and catalysis are occurring concurrently. Both factors cause K_v to be greater than K_m. Usually at high substrate concentration the value of V_s is almost equal to V_{max}. But if the cause of diffusion limitation is internal, then in practice, V_s appears to be lower than V_{max} (92).

Applications of Immobilized Enzymes

Enzyme Electrodes

The enzyme electrodes are devices capable of producing an electrical signal as a result of the reaction catalyzed by the immobilized enzyme deposited around the electrode. Four

important factors characterize an enzyme electrode: sensitivity, stability, response time, and cost.

Enzyme electrode began with immobilizing glucose oxidase in polyacrylamide gel that is attached by a piece of cellulose acetate around an oxygen electrode. Other enzyme electrodes have been developed such as ammonium-ion-sensitive electrode or urea enzyme electrode and the penicillin enzyme electrode (92).

Autoanalysis

The immobilized enzyme replaces the free enzyme in an automatic analyzer system. The analysis can be done repetitively in the case of small samples or continuously by using a stream in the case of larger samples. The major disadvantage of the repeated enzymatic analysis of the small samples is that the immobilized enzyme must be added to each sample. To solve this problem, a flow injection system can be used and the enzyme is immobilized on the inner wall of a column through which the sample stream will pass. In the case of big volumes of a substance analyzed continuously, a different detection method is used. The method is based on measuring the heat produced by the enzyme reaction (92).

Industrial Uses

The first immobilized enzyme that was employed in industry is amino acid acylase. It was used by a Japanese company for the resolution of racemic mixtures of amino acids into L-form (31). One of the immobilization techniques used for this application include ionic binding of enzyme to diethylaminoethyl (DEAE)-sephadex. Another industrial application is the use of immobilized glucose isomerase for the conversion of glucose syrups into high fructose syrups (45). Adsorption and cross-linking techniques are essentially used to immobilize this enzyme.

The immobilization of aspartase in a microbial cell is also considered important in the industrial field (99). It plays the role of a catalyst important for the production of L-aspartic acid from fumaric acid. L-aspartic acid is an important component in some medicines and it is also used as food additive (94).

Glucose Oxidase

Glucose oxidase is an important analytical reagent due to its use for determining the concentration of glucose, which is important in different fields such as biochemistry, clinical chemistry, and food analysis (100). Furthermore, glucose oxidase is not expensive and it has good stability, which makes the glucose oxidase biosensors very attractive in different fields. In enzyme immunossays, glucose oxidase is used as a marker for antigens. In the food industry, it is basically used to remove glucose from diabetic drinks. The feature that makes glucose oxidase more interesting is the possible use of the enzyme as an anticancer drug because it can help in the formation of hydrogen peroxide capable of harming cancerous cells (100).

Glucose oxidase is a dimeric protein with two polypeptide chains linked together by disulfide bonds. Each dimer of glucose oxidase possesses two molecules of flavine adenine dinucleotide (FAD) that works as a cofactor. Glucose oxidase works as a catalyst during the redox reaction. FAD is the first electron acceptor from the glucose substrate that is transformed into $FADH_2$. Then O_2 molecule oxidizes $FADH_2$ back to FAD and the O_2 reduced into H_2O_2 (100).

Two main reasons besides the usual benefits of enzyme immobilization encourage the immobilization of glucose oxidase. First, it is advantageous to employ the enzyme in flow

injection analysis. Second, it is the ease of immobilizing glucose oxidase. The immobilized glucose oxidase can be used as an external reactor important for flow injection system. It can also have the shape of internal reactor common in biosensor (100).

The techniques used for immobilizing glucose oxidase are really diverse. The following examples represent some techniques that were classified depending on the detection method. For spectrophotometric detection, glucose oxidase was immobilized in polyacrylamide gel (101), or covalently attached to the inner wall of a coiled diazotized polyaminostyrene tube (102), or it was physically entrapped between two dialysis membranes (103). For electrochemical detection, glucose oxidase was entrapped in a gel matrix made by polymerization of acrylamide and N,N methylbis-acrylamide (104), or covalently immobilized on porous alumina (105), or immobilized on controlled pore glass (106, 107). For luminescence detection, glucose oxidase was immobilized on Sepharose and packed in a Pyrex tube (108-110) or immobilized on the walls of a nylon tube (111-113). Many other techniques for immobilizing glucose oxidase were also use under different experimental conditions and for different applications (100).

Kinetic Study of Glucose Oxidase

A study has been done to determine the kinetic parameters to characterize the activity of immobilized glucose oxidase on β-D-glucose. It appears that the turnover number K_{cat} of immobilized glucose oxidase (encapsulated in sol-gel) is the same as K_{cat} for free glucose (≈250 s^{-1}). The K_m value for the glucose-glucose oxidase (GO_x) system is twice that found in the solution. It can be deduced from the above results that the formation constant of the glucose-GOx complex is reduced, and the probable reason for that is the limited mass transport or the

diffusion limitation caused by the nature of the sol-gel matrix. The value of the apparent association constant (K_{cat} / K_m) for the glucose-GOx system in the solution is bigger than in the gel, which implies that the rate of formation of the enzyme substrate complex decreased in the gel matrix almost by half (98).

CHAPTER 2

SOL-GELS FOR IMMOBILIZATION OF GLUCOSE OXIDASE

The sol-gel technique is successful for the immobilization of different molecules by microencapsulation especially enzymes as it can retain their activity. This method appeared to be one of the best techniques to immobilize glucose oxidase (GOx) (114).

Sol-gel matrix present an interesting surface with organized porosity, chemical inertness, stability, and ability to form different designs as well as ease of preparation making them attractive for immobilizing enzymes (4, 5). The sol-gel entrapment process is a simple chemical reaction that requires mild conditions of temperature and pH. For that reason most enzymes entrapped can survive in its matrix (8).

The sol-gel process is based on the hydrolysis of monomeric alkoxo precursors. If the sol-gel is a silica gel, the common precursors used are the tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS). Other organically modified precursors can be used such as $RSi(OCH_3)_3$ with R = alkyl, aryl or alkoxy (115). The condensation of the hydrolyzed precursors will form a polymeric oxo-bridged SiO_2 network (3). If the dopant enzyme is added to the initial reaction mixture, it can be trapped in the pores of the SiO_2 network. The advantage of this method is that immobilization will happen by physical encapsulation in the porous structure without introducing any covalent bond or modification of the enzyme structure. The isolation of the biomolecules in the porous structure will protect them from any microbial attack (98). Silica gels retain the tertiary structure of the enzyme and prevent their denaturation because of the existence of the trapped interstitial water in the gels (3, 98).

Chemistry of the Sol-Gel

In the making of the sol-gel, the first reaction that occurs is the hydrolysis where the Si–OR bond is converted to Si−OH bond. This reaction will produce an alcohol corresponding to the precursor used. The following reaction is the condensation reaction that leads to the formation of the oxo-bridged polymeric Si−O−Si structure. The continuity of the condensation reaction will increase the viscosity of the sol. After solidification, a porous gel that contains 70% of water is obtained. The structure and the physical properties of the gel are not constant as long as the polycondensation reactions continue. This phase is called the "aging" process where the cross-linking in the silicate network increases. Then, the pore liquid made of water and alcohol begins to dry causing a decrease in the gel volume and a spontaneous shrinkage of the gel. The xerogel formed is a solid glass material that has a high mechanical strength (98).

The chemical reactions that occur during the formation of the sol-gel are summarized below (98):

$(RO)_4Si + H_2O \leftrightarrow (RO)_3Si\text{-}OH + ROH$ (hydrolysis) [2.1]

$2(RO)_3SiOH \leftrightarrow (RO)_3Si\text{-}O\text{-}Si(OR)_3 + H_2O$ (condensation) [2.2]

$Si_xO_y(\mu\text{-}OH)_z(t\text{-}OH)_{4x-2y-2x}$ (gelation) [2.3]

Si_xO_{2x} (formation of xerogel) [2.4]

Factors Affecting the Sol-Gel Formation

The drying step is really critical for the formation of the sol-gel. Non-uniform drying can cause extensive internal stresses in the aged gels which contribute to a catastrophic fracture of the materials. For this reason, a slow drying process is really important to get stable xerogels.

In addition, the relative rates of hydrolysis and condensation could have a big impact on the gel morphology. A high rate of hydrolysis leads to an extensive condensation that results in a highly branched interconnected porous network. The relative rates are also affected by the pH of the medium. An acid catalyst can accelerate the hydrolysis reaction and prevents the formation of the oxo-bridges resulting from loss of protons that will cause the formation of material with smaller pores. A basic catalyst will give a material with larger pores because it can accelerate the condensation reaction (98). The pH effect will be explained in details later.

Characteristics of the Sol-Gel Glasses

The sol-gel glass can be used for the entrapment of different biomolecules and sensing agents. It is prepared under mild environmental and chemical conditions that helps in the immobilization of biomolecules with poor stability. The sol-gel glasses are physically and chemically stable and can be used in harsh environments. Finally, the sol-gel glass is optically transparent which makes it useful in the spectroscopic field. However, the sol-gel glass based sensors shows a slow response due to diffusion limitation depending on the size of the pores, thickness of the film, and concentration of the analyte. This kind of matrix can present a degree of leaching affected by the method of preparation of the sol-gel by the size of the pores and the entrapped reagent (116).

Sol-Gel Glass with TMOS

The methoxy precursor tetramethyl orthosilicate (TMOS) is a common reagent used for the formation of the silica sol-gel because the polarity of this reagent is close to water and it is higher than the polarity of other precursors that usually require the presence of alcohol as

solvent and the alcohol may alter the stability of the encapsulated enzyme. The use of buffer during the synthesis of the sol-gel is also important. The buffer increases the pH value to be close to the biological pH in order to avoid acid denaturation or aggregation of the enzyme (3).

Sol-Gel Glass with TEOS

TEOS (tetraethoxysilane) is a convenient co-precursor for the formation of hydrophobic sol-gel; it is less toxic and less expensive than TMOS (tetramethoxysilane) (117). TEOS possesses longer chain organic group that makes the reaction of formation of the sol-gel slower than the reaction conducted by the methoxy groups (118). For the sol-gel based on adding TEOS, alcohol, water, acid as catalyst and the buffer solution of the enzyme, different studies (118, 119) proved that pH and H_2O to Si molar ratio can have a big effect on the structure of the sol-gel (size of the pores, degree of porosity, and others). It was demonstrated that the favorable range of pH is between 5 and 8. It was also shown that a higher value of the H_2O to Si ratio is favorable for the enzyme, and that a value of this ratio between 4 and 20 will result in the formation of linear chain of molecules in the network. Furthermore, the relationship between H_2O to Si molar ratio and the activity of the enzyme (glucose oxidase) had also been determined. The immobilized glucose oxidase can retain 70% of its original activity if the hydrolysis degree (H_2O: Si molar ratio) is increased to a certain limit (119).

Effect of Acid/Base Catalysis on the Sol-Gel

As mentioned before, the rates of hydrolysis and condensation have a big impact on the morphology of the sol-gel. In the case of using acid (HCl) as catalyst, the first rapid step is the protonation of the alkoxide group. The mechanism of the hydrolysis reaction is the nucleophilic

substitution SN_2 and it results in the formation of a tetrahedral structure of silicon (120). In acidic solution, the first hydrolysis is easier than the subsequent hydrolysis of the same unit toward the formation of $Si(OH)_4$. This means that the condensation reaction is preferred to happen at the ends of the oligomer producing linear polymers (118, 120). The condensation reaction is the result of the reaction between Si-OH species and the silicon atoms in the protonated species $Si\text{-}OH_2^+$ (121).

When base (NH_4OH) is used as a catalyst, the mechanism of the hydrolysis involves the attack of the nucleophile (OH^-) on the silicon atom (121). The complete hydrolysis of the precursor TEOS is easily achieved and the condensation will happen preferentially in the center of the oligomer that contributes to the formation of a highly branched dense polymers (118, 120). In the basic medium, both hydrolysis and condensation are favored; the condensation will happen by the attack of ($Si\text{-}O^-$) species on the neutral species (Si-OR) (121). Consequently, small pores (< 2 nm) are usually obtained in the case of acid catalysis and larger pores are seen in the network structure during base catalysis (122).

Sol-Gel Enzyme-Modified Electrode

This kind of enzyme electrode has many applications in chemistry and biochemistry because of its high sensitivity and selectivity. This technique is based on immobilizing the enzymes on electrode surfaces using different methods like physical adsorption, solvent casting, covalent binding, electropolymerization, and Langmuir-Blodgett methods (method based on formation of monolayers of biological molecules) (123,124) as well as the dipping or spin coating method (125-128).

Electrochemical Deposition of Sol-Gel with Immobilized Glucose Oxidase

This method is known as the electrochemically induced sol-gel process. The mechanism (3) of the formation of the silica sol-gel where tetraethyl orthosilicate is used as precursor is represented by these reactions:

Reduction of water on the electrode: $H_2O + e^- \rightarrow H_2 + OH^-$ [2.5]

Hydrolysis in the diffusion layer: $Si(OC_2H_5)_4 + H_2O \xrightarrow{OH^-} (SiO_mH_n)_p + C_2H_5OH$ [2.6]

Condensation Reaction: $(SiO_mH_n)_p \rightarrow SiO_2 \bullet xH_2O + H_2O$ [2.7]

A cathodic current is applied to the working electrode immersed in the bulk solution containing the precursor, the solvent, water, and the enzyme. The current causes the reduction of water and formation of OH^- and hydrogen gas bubbles. The OH^- ions play the role of catalyst for the polycondensation reaction and the hydrogen bubbles will help in the formation of the three dimensional porous silica structure because the deposition of silica will happen only around the surface of the bubbles. The studies show that a uniform distribution of the pores of the silica network structure is obtained (4).

Advantages of the Electrochemical Deposition of Sol-Gel

Because of the typical porous structure of this kind of sol-gel, the inner stress of the gel materials can be released and consequently no cracking is observed. The existence of the favorable microstructures helps to decrease the diffusion resistance of the sol-gel for the glucose molecules. Further experiments using the sol-gel chemically deposited on the electrode show that a quick electrochemical signal can be obtained due to the rapid mass transport through the pores of the matrix. Comparing this method to the physical adsorption method used to generate the enzyme-modified electrode, it seems that the encapsulated

glucose oxidase in silica using the current method shows better stability and no loss in activity during the storage time (4).

Immobilization of Enzymes by Adsorption on Silica Nanoparticles

A great deal of interest in nanotechnology occured in recent years due to important physicochemical properties of nanoparticles that make them useful in many fields such as drug delivery, imaging, and diagnosis. Nanoparticles are small particles with dimension range from 1-100 nm. Consequently, they have a large surface to volume ratio (large surface area). Due to the high specific area, nanoparticles possess high adsorption capacities and they can attach to different molecules by covalent bonding or physical adsorption (129). Different reports about the adsorption of proteins including enzymes such as lysozyme, horseradish peroxidase, catalase, and trypsin on silica nanoparticles show that these enzymes can undergo a rapid conformational change at the secondary and tertiary structure and that they can lose significantly a part of their activity due to the decrease in thermodynamic stability of the enzymes. This result is contradictory to the concept that the proteins should conserve most of their original activity after binding to the high curvatures of nanoparticles. The degree of enzyme activity loss is also dependent on the size of the nanoparticles (129,130). An experimental study has been done on lysozyme adsorbed on silica nanoparticles showed the same surface chemistry but with size changing from 4 to 100 nm to investigate the effect of the nanoparticles size on the adsorption isotherms of the enzyme. The results demonstrated that nanoparticles with smaller size help the protein to keep its native structure. For that reason, the enzyme activity in the case of small nanoparticles is higher than the activity of the enzyme

adsorbed on larger nanoparticles that were found to perturb the protein structure to a significant extent (130).

