

SUPERCRITICAL CARBON DIOXIDE IMMOBILIZATION OF GLUCOSE OXIDASE
ON CONDUCTING POLYMERS TOWARDS BIOSENSOR PRODUCTION

by

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ABSTRACT

SUPERCritical CARBON DIOXIDE IMMOBILIZATION OF GLUCOSE OXIDASE ON CONDUCTING POLYMERS TOWARDS BIOSENSOR PRODUCTION

In this work, glucose oxidase (GOX) is immobilized on conducting polymer matrices (polypyrrole film, PPY; polyurethane/polypyrrole composite foam and film, PU/PPY) via Supercritical Fluid Immobilization (SFI) for further use in the preparation of biosensors. Initially, the effects of supercritical carbon dioxide (scCO₂) pressure (80-150 bar, 40 °C) and temperature (30-60 °C) on free-enzyme buffer-solution activity are investigated for 24 h scCO₂ exposure time. Results show that enzymatic activity of GOX after exposure to scCO₂ is retained. The enzymatic activity of GOX-buffer solution does not show a decrease between 30-50 °C, whereas 30 per cent and 94 per cent activity losses are noticed at 55 and 60 °C temperatures. In order to provide an insight into the relation between the scCO₂ and the GOX-buffer solution, the GOX-buffer solutions were subjected to scCO₂ and the protein amounts of the GOX-buffer solutions before and after scCO₂ exposure were determined at 80, 100 and 150 bar and 30, 40 and 50 °C for an exposure time of 24 h. Results showed that the protein amount in GOX-buffer solution decreased in all cases after exposure to scCO₂. The decreases in GOX amount are 18, 14.3 and 49.5 per cent compared to the original value at 100 bar, 30, 40 and 50 °C, respectively. The GOX immobilized polymer matrices are analyzed via Environmental Scanning Electron Microscopy (ESEM) and enzymatic activity methods. To determine the immobilization medium to be used on PPY films, four different immobilization mediums were tried. These were: Dry GOX and dry PPY (Dry); dry GOX and dry PPY with water vapor (Semi-Dry); GOX and PPY in water (Wet); GOX and PPY in buffer (Wet-b). In “Dry” and “Semi-Dry” procedures (up to 150 bar, 40 °C and 72 h), the PPY films showed no noticeable activity, whereas films processed in “Wet” and “Wet-b” methods showed enzymatic activity. In “Wet” method (150 bar and 40 °C), as the immobilization time increased (5-72 h) the activity also increased. The maximum activity is observed for GOX immobilized PPY film in the “Wet-b” medium. Thus, during GOX immobilization of PU/PPY, buffer solution is used as the

immobilization medium. The effects of immobilization time (4, 24, 72 h), immobilization solution concentration (6.25, 0.625 and 0.0625 mg/ml), washing procedures of samples prior to and after the immobilization process, temperature (30, 40 and 50 °C) and pressure (80, 100, and 150 bar) on the activity of PU/PPY samples are determined. The immobilized GOX amounts on the polymer samples are also measured. Decreasing the initial concentration of GOX solution two orders of magnitude enhances the specific activity of the immobilized on PU/PPY composite GOX by 25 fold. As the washing steps get longer, the activity of the sample increases. Washing the sample in scCO₂ before immobilization also increases the activity compared to non-CO₂ washing procedure. Increasing immobilization time from 4 to 24 h leads to the activity enhancement by 71.8 per cent, and an increase from 24 h to 72 h caused 16.4 per cent decrease in activity. The immobilized amount of GOX on PU/PPY composite increased as the temperature increased at all pressures and decreased as the pressure increased at all temperatures. The specific activity value (U/mg) increased as the pressure increased from 80 to 100 bar, however further increase to 150 bar did not cause any increase in specific activity. At constant pressure, as the temperature increased, the specific activity decreased. The highest activity and highest specific activity for the samples processed at supercritical conditions were obtained at 100 bar, 30 °C as 0.040 U/cm² and 4.9 U/mg, respectively. When immobilization was performed via SFI, the activity values were doubled compared to the immobilization at atmospheric conditions. This might mean using less enzyme to get same activity, thus cheaper biosensor of equal performance.

ÖZET

BİYOSENSÖR ÜRETİMİNDE KULLANILMAK ÜZERE KRİTİKÖTESİ KARBON DİOKSİT İLE