Synthesis of Silica Nanoparticles

Sol-gel technique is widely used for the synthesis of silica nanoparticles. The common reagents are the precursor TEOS, the alcohol as solvent, water, and the catalyst (usually ammonia) (131). The reactions involved in the synthesis of the silica nanoparticles are the hydrolysis of the precursor and then the condensation to have formation of Si-O-Si bond. The only difference that induces the formation of the silica nanoparticles instead of the silica network is the rate of the condensation step (132). The most important work in the synthesis of silica nanoparticles was presented by Stöber et al.(133) who were able to develop a series of chemical reactions that helped to prepare monodispersed nanometer-sized particles.

Different techniques under different reaction conditions can be used for the preparation of silica nanoparticles. A study (131) was done to investigate the effect of different solvents like ethanol and ethanol-glycerol on the structure and morphology of silica nanoparticles. The experimental procedure was conducted at room temperature but under dry air. The results showed that the molar ratio of the reagents, especially the solvent (ethanol) could affect the morphology of the nanoparticles. A lower molar ratio of the solvent will produce agglomerated silica particles (131). Another study (132) based on a sequential addition method was performed in order to obtain monodiperse and uniform silica nanoparticles with a size ranging from 20 to 460 nm. In order to obtain the silica nanoparticles, the reagents were mixed in an ultrasonication bath (132).

Oxidation of Glucose in the Presence of Glucose Oxidase

Glucose oxidase is a specific catalyst for the oxidation of glucose and its transformation into gluconic acid and H_2O_2. In an aqueous solution of glucose, there is double quantity of β-D-glucose than α-D-glucose at equilibrium. Glucose oxidase catalyzes only the oxidation of β-D-glucose and the rest of α-D-glucose in the solution will be transformed into form β (134). Figure 1 shows the oxidation reaction of β-D-glucose and the production of H_2O_2 (135).

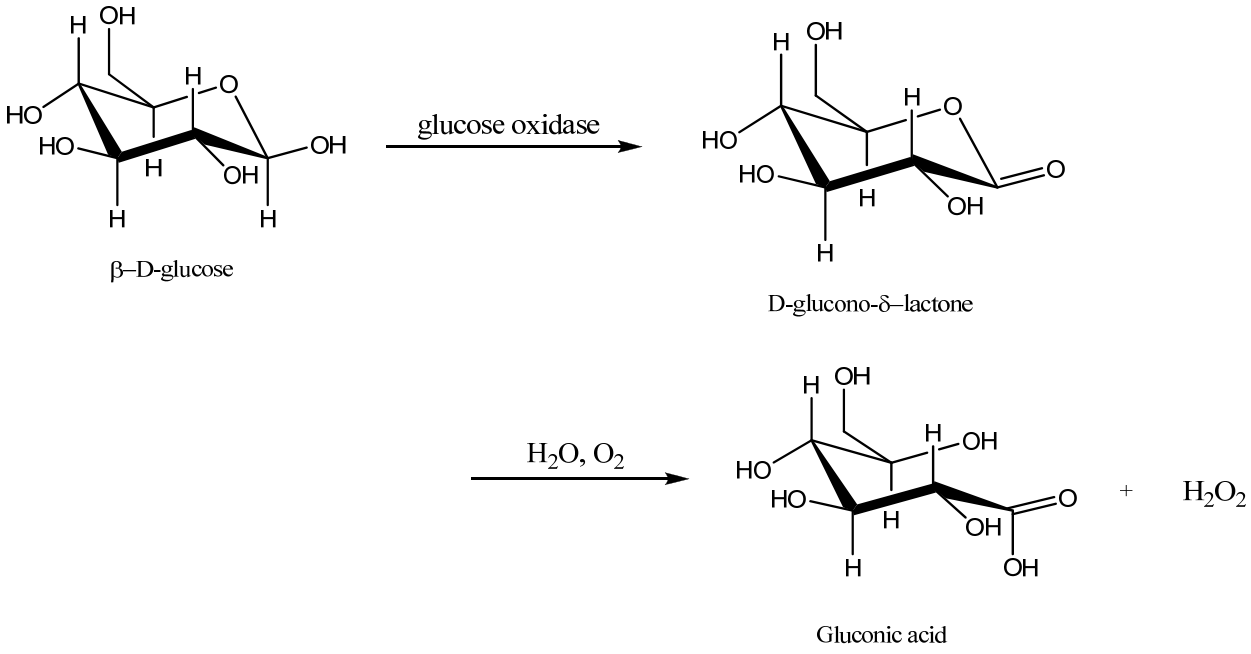

Figure 1. Oxidation reaction of β-D-glucose.

H_2O_2 is the product of the reduction of O_2 after transferring electron to $FADH_2$ which is the reducing agent of the system $FAD/FADH_2$. Figure 2 is the pictorial representation of the oxidation reduction reactions responsible of the production of H_2O_2 (136).

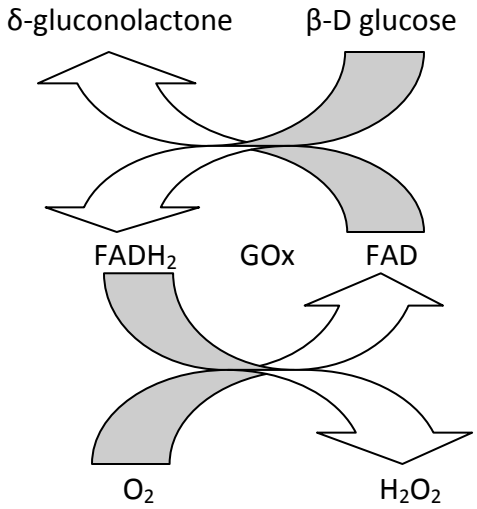

Figure 2. Representation of the oxidation reduction reactions of glucose.

Trinder Reaction

H_2O_2 formed from the oxidation of glucose can be determined using Trinder reaction. Trinder published in 1969 an article about the determination of blood glucose using an oxidase / peroxidase system with the help of a non-carcinogenic chromogen. The chromogen tested was dl-adrenaline and it was working as an oxygen acceptor (137). Later in the same year, Trinder wrote another article (138) where he replaced the dl-adrenaline with 4-aminoantipyrine and the method is three times more sensitive than the previous one.

Trinder reagents are widely used for the colorimetric determination of H_2O_2. Trinder reagents present many advantages like high water solubility and the stability of the dye formed (139). The dye formed is 4-(p-benzoquinone-monoimino) antipyrine and it is the result of an oxidative reaction of the working reagent made of phenol and 4-aminoantipyrine in the presence of hydrogen peroxide and peroxidase. The reaction occurs in the presence of peroxidase and it can be summarized thus:

$2H_2O_2$ + phenol + 4-aminoantipyrine → 4-(p-benzoquinone-monoimino)antipyrine + 4 H_2O

Based on some studies, it was found that the working reagent should be stored in a dark vial at 4°C to maintain its stability (140, 141). The change in absorbance of oxidized chromogen is measured at a wavelength of 500 nm (140).

The Detection Method

Ultraviolet-visible (UV-Vis) light absorption spectrophotometry is used as the detection method to measure the absorbance of the dye formed as a product of Trinder reaction. As a result of the interaction of the beam of light with the sample, part of the light is absorbed and the rest is transmitted to the detector (142). During the absorption, electronic transition occurs in the molecules called chromophores. Single bond systems show transitions from sigma bonding orbital to sigma antibonding orbital ($\sigma \rightarrow \sigma^*$) or from nonbonding orbital to antibonding orbital ($n \rightarrow \sigma^*$). Systems with double bonds can show electronic transitions like transition from pi bonding orbital to pi antibonding orbital ($\pi \rightarrow \pi^*$) or from nonbonding orbital to pi antibonding orbital ($n \rightarrow \pi^*$) (143).

The Beer-Lambert law usually applies to the absorption technique. It states that there is a linear relationship between the concentration of the analyte and the absorbance detected:

$$A = \xi bC \qquad [2.8]$$

where ξ is the molar absorptivity of the substance, b is the light path length, and C is the molar concentration. In some conditions, the linearity is lost; this can happen due to the instrument's stray light, or to sample scattering, as well as other causes related to the composition of the sample itself (142).

Research Objective

From the discussion in Chapters 1 and 2, it is clear that immobilizing enzyme is a popular subject in all fields related to enzymology for research and applications. The purpose of this research is to make a comparative study between different methods of immobilizing glucose oxidase in sol-gel and to evaluate each method for future applications. The sol-gel suitable for encapsulating glucose oxidase can be used to immobilize other molecules for different reactions serving different goals. What is learned from conducting this research will lead one towards immobilizing fluorophores with the enzyme in a convenient sol-gel. This allows chemical reaction and detection with luminescence that is known for its sensitivity to be done almost simultaneously.

The goals of this research are:

1. Preparing sol-gels encapsulating glucose oxidase by four different methods under suitable conditions.
2. Assess the stability of the enzyme activity with the number of time or frequency of uses of the sol-gel.
3. Assess the storage time or the lifetime of each sol-gel in retention of enzymatic activity.
4. Evaluating the enzyme activity in these sol-gels by evaluating the reproducibility of enzyme activity in these sol-gels.
5. Evaluate the linearity of enzyme activity, the recovery, and the application in commercial samples of these sol-gels.

CHAPTER 3

EXPERIMENTAL METHODS

Instrumentation

A Genesys 20 single beam spectrophotometer (Thermo Electron Corporation, Madison, WI) was used for all absorption measurements. The spectrophotometer has a stability ≤ 3 mA / hour drift, a wavelength repeatability of ± 0.5 nm, a wavelength accuracy of ± 2.0 nm, and a noise at 500 nm ≤ 1 mA at 0 A and 2 mA at 2 A where A is the absorbance unit.

A Heath Kit, laboratory bread broad kit ET-3300, was used to generate the cathodic current.

Reagents

1. Deionized Water, from US Filter Company (Pittsburg, PA).
2. Glucose oxidase Type II-S, Aspergillus niger (2.36 G solid; 21200 units / G solid), from Sigma Aldrich Company.
3. Glucose oxidase Type X, 50 KU, from MP Biomedicals, LLC, (Solon, OH).
4. Tetramethyl orthosilicate 99%, tetraethyl orthosilicate 98%, and 4-Aminoantypyrine 98%, from ACROS ORGANICS (New Jersey, USA).
5. Peroxidase Horseradish, from United States Biochemical Corporation (Cleveland, Ohio), 1.1447U/mg.

Phenol, sodium phosphate dibasic, potassium phosphate monobasic, absolute ethanol, hydrochloric acid, 6M, ammonium hydroxide, 14.8M, and N,N-dimethylformamide were all ACS certified reagents, obtained from Fisher Scientific Company (Fair Lawn, NJ).

Preparation of the Sol-Gels

Sol-Gel with TMOS as Precursor

To a mixture of TMOS solution and 0.01 M of phosphate buffer kept in ice-bath, 3 mL of a solution of glucose oxidase Type II-S (2.92 mg / mL) or glucose oxidase Type X (1.46 mg / mL) were added. The sol was transferred to the containers covered by a parafilm. Once the solution began to become a gel, it was in the refrigerator at 4°C. After a few days, some pin holes were made in the parafilm to let the sol-gel dry. The sol-gel can be used after 3 to 4 weeks when it is completely dry (98).

Sol-Gel with TEOS as Precursor

The acid-catalyzed sol was prepared by mixing 2 mL TEOS, 0.5 mL ethanol, 25 µL of 0.05 M HCl and 0.5 mL of water. After sonication for half hour, 0.4 mL of phosphate buffer solution of glucose oxidase Type II-S (23.56 mg / mL) or buffer of glucose oxidase Type X (6 mg / mL) was added to the sonicated sol solution. The mixture was kept in the refrigerator at 4 °C (119). The sol-gel was ready for use after 1 or 2 weeks.

Electrochemically Induced Sol-Gel

TEOS was mixed with ethanol in a 1: 1 volume ratio and the mixture was sonicated for half an hour. A glucose oxidase Type II-S solution with a concentration of 23.56 mg / mL or a solution of glucose oxidase Type X (6 mg / mL) was added to the mixture in the same volume ratio. The sol solution was sonicated again for 2 minutes. After sonication, a cathodic current of 100 mA was applied to a Pt electrode immersed in the sol solution, the hydrogen bubbles appeared around the working electrode and the gel began to form (4). A few hours were

enough to have the formation of a sol-gel encapsulating the enzyme. The sol-gel was kept at 4°C and ready for use the next day.

Sol-Gel Modified Silica Nanoparticles

Synthesis of the Nanoparticles. About 0.4 mL ethanol solution was sonicated for 10 minutes, 2 mL of TEOS was added to the alcohol and sonicated again for 20 minutes, then 10 drops of concentrated ammonium hydroxide was added to catalyze the condensation. The whole mixture is sonicated further for 60 minutes when a white suspension was formed (132).

Preparation of the Sol-Gel. A sol solution made with TEOS, ethanol, HCl, water, and a buffer solution of glucose oxidase Type II-S (23.56 mg / mL) was prepared. Then 0.4 mL of this solution was cast into 3.00 g of silica nanoparticles. The nanoparticles were stored at 4°C (119).

Preparation of the Phosphate Buffer Solutions

Two phosphate buffers solutions with different pH were prepared. The first one had a pH of 6 and it was used for the preparation of the sol-gels. To prepare 0.1 M of this buffer solution, 0.142 g of sodium phosphate was dissolved in 100 mL of deionized water and then the pH was adjusted to 6 by adding a few drops of 6 M hydrochloric acid or sodium hydroxide.

The second buffer was used to prepare glucose solutions. It had a pH of 7 and it was made by mixing 0.94 g of potassium phosphate and 1.57 g of sodium phosphate in 200 mL of deionized water. The pH was adjusted again by addition of 6 M HCl or NaOH.

Preparation of Reagents

Preparation of Glucose Stock Solution

To prepare a stock solution of 10 mg / mL concentration, 0.05 g of anhydrous glucose were dissolved in 50 mL of phosphate buffer.

Preparation of the Glucose Working and Standard Solutions

Two glucose working solutions were used. The first one was obtained by diluting 1 mL of the glucose stock solution in 10 mL of buffer in a volumetric flask. The other working solution was prepared by dissolving 100 µL of the stock solution in 10 mL of buffer in volumetric flasks.

The standard solutions used were prepared by diluting 1mL, 0.05 mL, 0.025 mL, and 0.01 mL of the working solution of 1 mg / mL glucose into 10-mL volumetric flasks diluted with buffer. The more diluted standard solutions were prepared from the second working solution with 0.01 mg / mL glucose concentration by diluting 0.025 mL and 0.01 mL of this working solution in 10-mLvolumetric flasks and diluted to the mark with buffer. These standard solutions were used to prepare the calibration curves.

Preparation of Trinder Reagent

Trinder reagent is prepared by dissolving 0.1 g of phenol, 0.05 g of 4-aminoantipyrine, and 0.001 g of peroxidase in phosphate buffer of pH 7 in a 100-mL volumetric flask. The prepared reagent was stored in the refrigerator in an amber bottle (138).

Experimental Procedures

Glucose Oxidation

To carry out the oxidation of glucose and the production of H_2O_2, a volume of the glucose solution varying from 4 mL to 8 mL was incubated with the sol-gel at 37°C for 25 minutes to 45 minutes.

Trinder Reaction

To determine the amount of hydrogen peroxide produced, 3 mL of the incubated glucose solution was taken and added to 3 mL of the Trinder reagent. After 15 minutes, the pink dye formed was detected at 500 nm.

Reproducibility of the Sol-Gels

Checking the possibility of using different sol-gels prepared in the same way to make the calibration curve to save time is important. Five to nine sol-gels from each kind were incubated with the same concentration of glucose solution and the measured absorbances were recorded to calculate the relative standard deviation between the values.

Solutions for Recovery Studies

To test the recovery yield relative to each kind of sol-gel, the standard addition method was applied on the commercial sample Pedialyte. Three different sets of solutions were prepared by diluting a stock solution from the commercial sample in 25-mL volumetric flasks. In set 1, no glucose was added, in set 2 a value of X volume of glucose from the working solution of known concentration used in the calibration curve was added, and in set 3, 2X volume was added from the same working solution. All three sets solutions were each prepared in triplicates.

Preparation of Commercial Samples

Three commercial samples containing dextrose (Pedialyte, Comforts, and Dex4) were used. Pedialyte and Comforts are pediatric electrolyte used as oral electrolytes maintenance solutions and Dex4, a glucose Liquid Blast. Two to three aliquots from each commercial product were obtained and each aliquot was then prepared in triplicates and reacted with each kind of sol-gel. The average dextrose value found was compared to the value labeled on the commercial product and the relative standard deviation and percent error were also calculated.

CHAPTER 4

RESULTS AND DISCUSSION

In this chapter, the results for different experiments made on the sol-gels are presented. These experiments were performed to assess the quality of the sol-gels in terms of how the sol-gels were prepared and the activity of the encapsulated enzyme. Then the stability of the enzyme, retention of its activity with storage time and number of use were evaluated. The analytical figures of merits were also studied to assess the quality of the sol-gel and to evaluate their feasibility in applications.

Preparation of the Sol-Gels

The sol-gels were prepared as mentioned in the literature with a few modifications in some cases. Sol-gels prepared with TMOS did not give successful results at the beginning due to cracking of the sol-gel during the drying stage. Such problem could not be ignored because the resulting pieces of sol-gels were losing the activity from time to time and at times some pieces were not showing any enzymatic activity at all. Many trials to solve the problem by changing the storage conditions were not successful. The efforts were later focused on the way of preparing the sol-gels such as adding reagents that could probably solve the cracking problem. Titanium dioxide nanoparticles were added assuming that the polarity of these particles could solve the problem, but they did not help in improving the texture of the sol-gel. The drying agent dimethylformamide was also added in order to help the evaporation of the water and alcohol from the sol-gel, but the drying agent did not have any effect on the sol-gel.

Table 1 is an example showing the loss of enzymatic activity in one piece of sol-gel after each use.

Table 1. Loss of enzymatic activity with time of uses in the same piece of the sol-gel.

Times of use	First time	Second time	Third time	Fourth time
Absorbance	0.324	0.217	0.189	0.169

.

In Table 1, the results showed that with each subsequent use of the sol-gel, the loss of activity was about 33.0%, 12.9%, 10.6%, respectively, at the second, third, and fourth use from the previous usage. The absorbance was only 52.2% of the original value at the fourth use. These values were significant and gave rise to non-consistent measurements. It was possible that the enzyme was getting denatured or deactivated because of the cracking.

Finally, mixing the solutions well during the preparations and using containers with a specific shape helped in diminishing the cracking and led to the formation of more compact sol-gels. These sol-gels were used later to study the enzyme activity and to apply to analysis of the commercial samples. Most of the sol-gels prepared with TEOS as precursor and HCl as catalyst resulted in sol-gels glass of good shape and the ones that had some cracking did not show any abnormality in their enzymatic activity.

As mentioned in the literature review, the sol-gels induced electrochemically were usually deposited around the electrode and used for electrochemical detection. In the experimental part done in this research, the sol-gels were induced electrochemically. However, they were separated from the Pt electrode and then employed as regular sol-gels instead of leaving them on the electrode for electrochemical analysis.

The methodology used in the case of the sol-gels modified silica nanoparticles was based on an article published by Qingwen et al. (119). The enzyme encapsulated in the sol-gel was adsorbed on molecular sieve powders instead of silica nanoparticles. Trying to immobilize glucose oxidase in a sol-gel modified nanoparticles was aimed at studying the effect of the particular physical properties of the nanoparticles on enzyme activity.

Reproducibility of the Sol-Gels

Hou et al. (144) showed in a study how the activity of immobilized glucose oxidase varies with incubation time. They showed that the absorbance of the product increased significantly within the first 15 minutes of incubation, and then the change in absorbance slowed. These observations were justified by the decrease of the amount of substrate after 15 minutes of incubation and by the conformational changes of glucose oxidase molecules after the production of H_2O_2. Based on this study the preferred incubation time of glucose solution when using different sol-gels should be the one at which the variation of absorbance becomes slow. At this critical incubation time different sol-gels prepared in the same way should give the same absorbance for the same amount of substrate. The experimental studies in this project began at an incubation time longer than 15 minutes.

The variation of the absorbances from the product of glucose oxidation conducted on different sol-gels made with tetramethyl orthosilicate (TMOS) as a function of incubation time is presented in Table 2 and Table 3. The concentration of the glucose solution was 0.1 mg / mL. All the studies on the reproducibility of results obtained from different sol-gels were performed using this same glucose concentration.

Table 2. The reproducibility of results from the use of sol-gels prepared with TMOS at 25 min of incubation.

Incubation time = 25 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5
Absorbance	0.993	1.032	0.990	0.946	1.033
Average	0.999				
RSD	3.60%				

Table 3. The reproducibility of results from the use of sol-gels prepared with TMOS at 30 min of incubation.

Incubation time = 30min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5
Absorbance	1.024	1.047	1.032	0.989	1.009
Average	1.02				
RSD	2.18%				

For the sol-gels prepared by using TMOS as precursor and by encapsulating glucose oxidase Type II-S, an incubation time of 30 min seems to be sufficient to ensure that the use of these sol-gels will yield the same absorbance for the same glucose solution concentration and under the same conditions.

The reproducibility of nine sol-gels prepared with tetraethyl orthosilicate (TEOS) as precursor and HCl as catalyst at different incubation times is presented in Table 4 and Table 5. The results show that the sol-gels with TEOS can be favorably used at an incubation time varying from 30 to 35 minutes.

Table 4. The reproducibility of results from the use of sol-gels made with TEOS at incubation time of 25 minutes.

Incubation time= 25 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5	Sol-gel 6	Sol-gel 7	Sol-gel 8	Sol-gel 9
Absorbance	0.552	0.553	0.564	0.561	0.565	0.585	0.403	0.576	0.580
Average	0.549								
RSD	10.2%								

Table 5. The reproducibility of results from the use of sol-gels made with TEOS at incubation time of 30 minutes.

Incubation time=25 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5	Sol-gel 6	Sol-gel 7	Sol-gel 8	Sol-gel 9
Absorbance	0.530	0.576	0.558	0.543	0.519	0.546	0.589	0.570	0.573
Average	0.556								
RSD	4.17%								

Table 6 summarizes the reproducibility of absorbances from the glucose solution of concentration 0.1 mg / mL from the use of five sol-gels prepared using sol-gels induced electrochemically with different incubation time.

Table 6. Variation of the relative standard deviation of results obtained from the use of sol-gels induced electrochemically with incubation time.

Incubation time	25 min	30 min	35 min	40 min	45 min
Sol-gel 1	0.467	0.426	0.742	0.740	0.805
Sol-gel 2	0.810	0.738	0.811	0.741	0.828
Sol-gel 3	0.792	0.763	0.837	0.739	0.845
Sol-gel 4	0.414	0.479	0.610	0.657	0.850
Sol-gel 5	0.176	0.367	0.363	0.685	0.854
Average	0.532	0.555	0.673	0.712	0.836
RSD	50.6%	33.1%	28.9%	5.48%	2.41%

For the reproducibility of results obtained from the sol-gels induced electrochemically, the relative standard deviation is acceptable for an incubation time of 40 min. The reason is that the absorbance of sol-gels 4 and 5 did not reach the absorbances of the other sol-gels until a longer incubation period. Although these sol-gels were made in the same way, the possibility of obtaining an enzyme encapsulated differently in the medium of the sol-gel was there and could affect the interaction between the enzyme and the substrate and thus affecting the kinetics of the enzymatic process. For example, sol-gel 5 seemed to be one that required a longer incubation period.

In some cases the reproducibility of the sol-gels was totally lost like the case of one batch of TMOS sol-gels prepared. The loss of reproducibility is shown in Table 7.

Table 7. The reproducibility of results obtained from sol-gels prepared with TMOS after 30 min incubation.

Incubation time= 30 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5	Sol-gel 6	Sol-gel 7	Sol-gel 8	Sol-gel 9
Absorbance	0.731	1.198	0.679	0.839	0.887	0.598	1.198	0.571	0.680
Average	0.820								
RSD	28.9%								

Some Sol-gels made with TEOS and HCl lost also their reproducibility after a few times of use as shown in Table 8.

Table 8. The reproducibility of results obtained from sol-gels made with TEOS and HCl after 45 min of incubation.

Incubation time = 45 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5	Sol-gel 6
Absorbance	0.620	0.075	0.116	0.416	0.242	0.330
Average	0.300					
RSD	67.5%					

Another example concerning the loss of the reproducibility in the results from the use of electrochemically induced sol-gels is presented in Table 9.

Table 9. The reproducibility of results obtained from the use of sol-gels induced electrochemically after 45 min incubation.

Incubation time = 45 min	Sol-gel 1	Sol-gel 2	Sol-gel 3	Sol-gel 4	Sol-gel 5	Sol-gel 6	Sol-gel 7	Sol-gel 8	Sol-gel 9
Absorbance	0.678	0.48	0.337	0.523	0.577	0.360	0.284	0.433	0.725
Average	0.489								
RSD	31.2%								

The common fact of the above cases where the reproducibility was poor was that these sol-gels were prepared at the same period of time from the same glucose oxidase stock solution. The enzyme stock solution of enzyme was prepared 9 months prior and stored in the freezer. It was possible that some bottles of the enzyme solution had deteriorated over time. Another possible cause could be the loss of enzymatic activity to a different extent from one sol-gel to another. The effect of storage time on enzymatic activity will be discussed later. Due to the loss of reproducibility, new sol-gels were prepared continuously to continue the experiments.

Calibration Curves

Five sol-gels were used in preparing the calibration curve shown in Figure 3. These sol-gels were prepared with TMOS as precursor and the enzyme used is glucose oxidase Type II-S.

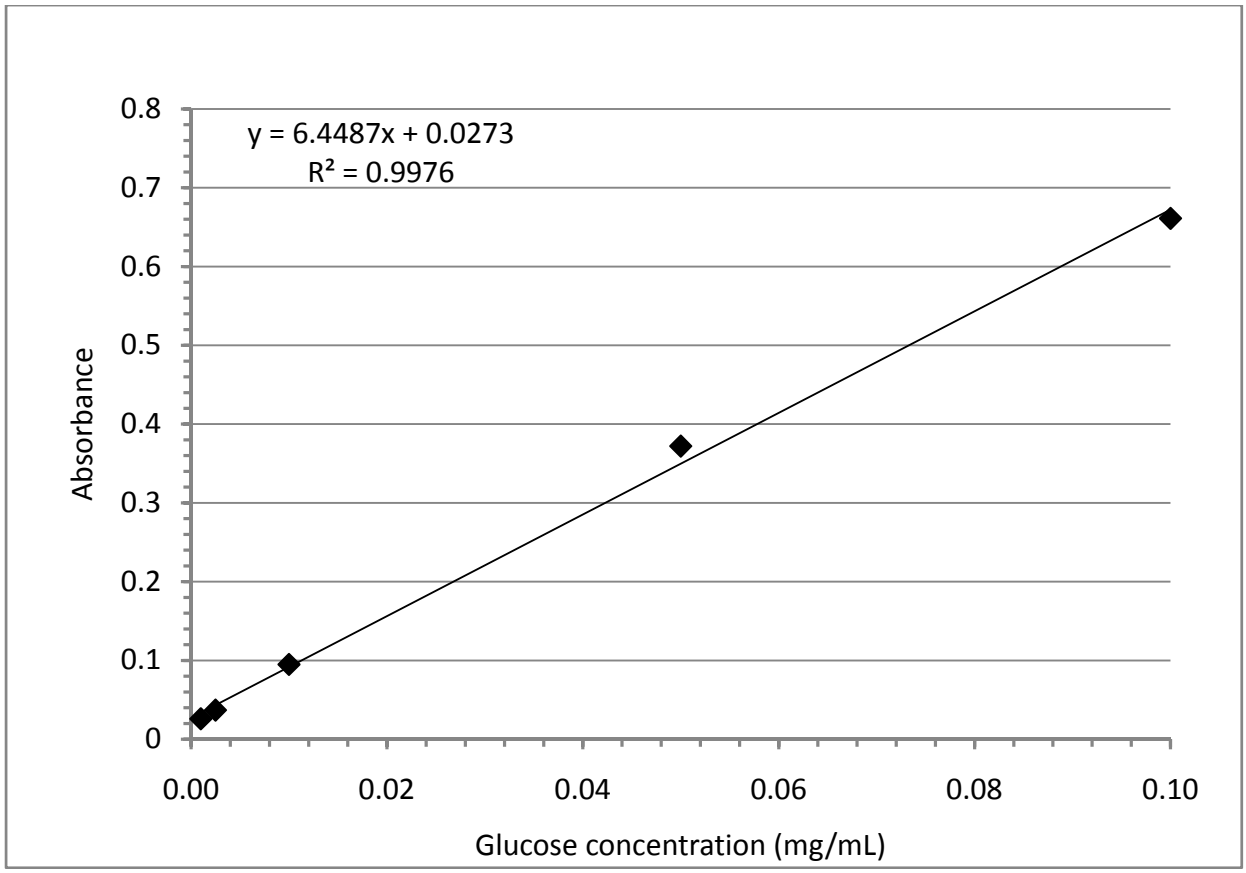

Figure 3. Calibration curve made with five sol-gels with TMOS.

Figure 3 shows a good linear dynamic range of two orders of magnitude between 0.1 mg / mL and 0.001 mg / mL of glucose. According to the slope of the line, these sol-gels have a good sensitivity in the determination of glucose. The equation of the regression line and the correlation coefficient are given on the plot

Although not shown, sol-gels made with TEOS and with glucose oxidase Type II-S also present a linearity ranging from 0.1 mg / mL to 0.01 mg / mL with good sensitivity. The equation of the regression line obtained was y = 8.5943x + 0.0456 with an R^2 of 0.9974.

Figure 4 and Figure 5 are dynamic range studies of sol-gels induced electrochemically. In Figure 4, encapsulated glucose oxidase type II-S was used while in Figure 5 encapsulated glucose oxidase Type X was used in the sol-gels.

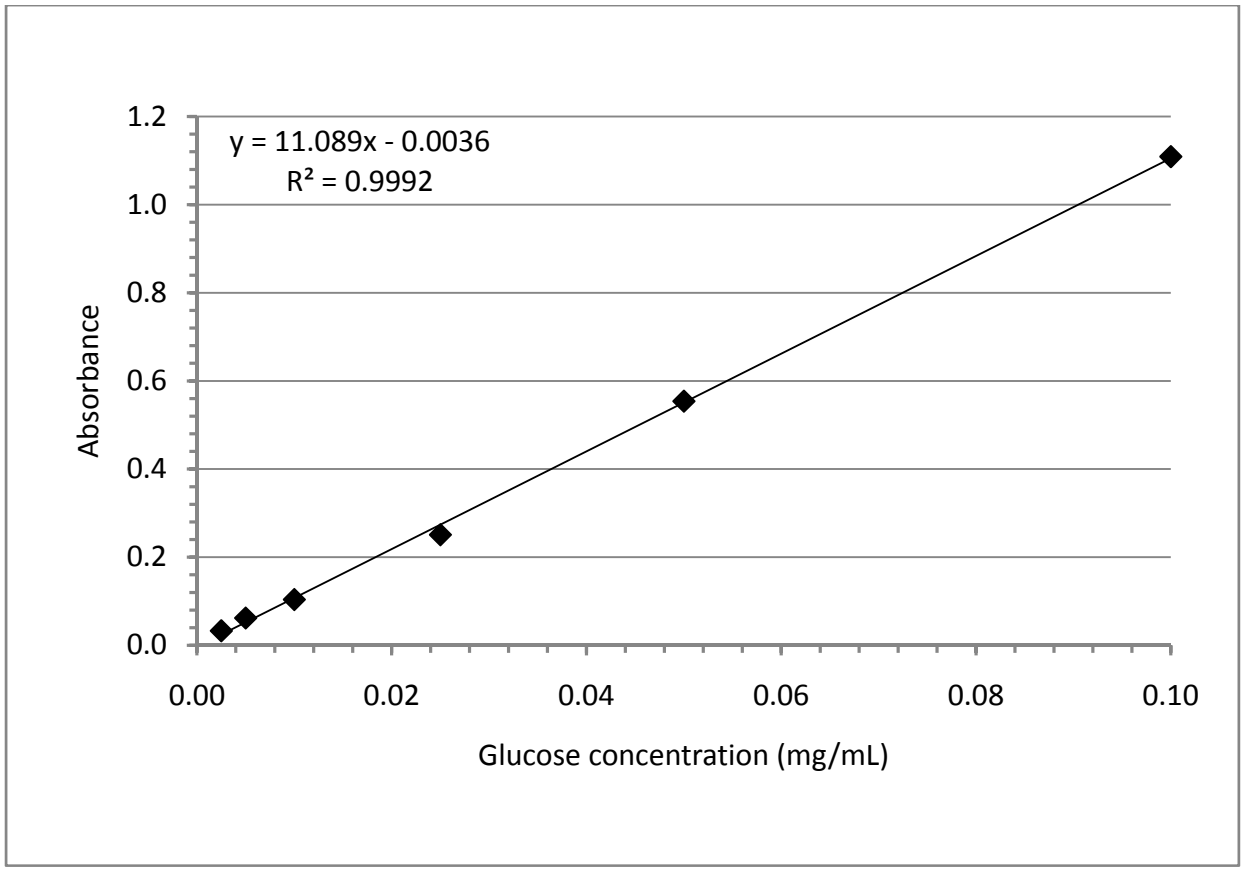

Figure 4. Calibration curve made with one sol-gel induced electrochemically.

In this experiment, instead of using different sol-gels, a single sol-gel was used for obtaining the six data points. Figure 4 showed that the same sol-gel can be used to get a linear calibration curve without worrying about a decrease in the enzymatic activity going from solution to solution, Figure 4 showed also that a linear dynamic range from 0.1 mg / mL to 0.025 mg / mL while that in Figure 5 showed linearity from 0.1 mg / mL to 0.01 mg / mL were possible. One specific feature about these sol-gels was the high value of the slope which implied high sensitivity in detection of glucose.

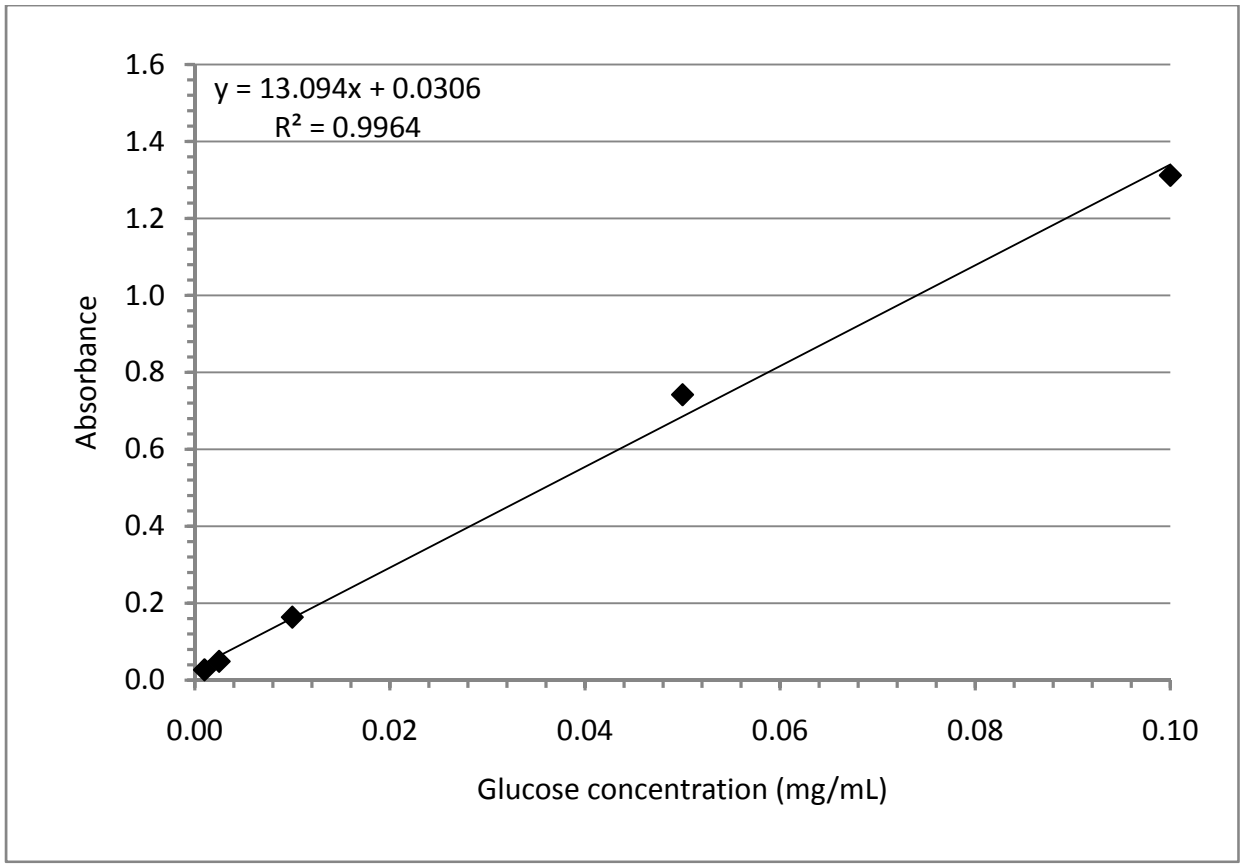

Figure 5. Calibration curve made with five sol-gels induced electrochemically.

Activity of the Enzyme with Frequency of Use

The activity of the enzyme encapsulated in different sol-gels was studied by measuring the absorbance of a glucose solution of concentration 0.1 mg / mL as a function of number of uses of the sol-gels. The solutions were incubated enough time and the oxidation product then reacted with Trinder reagent for 15 minutes. The absorbances were measured at 500 nm.

For the sol-gel prepared with TMOS, it showed a stability of enzymatic activity for at least 11 times of use during 1 month. The first 8 points were obtained during the 1st week and the other three data points were obtained in the following 3 weeks. The results are presented in Figure 6:

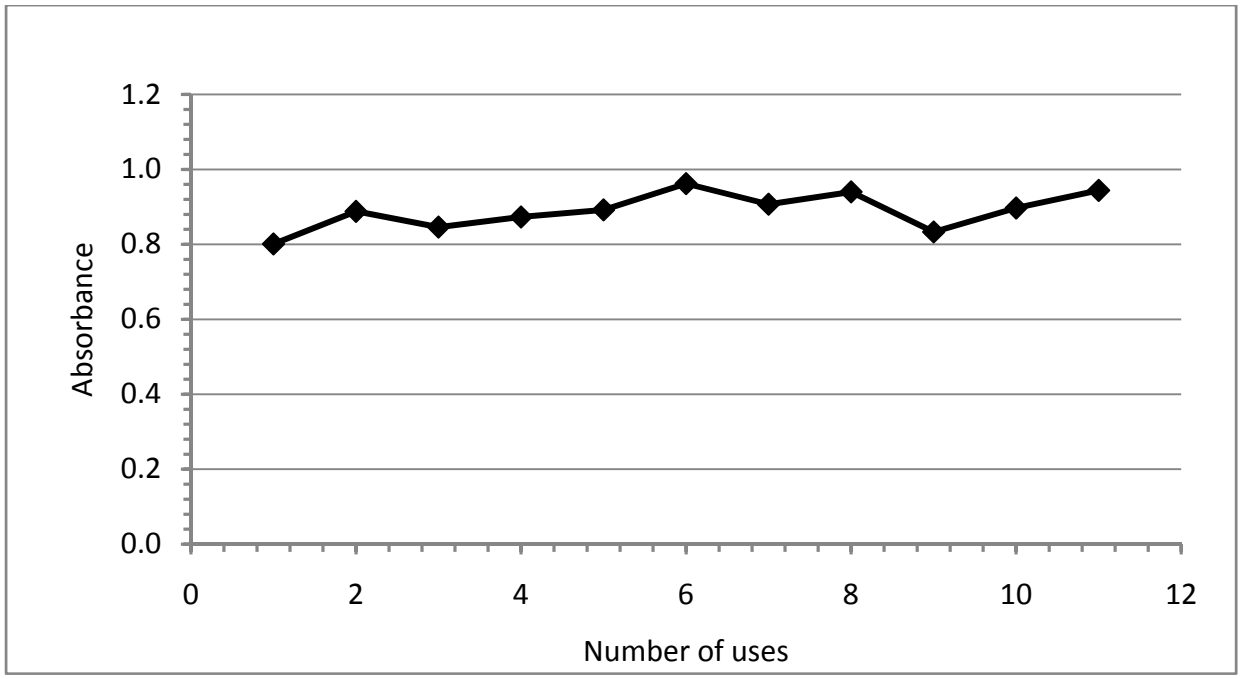

Figure 6. Stability of the enzymatic activity in the sol-gels with TMOS precursor.

For the sol-gel made with TEOS and HCl, it showed enzyme stability for 11 times of use during 3 days of experimentation but the big loss of enzyme activity appeared when testing them for the 12th time after 3 weeks as shown in Figure 7.

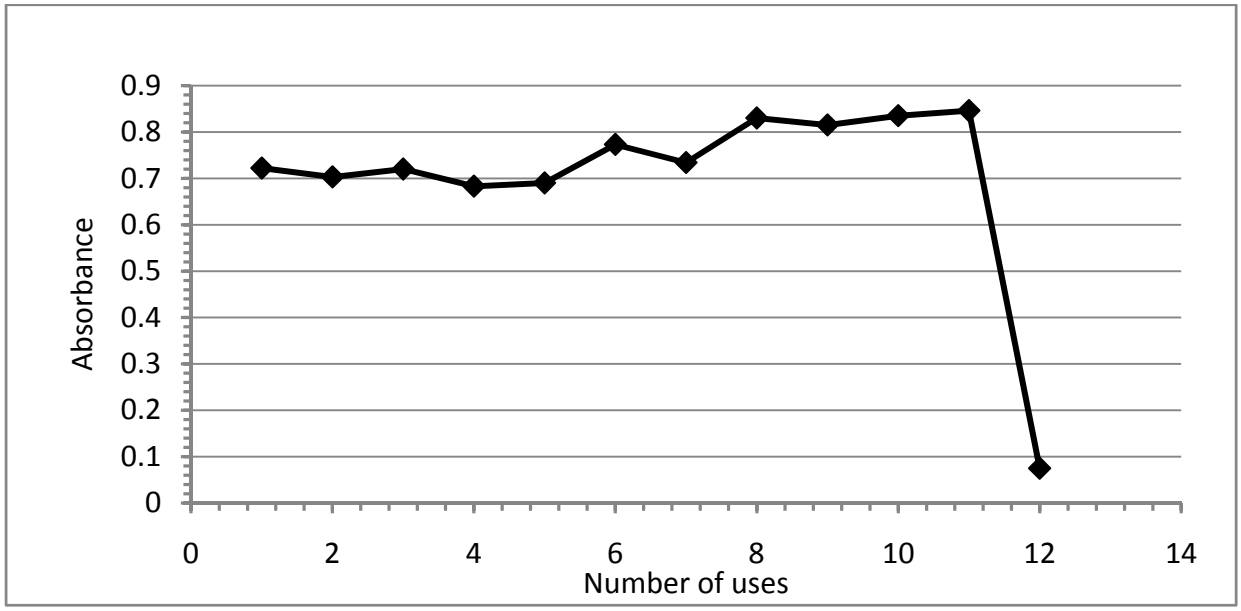

Figure 7. Stability of the enzymatic activity in the sol-gels prepared with TEOS and HCl.

For the sol-gel induced electrochemically, it showed stability in the enzymatic activity for the seven first tests and then the activity began to degrade. It increased later at the 10th trial but never got back to the initial value again. The first 11 points were obtained during 3 consecutive days of experiments. This sol-gel was again tested after 3 weeks and the value was not significantly lower from the previous one. The results are represented in Figure 8.

Not all the sol-gels induced electrochemically present the same pattern of enzymatic activity. One of these sol-gels was used 12 times in 2 days to make two calibration curves and used on some real samples. These results showed that not all the sol-gels present the same stability of enzymatic activity. However, it is reasonable to state that they can be used a certain number of times without losing activity.

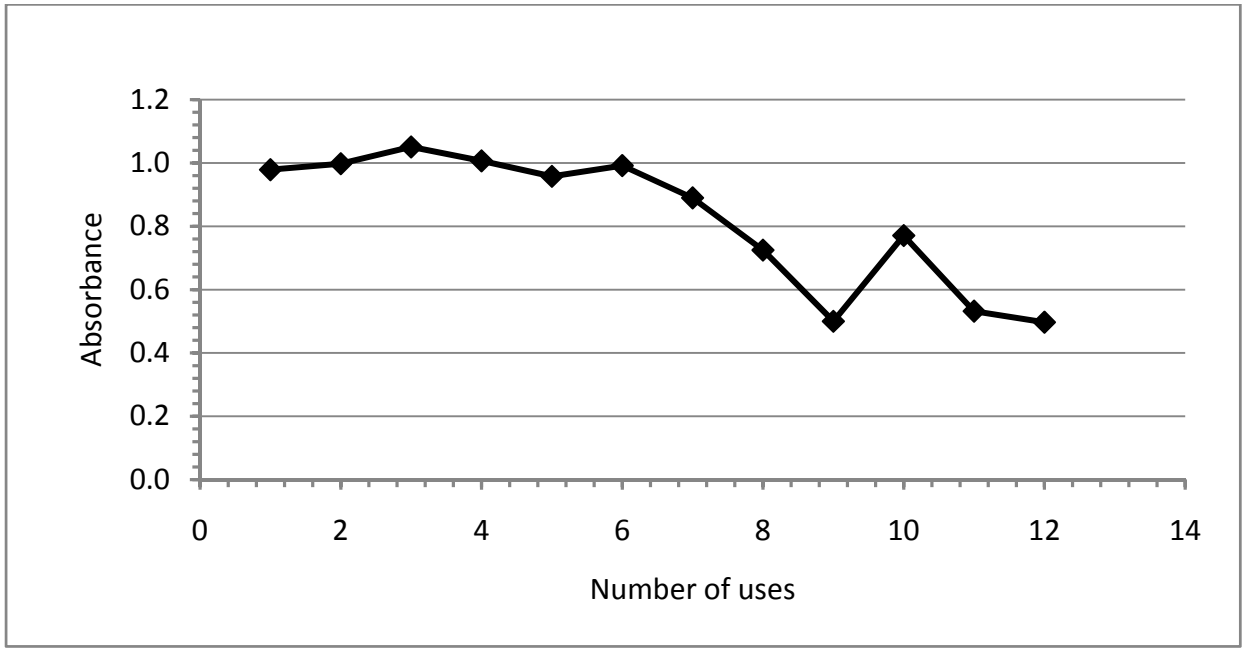

Figure 8. Stability of enzyme activity in a sol-gel induced electrochemically.

For the modified nanoparticles sol-gels, the stability of enzymatic activity was studied with 8 uses during 4 consecutive days and then tested once after 3 weeks. Figure 9 shows that the activity began decreasing at the second time of use and continued decreasing until reaching

38.78% of the original enzymatic activity at the 8th time of use. After 3 weeks the activity was almost completely gone. This result can be attributed to the fact that the enzyme is encapsulated in the sol solution and then adsorbed on the silica nanoparticles and the weak forces of the adsorption can be the reason of the activity decreasing. But according to the literature (129,130), it is a common feature of the enzyme adsorbed on the nanoparticles to become denatured for unknown reason. This could explain the instability of the activity of glucose oxidase immobilized in sol-gels modified silica nanoparticles.

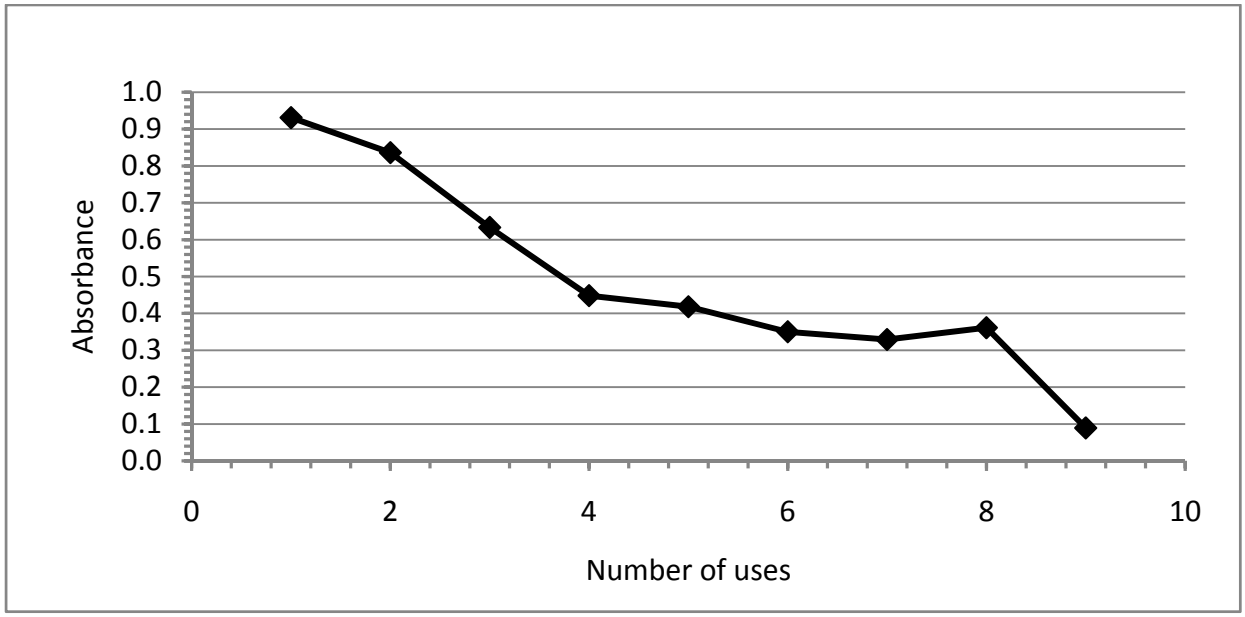

Figure 9. Stability of enzyme activity in the sol-gel modified silica nanoparticles.

Sol-Gel Storage Time and Activity of Immobilized Enzyme

An estimation of the lifetime of the three sol-gels (sol-gel with TMOS, sol-gel with TEOS and HCl, and sol-gel induced electrochemically) was taken into consideration. In the case of the sol-gel with TMOS and the sol-gel induced electrochemically, the activity of glucose oxidase in a sol-gel from each kind was tested from time to time to check if the enzyme was still active. For

the sol-gel made with the TEOS and HCl, the lifetime of the sol-gel was checked once after a certain period of storage.

A sol-gel prepared with TMOS at the beginning of March was tested for the first time at the end of the 3rd week of March where it had a good enzymatic activity. The sol-gel was tested again by the end of the 2nd week of April where it showed a moderate enzymatic activity. The lifetime of the sol-gel was estimated to be at least 6 weeks of storage.

A sol-gel induced electrochemically made at the end of February was tested in the beginning of March where it showed a good enzymatic activity. The activity was still high by the 3rd of week of March. After this test, the sol-gel was not used again until the end of the 2nd week of April, the sol-gel presented a moderate enzymatic activity. It thus can be concluded that the lifetime of this sol-gel was at least 6 weeks of storage.

A sol-gel made with TEOS and HCl was prepared in mid April. It was tested for the first time at the end of May where it showed a really high enzymatic activity. The storage time of the sol-gel was again shown to be at least around 6 weeks.

Although no specific values were tabulated in the study of the effect of the storage on the stability of the enzymatic activity because of the use of different Trinder reagents each time, it is evident that storing the sol-gel for a certain period was possible. But an enzyme would eventually show a decrease in enzymatic activity after prolonged storage.

Effect of Trinder Reagent

Sometimes the Trinder reagent can be the reason of obtaining irreproducible results. An example of this was obtained from three sol-gels induced electrochemically. The

experiment was done twice and the kind of calibration curve obtained is shown in Figure 10. The correlation coefficient was about 0.88 indicating the linearity was not as good as it should be. However, when a new Trinder reagent was prepared and the experiment repeated, the data obtained was much improved and the calibration curve was linear as shown in Figure 11.

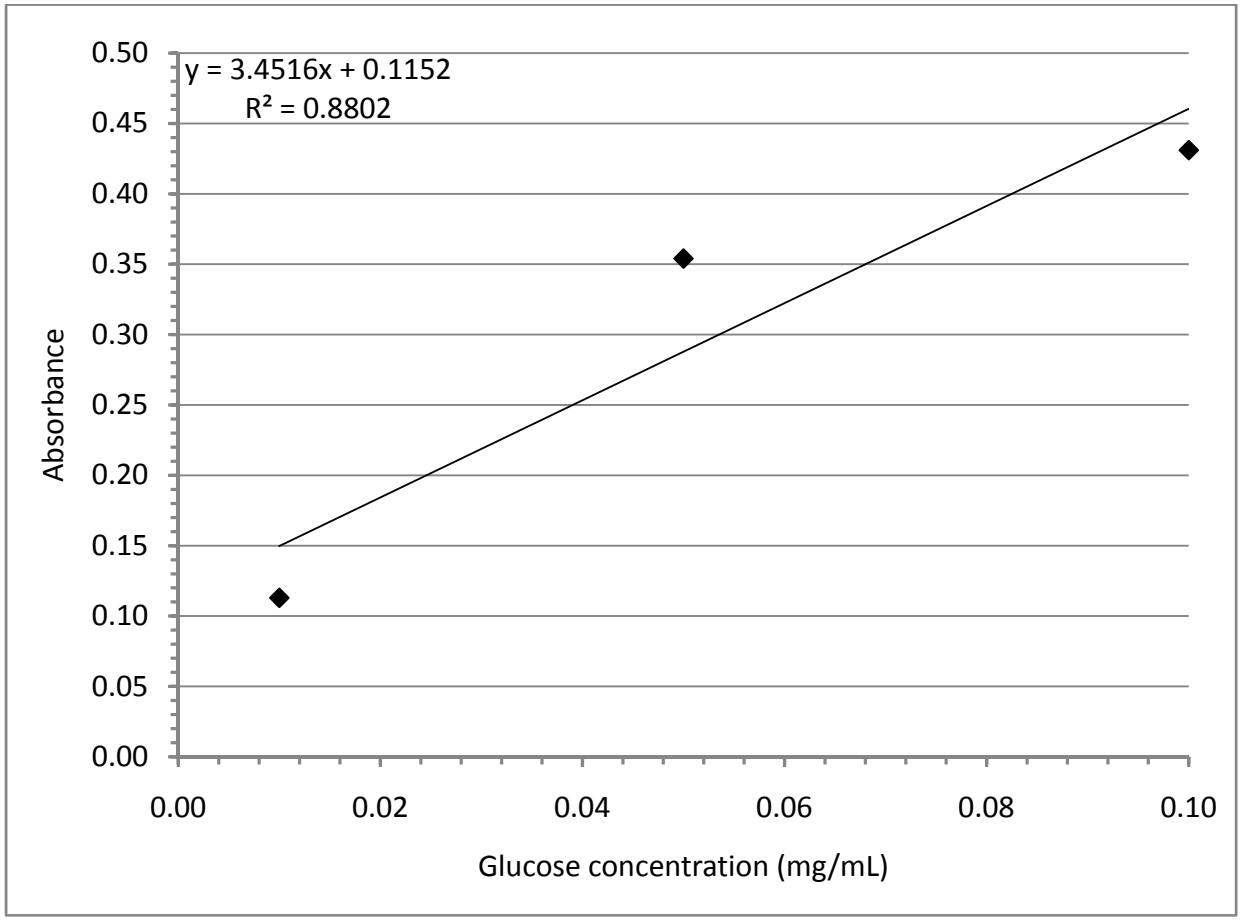

Figure 10. Calibration curve showing the degraded Trinder reagent giving rise to poor linearity.

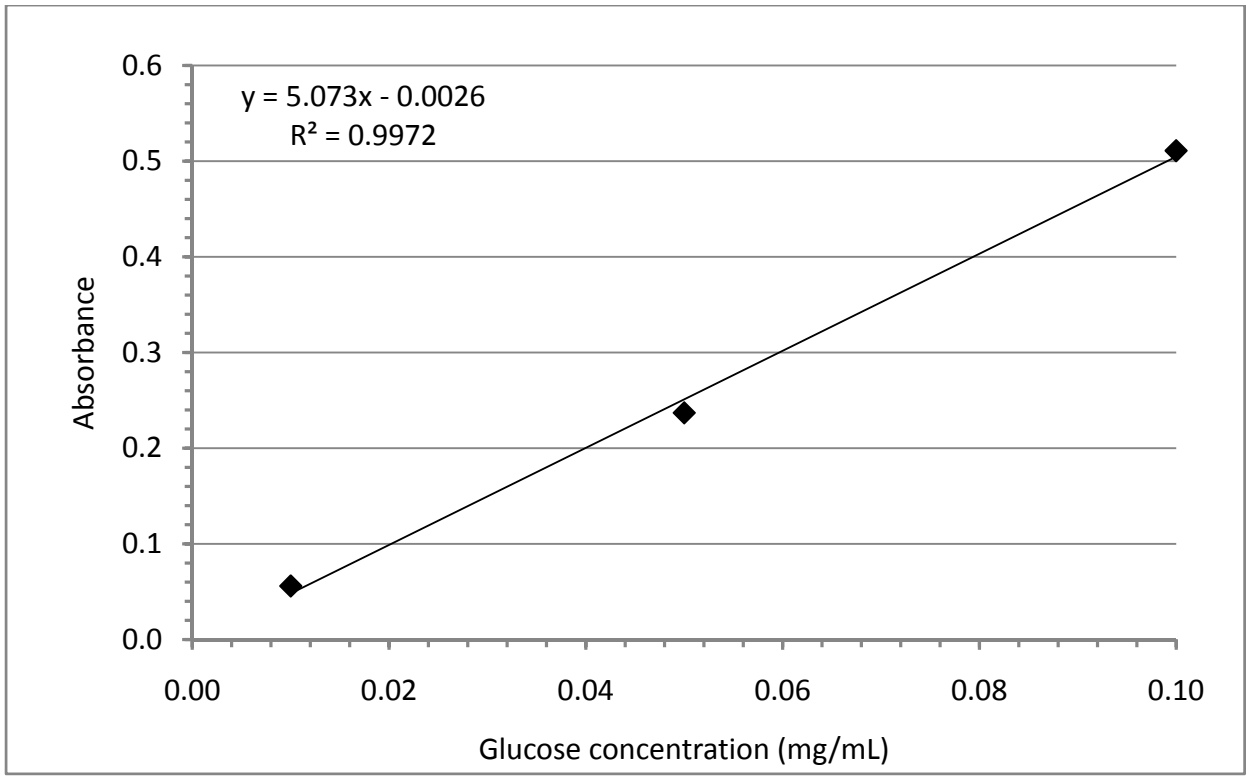

Figure 11. Calibration curve showing good linearity with a new different Trinder reagent.

Trinder reagent can also affect the precision of the results. The data obtained from using sol-gels prepared with TMOS to analyze the real sample Comforts showed this. The triplicate results from the experiment on the sample are shown in Table 10.

Table 10. Triplicate results in analysis of Comforts with degraded Trinder reagent.

Experimental absorbances	Found concentration	Expected concentration	RSD
0.140	0.026	0.025	12.01%
0.163	0.031		
0.169	0.033		

Thus it is important to monitor Trinder reagent as its stability can be degraded with time and hence affecting the results of analysis. It is important to date the preparation of Trinder reagent and prepare fresh reagent whenever needed.

Recovery Yield

Recovery studies are often conducted to ascertain the absence of interferences and systematic errors. Thus recovery studies indicate the accuracy of a proposed analytical procedure. Recovery experiments are done by the so called "spiking" procedure. Aliquots of a sample are obtained and separated into groups. One group of aliquots (usually triplicates) has no known amount of the analyte standard solutions added while other groups of the samples are "spiked", i.e. with known amounts of the standard solutions with known concentration added. By using an appropriate calibration curve, the amount of "spikes" or added analyte standard are found and compared to the actually added amount. Hence, percent recovery is calculated:

Recovery yield = (mass found / mass added) * 100

where the mass founded is the mass of glucose obtained experimentally and the mass added is the mass of glucose added from volume of the glucose working solution of known concentration.

The sol-gels used in this experiment encapsulated glucose oxidase Type X with TMOS as precursor. Table 11 shows the results of the recovery experiment using Pedialyte and sol-gels prepared with TMOS.

The recovery yields on the Pedialyte commercial samples shown in Table 11 indicated that the recovery for the larger spikes were very good and consistent compared to the smaller spikes, and the overall results range from 84% to 102%. The most likely reason for the poorer precision could be the variation in the enzymatic activity in the different sol-gels used.

Table 11. Recovery yield of glucose on Pedialyte sample using the sol-gels prepared with TMOS.

Solution	Absorbance	Total Glucose found (mg)	Glucose found (mg)	Glucose added (mg)	Recovery yield (%)
1	0.305	0.68	0.63 (average)	0.00	
	0.283	0.62			
	0.271	0.59			
2	0.441	1.05	0.42	0.50	84.0%
	0.455	1.09	0.46	0.50	92.0%
	0.473	1.14	0.51	0.50	102.0%
3	0.656	1.63	1.00	1.00	100.0%
	0.657	1.63	1.00	1.00	100.0%
	0.668	1.66	1.03	1.00	103.0%

The calibration curve used in the calculation for the results obtained was linear ($y = 9.2455 x + 0.0526$) and with good correlation coefficient of 0.9991 shown in Figure 12. Thus the disparity in the two sets of spike most likely was due to the above mentioned reasons.

The results of the recovery yield for the sol-gels with TEOS and HCl are presented in Table 12. The recovery yield of the added glucose to Pedialyte solutions using sol-gels prepared with TEOS and HCl again showed reasonably consistent good results at 92.0% for the larger spiked while the results for the lower spikes were inconsistent and much larger. The calibration

curve (not shown) as before was linear with good correlation coefficient of 0.9947. This further confirms that the lower spikes aliquots seem to present greater problems than the bigger spikes. Also, for this recovery study, only duplicates were used because of the limited number of reproducible sol-gels available.

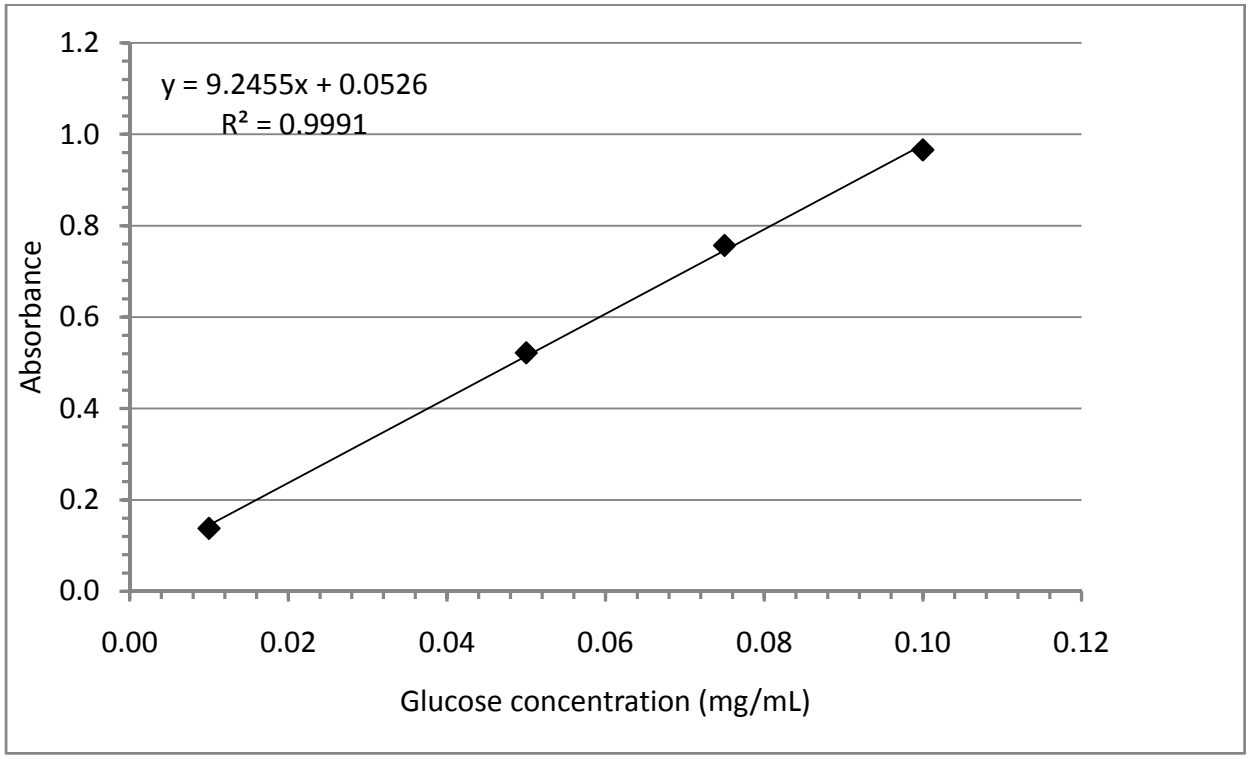

Figure 12. Calibration curve made with sol-gels with TMOS for the recovery yield.

Table 12. Results for the recovery yield of the sol-gels with TEOS and HCl.

Solution	Absorbance	Total Glucose found (mg)	Glucose found (mg)	Glucose added (mg)	Recovery yield (%)
1	0.370	0.62	0.57 (average)	0.00	
	0.321	0.52			
2	0.708	1.33	0.76	0.50	152.0%
	0.673	1.25	0.62	0.50	124.0%
3	0.811	1.55	0.92	1.00	92.0%
	0.819	1.55	0.92	1.00	92.0%

The calculated recovery yield of glucose in the case of using sol-gels induced electrochemically is shown in Table 13. These results again showed that the higher spike aliquot gave a recovery yield consistently around 100% and reasonably good. The lower spike aliquots again showed inconsistent and poor results. The calibration curve used in this study was good and linear with a correlation coefficient of 0.9992. It now became more puzzling because we cannot continually attribute the poorer results to variation in sol-gels' enzymatic activity. There were other unknown reasons at play.

Table 13. Recovery yield of the sol-gels induced electrochemically.

Sample	Absorbance	Total glucose found (mg)	Glucose found (mg)	Glucose added (mg)	Recovery yield
1	0.342	0.52	0.52 (average)	0.00	
	0.355	0.55			
	0.324	0.49			
2	0.501	0.83	0.31	0.50	62.0%
	0.536	0.90	0.38	0.50	76.0%
	0.575	0.98	0.43	0.50	86.0%
3	0.819	1.46	0.94	1.00	94.0%
	0.913	1.64	1.12	1.00	112.0%
	0.832	1.48	0.96	1.00	96.0%

Application to Commercial Samples

Thus far, it was shown that the activity of the enzymes encapsulated in the different sol-gels prepared gave good linear calibration curve, reproducibility, and recovery. The next set of experiment to be done was the application of these sol-gels encapsulating glucose oxidase on

commercial samples containing glucose. For this set of experiments, glucose oxidase Type X was used in the sol-gels prepared.

Pedialyte Samples

Table 14 presents the results of glucose determination of Pedialyte using three different kinds of sol-gels. The results are done on aliquots of Pedialyte all containing the expected concentration of glucose of 0.025 mg / mL.

Table 14. Results of glucose found in Pedialyte using glucose oxidase in three kinds of sol-gels.

Sol-gels with TMOS			Sol-gels with TEOS and HCl			Sol-gels induced electrochemically		
Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)
0.025	0.025 (4.00%)	0.0	0.028	0.027 (7.62%)	8.0	0.027	0.028 (3.57%)	12.0
0.026			0.025			0.028		
0.024			0.029			0.029		
0.024	0.025 (6.03%)	0.0	0.024	0.025 (4.00%)	0.0	0.029	0.027 (7.41%)	8.0
0.025			0.025			0.025		
0.027			0.026			0.027		
0.030	0.030 (5.15%)	20.0				0.025	0.026 (10.2%)	4.0
0.031						0.029		
0.028						0.024		

Two aliquots only from the sample Pedialyte were tested on the sol-gels prepared with TEOS and HCl because of the limited number of the available reproducible sol-gels. The glucose concentrations were determined using to the calibration curves prepared with different sol-gels. The calibration curves were all linear with a correlation coefficient of 0.99. Therefore they are not displayed as they have been shown a good number of times. The results obtained were all with good accuracy with a low relative error ranging from 0% to 20%

using all the different sol-gels. The errors in all cases were most likely due to experimental errors and did not seem to be systematic.

If the total average of glucose concentrations given by all the three kinds of sol-gels, the value obtained is 0.027 mg / mL with the relative error of 8%. Thus the results on Pedialyte using glucose oxidase encapsulated in all three kinds of sol-gels were satisfactory.

Comforts Samples

Table 15 shows the results on glucose determination in Comforts using glucose oxidase encapsulated on the three different kinds of sol-gels. The expected glucose concentration was again 0.025 mg / mL.

Table 15. Results of glucose found in Comforts using glucose oxidase in three kinds of sol-gels.

Sol-gels with TMOS			Sol-gels with TEOS and HCl			Sol-gels induced electrochemically		
Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)
0.030	0.030 (5.15%)	20.0	0.030	0.027 (11.2%)	8.0	0.026	0.025 (4.00%)	0.0
0.031			0.028			0.025		
0.028			0.024			0.024		
0.023	0.025 (8.00%)	0.0	0.022	0.024 (10.3%)	-4.0	0.027	0.026 (3.85%)	4.0
0.025			0.027			0.025		
0.027			0.024			0.026		
0.027	0.027 (2.11%)	8.0				0.016	0.015 (26.4%)	-40.0
0.028						0.019		
0.027						0.011		

The calibration curves used to obtain the results are again not displayed here as they are linear and with similarly good correlation coefficients. The results obtained were satisfactory compared to the expected glucose concentration. The percent error ranged from – 4.0% to 20%

with an anomalous – 40% error for the third aliquot of the sample (analyzed in triplicates) using electrochemically induced sol-gels. One possible reason for the result is the non-uniform distribution of glucose in the real sample obtained for this set of analysis. The other possible source of error was experimental and that could happen during the preparation of the solutions. The third possibility was the change of enzyme activity in that sol-gel used but the reason of this decrease so large that it was not understood. Also, the third aliquot was the only aliquot with low results.

Dex4 Samples

The results for the glucose determination in commercial samples of Dex4 on these sol-gels are presented in Table 16 and the results are compared to the expected glucose concentration of 0.025 mg / mL.

Table 16. Results of glucose found in Dex4 using glucose oxidase in three kinds of sol-gels.

Sol-gels with TMOS			Sol-gels with TEOS and HCl			Sol-gels induced electrochemically		
Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)	Glucose (mg/mL)	Average	Error (%)
0.026	0.025 (4.00%)	0.0	0.022	0.023 (6.55%)	-8.0	0.028	0.027 (4.23%)	8.0
0.025			0.023			0.026		
0.024			0.025			0.028		
0.028	0.029 (4.03%)	16.0	0.022	0.020 (14.2%)	-20.0	0.029	0.028 (4.17%)	12.0
0.028			0.017			0.027		
0.030			0.022			0.027		
0.036	0.036 (0.00%)	44.0				0.026	0.026 (4.00%)	4.0
0.036						0.026		
0.036						0.026		

The results obtained were similarly satisfactory ranging in percent error from – 20% to 16% with an anomalous 44% relative error for the third aliquot of the sample Dex4 using sol-gels with TMOS. The same reasons of the error mentioned in the previous application on Comforts can apply also this time.

Thus overall the proposed procedure established that the sol-gels prepared were capable of yielding good results on real samples. Considering the number of samples prepared and sol-gels involved, one set of slightly large or low results while the majority of the results were resonable was a good sign. Also, the larger error tends to be on the low side except one indicating the activity of the enzyme may be suspect.

CHAPTER 5

CONCLUSION

A comparative study of glucose oxidase encapsulated in four different kinds of sol-gel were done with the goals to study the efficacy of each method, the stability of the enzyme encapsulated, and the suitable conditions for the determination of glucose in commercial samples.

Some sol-gels were prepared easily without any significant problems and in a very short period of time like the sol-gel induced electrochemically that can be ready for use with 1 day of preparation. The major problem in preparing the sol-gels was confronted in the case of sol-gels prepared with TMOS that showed severe cracking in some cases before any use. Cracking caused the loss of enzyme activity. This problem had to be solved by using different experimental procedures like adding titanium dioxide nanoparticles, adding the drying agent dimethylformamide, or using specific containers and mixing the solutions during the preparation of sol-gels, and this final procedure was successful in diminishing the cracking problem.

All the different kinds of sol-gels prepared except the sol-gel modified nanoparticles showed enzyme stability for several uses and reasonable storage time. The experiments that were done imply that the enzymatic stability is affected by two main factors. They were the number of uses and the length of time of storage. Applications of these sol-gels for analysis should not exceed a given number of uses and to be used within only a few weeks.

Trinder reagent did not cause any complications as the method for the detection of hydrogen peroxide produced when glucose was oxidized. However, the stability of this reagent

should be monitored because this reagent employs the peroxidase enzyme as a catalyst for Trinder reaction. Hence it is prudent to use the reagent that is as freshly made as possible.

Using sol-gels to determine the glucose in three different kinds of commercial samples was successful although few problems were encountered especially when the sol-gel was used for a number of times before or when it was stored in the refrigerator for a period of time. Good results were obtained with different sol-gels when these sol-gels were reproducible and this was possible if the sol-gels were prepared under the same conditions and they were freshly used. Intention to store the sol-gels for later repetitive uses should be done under suitable and controllable conditions to avoid inconsistent or poor results in the applications of the sol-gels as the loss of reproducibility in activity can occur.

In summary, the sol-gels prepared with TMOS, TEOS in the presence of HCl, and the sol-gels induced electrochemically were all suitable for immobilizing glucose oxidase because the enzyme was not denatured and yield good linearity of calibration curves, good reproducibility, and recovery. Their use in the determination of glucose in several commercial samples also gave accurate results with relatively low percentage errors from the expected values.

The best sol-gel is the one that fits with the future applications that could require specific shape, or specific time, or a determined size of the pores of the network if other molecules or enzymes need to be immobilized with glucose oxidase in the same sol-gel matrix.

REFERENCES

1. Scouten W. H., Luong J. H.T., and Brown R. S., Enzyme or protein immobilization techniques for applications in biosensor design, *Trends in Biotechnology,* May **1995**, 13 (5), 178-185.

2. Yang, Z. Zhou X., Xiao D., and Choi M. M. F., A fluorescent glucose biosensor based on immobilized glucose oxidase on bamboo inner shell membrane, *Biosensors and Bioelectronics,* 15 February **2006**, 21 (8),1613-1620.

3. Dave B., Dunn B., Valentine J. S., and ZinK J. l., Sol-Gel Encapsulation Methods for Biosensors, *Analytical Chemistry***,** November 15 **1994,** 66 (22), 1120A–1127A.

4. Jia W., Wang K., Zhu Z., Song H., and Xia X., One-Step Immobilization of Glucose Oxidase in a silica Matrix on a Pt Electrode by an Electrochemically Induced Sol-Gel Process, *Langmuir,* **2007**, 23**,** 11896-11900.

5. Dudas Z., Oros B., Preda G., Dragomirescu M., Halasz J., and Chiriac A., Comparison between hybrid organic/inorganic bioceramics containing microbial hydrolases, synthesized via the sol-gel process, *Journal of Biotechnology,* September **2007**, 131 (2) 1, S111-S112.

6. Mateo C., Palomo J. M., Fernandez-Lorente G., Guisan J. M., and Fernandez-Lafuente R., Improvement of enzyme activity, stability and selectivity via immobilization techniques, *Enzyme and Microbial Technology ,* 2 May **2007**, 40 (6) , 1451-1463.

7. Eijsink V.G.H., Gaseidnes S., Borchert T. V., and Van den Burg B., Directed evolution of enzyme stability, *Biomolecular Engineering,* June **2005**, 22 (1-3), 21-30.

8**.** Li F., Xing Y., and Ding, X. Immobilization of papain on cotton fabric by sol-gel method, *Enzyme and Microbial Technology*, 1 June **2007**, 40 (7), 1692-1697.

9. Nelson J. M., and Griffin E. G., Adsorption of Invertase, *J. Am. Chem. Soc.*, **1916**, 38, 1109-1115.

10. Harkins W. D., Fourt L., and Fourt P. C., Immunochemistry of catalase II. Activity in multilayers, *J. Biol. Chem.*, **1940**, 132, 111–118.

11. Gale E. F., and Epps M.R., Studies on bacterial amino-acid decarboxylases 1. 1-(+)-lysine Decarboxylase, *Biochem. J.,* **1944**, 38, 232–242.

12. Langmuir I., and Schaefer V. J., Activities of urease and monolayers, *J. Am. Chem. Soc.,* **1938**, 60, 1351–1360.

13. Stone I., Method of making dextrose using starch glucogenase, *US patent,* **1955**, 2, 717-852.

14. McLaren A. D., The Adsorption and Reactions of Enzymes and Proteins on Kaolinite. II[1] , The Action of Chymotrypsin on Lysozyme, *Soil Sci. Soc. Am. J.,* **1954**, 18, 170-174.

15. Mclaren A.D., Concerning the pH dependence of enzyme reactions on cells, particulates and in solution, *Science*, **1957**, 125, 697-697.

16. Fletcher G.L., and Okada S., Protection of deoxyribonuclease from ionizing radiation by adsorbents, *Nature,* **1955**, 176, 882–883.

17. Fletcher G.L., and Okada S., Effect of adsorbing materials on radiation inactivation of deoxyribonuclease, *I.Radiation Res,* **1959**, 11, 291–298.

18. Brandenberg H., Methods for linking enzymes to insoluble carriers, *Angew Chem,* **1955**, 67, 661–661.

19. McLaren A.D., and Estermann E.F., The adsorption and reactions of enzymes and proteins after filtration on (and through) a microcellulose membrane, *Biochem. Biophys. Res. Commun.,* **1956**, 61, 158–173.

20. Isliker H.C., Purification of antibodies by means of antigens linked to ion-exchange resin, *Ann. NY Acad. Sci.,* **1953**, 3, 225–238.

21. Grubhofer N., and Schleith L., Modified ion-exchange resins as specific adsorbent, *Naturwissenschaften*, **1953**, 40, 508–508.

22. Manecke G., and Gillert K. E., Serologich Spezifische Adsorbentien, *Naturwissenschaften*, **1955**, 42, 212–213.

23. Gyenes L., Rose B., and Sehon A. H., Isolationof antibodies on antigen–polystyrene conjugates, *Nature*, **1958**, 181, 1465–1466.

24. Mitz M. A., and Summaria L. J., Synthesis of biologically active cellulose derivatives of enzymes, *Nature,* **1961**, 189, 576–560.

25. Newirth T. L., Diegelman M. A., Pye E. K., and Kallen R. G., Multiple immobilized enzyme reactors. Determination of pyruvate and phosphoenolpyruvate concentrations using immobilized lactate dehydrogenase and pyruvate kinase, *Biotechnol. Bioeng.,* **1973**, 15, 1089–1100.

26. Dickey F. H., Specific adsorption, *J. Phys. Chem.,* **1955**, 59, 695-707.

27**.** Cao L., Carrier-bound immobilized enzymes: principles, applications and design, Copyright © **2005**, WILEY-VCH., 5-20.

28. Bernfeld P., and Wan J., Antigens and enzymes made insoluble by entrapping them into lattice of synthetic polymers, *Science*, **1963**, 142, 678–679.

29. Hicks G. P., and Updike S. J., The preparation and characterisation of lyophilized polyacrylamide enzyme gels for chemical analysis, *Anal. Chem.*, **1966**, 38, 726–729.

30. Mosbach K., and Mosbach R., Entrapment of enzymes and microorganisms in synthetic cross-linked polymers and their applications in column techniques, *Acta Chem. Scand.,* **1966**, 20, 2807–2810.

31. Tosa T., Mori T., Fuse N., and Chibata I., Studies on continuous enzyme reactions Part V Kinetic and industrial application of aminoacylase column for continuous optical resolution of acyl-dl-amino acids, *Biotechnol. Bioeng.,* **1967**, 9, 603–615.

32. Leuschner F., Shaped structures for biological processes, *German Patent,* **1966,** 1, 227-855.

33. Brown H. D., Patel A. B., and Chattopadhyay S. K. , Enzyme entrapment within hydrophobic and hydrophilic matrices, *J. Biomed. Mater. Res.,* **1968**, 2, 231–235.

34. Pennington S. N., Brown H. D., Patel A. B., and Chattopadhyay S. K., Silastic entrapment of glucose oxidase-peroxidase and acetylcholine esterase, *J. Biomed. Mater. Res.,* **1968**, 2, 443–446.

35. Quiocho F. A., and Richards F. M., Inter-molecular cross-linking of a protein in the crystalline state: carboxypeptidase A, *Proc. Natl. Acad. Sci. USA,* **1964**, 52, 833–839.

36. Quiocho F. A., and Richards F. M., Enzyme behavior of carboxypeptidase-A in the solid state. *Biochemistry,* **1966**, 5, 4062–4076.

37. Quiocho F. A., and Richards F. M., Intermolecular cross Linking of a protein in the crystalline state Carboxypeptidase-A, *Proc. Natl. Acad. Sci. U S A,* Sep **1964,** 52, 833–839.

38. Habeeb A. F., Preparation of enzymically active, water-insoluble derivatives of trypsin, *Archives of Biochemistry and Biophysics,* Mar **1967**, 119 (1), 264-268.

39. Ogata K., Ottesen M., and Svendsen I., Preparation of water-insoluble, enzymatically active derivatives of subtilisin type Novo by cross-linking with glutaraldehyde., *Biochim Biophys Acta.*, 4 Jun **1968**, 159(2), 403-405.

40. Jansen E. F., and Olson A. C., Properties and enzymatic activities of papain insolubilized with glutaraldehyde, *Arch. Biochem. Biophys.*, Jan **1969**, 129 (1), 221-227.

41. Clark Jr. L. C., and Lyons C., Electrode systems for continuous monitoring in cardiovascular surgery, *Ann. NY Acad. Sci.*, **1962**, 102, 29–45.

42. Glazer A. N., Bar-Eli A., and Katchalski E., Preparation and characterization of polytyrosyl trypsin, *J. Biol. Chem.,* **1962**, 237, 1832–1838.

43. Vretblad P., Immobilisation of ligands for biospecific affinity chromatography via their hydroxyl groups. The cyclohexa-amylose-α-amylase system, *FEBS Lett.,***1974**, 47, 86–89.

44. Kamogashira T., Mihara S., Tamaoka H., and Doi T., 6-Aminopenicillanic acid by penicillin hydrolysis with immobilized enzyme, *Jpn Kokai Tokkyo Koho*, **1972**, JP 47028187.

45. Messing R. A., and Filbert A. M. , Immobilized glucose isomerase for the continuous conversation of glucose to fructose, *J. Agric. Food Chem.*, **1975**, 23, 920–923.

46. Klibanov A. M., Why are enzymes less active in organic solvents than in water?, *Trends Biotechnol,* **1997**, 15, 97–101.

47. Schmitke J. L., Stern L. J., and Klibanov A. M., The crystal structure of subtilisin Carlsberg in anhydrous dioxane and itscomparison with those in water and acetonitrile, *Proc. Natl. Acad. Sci. USA*, **1997**, 94, 4250–4255.

48. Zaks A., and Kllbanov A. M., Enzyme-catalyzed processes in organic solvents, *Proc. Natl. Acad. Sci. USA,* **1985**, 82, 3192–3196.

49. Kise H. and Hayakawa A., Immobilisation of protease to porous chitosan beads and their catalysis for ester and peptide synthesis in organic solvents, *Enzyme Microb. Technol.,* **1991**, 13, 584–588.

50. St Clair N. L., and Navia M. A., Cross-linked enzyme crystals as robust biocatalysts, *J. Am. Chem. Soc.*, **1992**, 114, 7314–7316.

51. Lalonde J., Practical catalysis with enzyme crystals, *Chemtech,* **1997**, 27, 38–45.

52. Liu Z., Deng J., and Li D., A new tyrosinase biosensor based on tailoring the porosity of Al_2O_3 sol–gel to co-immobilize tyrosinase and the mediator, *Anal. Chim. Acta*, **2000**, 407, 87–96.

53. Niculescu M., Gáspár S., Schulte A., Csöregi E., and Schuhmann W., Visualization of micropatterned complex biosensor sensing chemistries by means of scanning electrochemical microscopy, *Biosen. Bioelectron.,* **2004**, 19, 1175–1184.

54. Doretti L., Ferrara D., Gattolin P., and Lora S., Covalently immobilized enzymes on biocompatible polymers for amperometric sensor applications, *Biosen. Bioelectron.,* **1996**, 11, 365–373.

55. Li Z. F., Kang E. T., Neoh K. G and Tan K. L., Covalent immobilization of glucose oxidase on the surface of polyaniline films graft copolymerized with acrylic acid, *Biomaterials*, **1998**, 19, 45–53.

56. Chiu S., Chung T., Giridhar R., and Wu W., Immobilization of β-cyclodextrin in chitosan beads for separation of cholesterol from egg yolk, *Food Res. Int.,* **2004**, 37, 217–223.

57. Yang Z., Sasaki S., Karube I., and Suzuki H., Fabrication of oxygen electrode arrays and their incorporation into sensors for measuring biochemical oxygen demand, *Anal. Chim. Acta,* **1997**, 357, 41–49.

58. Peter J., Hutter W., Stöllnberger W., and Hampel W., Detection of chlorinated and brominated hydrocarbons by an ion sensitive whole cell biosensor, *Biosens. Bioelectron.,* **1996**, 11, 1215–1219.

59. Schmidt A., Standfuss-Gabisch C., and Bilitewski U., Microbial biosensor for free fatty acids using an oxygen electrode based on thick film technology, *Biosens. Bioelectron.,* **1996**, 11, 1139–1145.

60. Yang X., Hua L., Gong H., and Tan S. N., Covalent immobilization of an enzyme glucose oxidase onto a carbon sol–gel silicate composite surface as a biosensing platform, *Anal. Chim. Acta,* **2003**, 478, 67–75.

61. Tag K., Lehmann M., Chan C., Renneberg R., Riedel K., and Kunze G., Measurement of biodegradable substances with a mycelia-sensor based on the salt tolerant yeast Arxula adeninivorans LS3, *Sens. Actuators B,* **2000***,* 67, 142–148.

62. Kwan R. C. H., Hon P. Y. T., Mak K. K. W., and Renneberg R., Amperometric determination of lactate with novel trienzyme/poly(carbamoyl) sulfonate hydrogel-based sensor, *Biosens. Bioelectron.,* **2004**, 19, 1745–1752.

63. Wang S., Zhang Q., Wang R., Yoon S. F., Ahn J., Yang D. J., Tian J. Z., Li J. Q., and Zhou Q., Multi-walled carbon nanotubes for the immobilization of enzyme in glucose biosensors, *Electrochem. Commun.,* **2003**, 5, 800–803.

64. Yu J., Liu S., and Ju H., Mediator-free phenol sensor based on titania sol–gel encapsulation matrix for immobilization of tyrosinase by a vapor deposition method, *Biosens. Bioelectron.,* **2003**, 19, 509–514.

65. Hasse U., and Scholz F., In situ AFM evidence of the involvement of an oversaturated solution in the course of oxidation of silver nanocrystals to silver iodide and bromide crystals, *Electrochem. Commun.,* **2004**, 6, 409–412.

66. Luo X. L., Xu J. J., Zhao W., and Chen H.Y., Glucose biosensor based on ENFET doped with SiO_2 nanoparticles, *Sens. Actuators B,* **2004**, 97, 249–255.

67. Wang H. and Mu S., Bioelectrochemical characteristics of cholesterol oxidase immobilized in a polyaniline film, *Sens. Actuators B,* **1999**, 56, 22–30.

68. Puig-Lleixà C., Jiménez C. and Bartrolí J., Acrylated polyurethane — photopolymeric membrane for amperometric glucose biosensor construction, *Sens. Actuators B*, **2001**, 72, 56–62.

69. Kok F.N., and Hasirci V., Determination of binary pesticide mixtures by an acetylcholinesterase–choline oxidase biosensor, *Biosens. Bioelectron.,* **2004**, 19, 661–665.

70. Qian J., Liu Y., Liu H., Yu T., and Deng J., Immobilization of horseradish peroxidase with a regenerated silk fibroin membrane and its application to a tetrathiafulvalene-mediating H_2O_2 sensor, *Biosens. Bioelectron.,* **1997**, 12, 1213–1218.

71. Michel P. E., Gautier-Sauvigné S. M., and Blum L. J., A transient enzymatic inhibition as an efficient toll for the discriminating bioluminescent analysis of three adenylic nucleotides with a fiberoptic sensor based on a compartmentalized tri-enzymatic sensing layer, *Anal. Chim. Acta*, **1998**, 360, 89–99.

72. Choi M. M. F., Pang W. S. H., Xiao D., and Wu X., An optical glucose biosensor with eggshell membrane as an enzyme immobilization platform, *Analyst,* **2001**, 126, 1558–1563.

73. Xiao D., and Choi M. M. F., Aspartame optical biosensor with bioenzyme-immobilized eggshell membrane and oxygen-sensitive optode membrane, *Anal. Chem.,* **2002**, 74, 863–870.

74. Choi M. M. F., and Yiu T. P., Immobilization of beef liver catalase on eggshell membrane for fabrication of hydrogen peroxide biosensor, *Enz. Microb. Technol.,* **2004**, 34, 41–47.

75. Wu B., Zhang G., Shuang S., and Choi M.M.F., Biosensors for determination of glucose with glucose oxidase immobilized on an eggshell membrane, *Talanta,* **2004**, 64, 546–553.

76. Braun S., Rappoport S., Zusman R., Avnir D., and Ottolenghi M., *Mater. Lett.,* Biochemically active sol-gel glasses: the trapping of enzymes, **1990**, 10 (1-2), 1-5.

77. Braun S., Shtelzer S., Rappoport S., Avnir D. J., and Ottolenghi M., Biocatalysis by sol-gel entrapped enzymes, *J. Non-Cryst. Solids*, **1992,** 147-148, 739-743.

78. Yamanaka S., F. Nishida, Ellerby L., C. Nishida, Dunn B., J. Valentine, and Zink J., Enzymatic activity of glucose oxidase encapsulated in transparent glass by the sol-gel method, *J. Chem. Mater*., **1992**, 4 (3), 495-497.

79. Sosnitza P., Farooqu M. i, Saleemuddin M., Ulber R. and Scheper T., Application of reversible immobilization techniques for biosensors, *Analytica Chimica Acta*, **1998**, 368, 197-203.

80. Ramanathan K., Shyam S., Pandey S. S., Kumar R., Gulati A., Surya A., Murthy N., and Malhotra B. D., Covalent immobilization of glucose oxidase to poly(*O*-amino benzoic acid) for application to glucose biosensor, *Journal of Applied Polymer Science*, 27 Jul **2000**, 78 (3), 662-667.

81. Tirkeş S., Toppare L., Alkan S., Bakır U., Önen A., and Yağcı Y., Immobilization of glucose oxidase in polypyrrole / polytetrahydrofuran graft copolymers, *Int. J. Biol. Macromol*., **2002**, 30 (2), 81.

82. Sarath Babu V. R., Kumar M. A., Karanth N. G., and Thakur M. S., Stabilization of immobilized glucose oxidase against thermal inactivation by silanization for biosensor applications, *Biosens. Bioelec.,* **2004**, 19, 1337–1341.

83. Foulds N. C. and Lowe C. R., Enzyme entrapment in electrically conducting polymers, *J. Chem. Soc. Faraday Trans 1,* **1986***,* 82, 1259–1264.

84. Umana M., and Waller J., Protein-modified electrodes. The glucose oxidase/polypyrrole system, *Anal. Chem.,* **1986**, 58, 2979–2983.

85. Bartlett P.N., and Whitaker R., Electrochemical immobilization of enzymes. Part I: Theory. *J. Electroanal. Chem.,* **1987**, 224, 27–35.

86. Shinohara H., Chiba T., and Aizawa M., Enzyme microsensor for glucose with an electrochemically synthesized enzyme-polyaniline film, *Sensors and Actuators,* **1988**, 13, 79–86.

87. Pandey P.C., A new conducting polymer-coated glucose sensor, *J. Chem. Soc. Faraday Trans.*, **1988**, 84, 2259–2265.

88. Pan D., Chen J., Yao S., Tao W., and Nie L., An Amperometric Glucose Biosensor Based on Glucose Oxidase Immobilized in Electropolymerized Poly(*o*-aminophenol) and Carbon Nanotubes Composite Film on a Gold Electrode, *Analytical Sciences,* **2005**, 21 (4), 367.

89. Li M., Deng C., Xie Q., Yang Y., and Yao S., Electrochemical quartz crystal impedance study on immobilization of glucose oxidase in a polymer grown from dopamine oxidation at an Au electrode for glucose sensing , *Electrochimica Acta*, 28 July **2006**, 51 (25), 5478-5486.

90. Zhao S., Zhang K., Bai Y., Yang W., and Sun C., Glucose oxidase/colloidal gold nanoparticles immobilized in Nafion film on glassy carbon electrode: Direct electron transfer and electrocatalysis, *Bioelectrochemistry*, October **2006**, 69 (2), 158-163.

91. Salimi A., Sharifi E., Noorbakhsh A., and Soltanian S., Immobilization of glucose oxidase on electrodeposited nickel oxide nanoparticles: Direct electron transfer and electrocatalytic activity, *Biosensors and Bioelectronics*, 15 June **2007**,22 (12), 3146-3153.

92. Trevan M.D., Immobilized Enzymes An Introduction and Applications in Biotechnology, *JOHN WILEY & SONS*, **1980**, 1-21, 35-40, 56-71.

93. Guisan J.M., Immobilization of enzymes and cells Second Edition, Humana Press, **2006**, 18-25.

94. D'Souza S.F., Immobilized enzymes in bioprocess, *Curr. Sci.,* **1999**, 77, 69–79.

95. Bickerstaff G.F., Immobilization of enzymes and cells, *Humana press*, **1997**, 2-10.

96. Mateo C., Palomo J. M., Fernandez-lorente G., Guisan J. M., and Fernandez-Lafuente R., Improvement of enzyme activity, stability and selectivity via immobilization techniques, *Enzyme and Microbial Technology,* 2 May **2007,** 40 (6) ,1451-1463.

97. Yon J. M., Perahia D., and Ghélis C., Conformational dynamics and enzyme activity, *Biochimie ,* **1998**, 80, 33-42.

98. Cass T., and Ligler F. S., Immobilized Biomolecules in Analysis A Practical Approach, Oxford University Press, **1998**, 113-118, 126-127.

99. Kubal, B. S., Sankaran, K., and D'Souza, S. F., Immobilization of yeast by invertase adsorption on magnetite, *J. Microbiol. Biotechnol*., **1990**, 4, 98–102.

100. Raba J., and Mottola H. A., Glucose Oxidase as an Analytical Reagent, *Critical Reviews in Analytical Chemistry,* **1995**, 25 (1), 1–42.

101. Hicks G. P., and Updike S. J., The Preparation and Characterization of Lyophilized Polyacrylamide Enzyme Gels for Chemical Analysis, *Anal. Chem.,* **1966**, 38, 726–730.

102. Hornby W. E., Fillippusson H., and McDonald A., Preparation of glucose oxidase chemically attached to polystyrene and its use in the automated analysis of glucose, *FEBS Lett.*, **1970**, 9, 8–10.

103. Campbell J., Chawla A.S., and S.Chang T. M., The use of immobilized enzyme-membrane sandwich reactors in automated analysis, *Anal. Biochem*., **1977**, 83, 330–335.

104. Updike S. J., and Hicks G. P., Reagentless Substrate Analysis with Immobilized Enzymes, *Science,* **1967**, 158, 270–272.

105. Watson B., Stifel D. N., and Semersky F. E., Development of a glucose analyzer based on immobilized glucose oxidase, *.Anal. Chim. Acta*., **1979**, 106, 233–242.

106. Masoom M., and Townshend A., Determination of glucose in blood by flow injection analysis and an immobilized glucose oxidase column, *Anal. Chim. Acta.,* **1984**, 166, 111–118.

107. Masoom M., and Townshend A., Simultaneous determination of sucrose and glucose in mixtures by flow-injection analysis with immobilized enzymes, *Anal. Chim. Acta*., **1985**, 171, 185–194.

108. Bostick D. T., and Hercules D.M, Enzyme-induced chemiluminescence determination of blood glucose using luminol, *Anal. Lett.*, **1974**, 7, 347–353.

109. Bostick D.T., Hercules D. M., Quantitative determination of blood glucose using enzyme induced chemiluminescence of luminol, *Anal. Chem.,* **1975**, 47, 447–451.

110. Williams D. C., Huff G. F., and Seitz W .R., Glucose oxidase chemiluminescence measurement of glucose in urine compared with the hexokinase method, *Clin. Chem*, **1976**, 22, 372–374.

111. Nielsen J., Nikolajsen K., Benthin S., and Villadsen J., Application of flow-injection analysis in the on-line monitoring of sugars, lactic acid, protein and biomass during lactic acid fermentations, *Anal., Chim. Acta.*, **1990**, 237, 165–175.

112. Benthin S., Nielsen J., and Villadsen J., Characterization and application of precise and robust flow-injection analysers for on-line measurement during fermentations**,** *Anal. Chim. Acta.,* **1991**, 247, 45–50.

113. Benthin S., Nielsen J., and Villadsen J., Flow-injection analysis of micromolar concentrations of glucose and lactate in fermentation media, *Anal. Chim. Acta.,* **1992**, 261, 145–153.

114. Wu B., Zhang G., Shuang S., and Choi M. M. F., Biosensors for determination of glucose with glucose oxidase immobilized on an eggshell membrane, *Talanta,* **2004**, 64, 546-553.

115. Noureddini H., and Gao X., Characterization of sol-gel immobilized lipases, *J. Sol-Gel Sci. Techn.,* **2007**, 41, 31-41.

116. Lin J., and Brown C. W., Sol-gel glass as a matrix for chemical and biochemical sensing, *trends in analytical chemistry,* **1997**, 16 (4), 200-211.

117. Rao A.V., and Kalesh R.R., Comparative studies of the physical and hydrophobic properties of TEOS based silica aerogels using different co-precursors, *Science and Technology of Advanced Materials,* **2003**, 4, 509-515.

118. Klein L.C., Sol-Gel Processing of silicates, *Ann. Rev. Mater.Sci.,* **1985**, 15, 227-248.

119. Qingwen L., Guoan L., Yiming W., and Xingrong Z., Immobilization of glucose oxidase in sol-gel matrix and its application to fabricate chemiluminescent glucose sensor, *Materials Science and Engineering: C,* 130 June **2000**, 11 (1), 67-70.

120. Tilgner I. C., Fisher P., Bohnen F. M., Rehage H., and Maier W.F., Effect of acidic, basic and fluoride-catalysed sol-gel transitions on the preparation of sub-nanostructured silica, *Microporous Materials,* **1995**, 5, 77-90.

121. Sanchez C., and Ribot F., Design of hybrid organic-inorganic materials synthesized via sol-gel chemistry, *New Journal of Chemistry,* **1994**, 18 (10), 1010-1011.

122. Aegerter M.A., and Mennig M., Sol-gel technologies for glass producers and users, *Springer,* **2004**, 314.

123. Liang R., Jiang J., and Qiu J., An Amperometric Glucose Biosensor Based on Titania Sol-gel/Prussian Blue Composite Film, *ANALYTICAL SCIENCES,* November **2008**, 24, 1425-1430.

124. Lvov Y., and Mohwald H., Protein Architecture: Interfacing Molecular Assemblies and Immobilization Biotechnology, *CRC press*, **1999**, 99-124.

125. Pandey P. C., Upadhyay, S. , and Pathak H. C., A new glucose sensor based on encapsulated glucose oxidase within organically modified sol–gel glass, *Sens. Actuators B*, **1999**, 60, 83.

126. Walcarius A., Analytical Applications of Silica-Modified Electrodes -A Comprehensive Review, *Electroanalysis*, **1998**, 10 (18), 1217.

127. Wang B. Q., Li B., Wang Z. X., Xu G. B., Wang Q., and Dong S., Sol−Gel Thin-Film Immobilized Soybean Peroxidase Biosensor for the Amperometric Determination of Hydrogen Peroxide in Acid Medium, *J. Anal. Chem.,* **1999**, 71, 1935.

128. Iwouoha E. I., Kane S., Ania C. O., Smyth M. R., Ortiz de Montellano P. R., and Fuhr U., Reactivities of Organic Phase Biosensors 3: Electrochemical Study of Cytochrome $P450_{cam}$ Immobilized in a Methyltriethoxysilane Sol-Gel, *Electroanalysis* **2000**, 12, 980.

129. Fei L., and Perrett S., Effect of Nanoparticles on Protein Folding and Fibrillogenesis, *International Journal of Molecular Sciences* 10 **2009,** 10, 646-655.

130. Vertegel A. A., Siegel R. W., and Dordick J.S., Silica Nanoparticle Size Influences the Structure and Enzymatic Activity of Adsorbed Lysozyme, *Langmuir* **2004**, 20, 6800-6807.

131. Tabatabaei S., Shukohfar A., Aghababazadeh R., and Mirhabibi, A., Experimental study of the synthesis and characterisation of silica nanoparticles via the sol-gel method, *Journal of Physics: Conference Series,* **2006**, 26, 371-374.

132. Rao K.S., El-Hami K., Kodaki T., Matsushige K., and Makino K., A novel method for synthesis of silica nanoparticles, *Journal of Colloid and Interface Science,* **2005**, 289, 125-131.

133. Stober W., Fink A., and Bohn E., A novel method for synthesis of silica nanoparticles, *J. Colloid Interface Sci*., **1968**, 26, 62.

134. Anderson S. C., and Cockayne S., Clinical Chemistry: concepts and applications, *McGraw-Hill Professional*, **2003**, 172.

135. Witt S., Wohlfahrt G., Schomburg D., Hecht H., and Kalisz H., Conserved arginine-516 of *Penicillium amagasakiense* glucose oxidase is essential for the efficient binding of β-d-glucose, *J Biochem.,* **2000**, 347, 553–559.

136. Wilson C. L., Wilson D. W., Svehla G., Weber S. G., Barceló D., and Gorton L., Comprehensive analytical chemistry, *Elsevier*, **1959**, 133.

137. Trinder P., Determination of blood glucose using an oxidase-peroxidase system with a non-carcinogenic chromogen, *J.Clin.Path.***, 1969**, 22, 158-161.

138. Trinder P., Determination of blood glucose using 4-amino phenazone as oxygen acceptor, *J.Clin.Pathol.,* **1969**, 22, 246.

139. Yazawa N., Ono J., Yoshimura H., and Kimura S., Influence of nonspecific reaction on determination of H_2O_2 using Trinder reagents, *Clinica Chimica Acta,* **2005**, 360**,** 52-59.

140. Deeg R., and Ziegenhom J., Kinetic enzymatic Method for Automated Determination of Total Cholesterol in Serum, *CLIN. CHEM.,* **1983**, 29 (10), 1798-1802.

141. Reljić R., Ries M., Anić N., and Ries B., New Chromogen for Assay of Glucose in Serum, *CLIN. CHEM.,* **1992**, 38 (4), 522-525.

142. Upstone S. L., Ultraviolet/Visible Light Absorption Spectrophotometry in Clinical Chemistry, *Encyclopedia of Analytical Chemistry*, John Wiley &Sons, **2000**, 1699-1714.

143. Settle F., Handbook of Instrumental Techniques for analytical chemistry, *Prentice Hall*, **1997**, 485.

144. Hou X., Liu B., Deng X., Zhang B., Chen H., and Luo R., Covalent immobilization of glucose oxidase onto poly(styrene-co-glycidyl methacrylate) monodisperse fluorescent microspheres synthesized by dispersion polymerization, *Analytical biochemistry*,**2007**, 368 (1), 100.

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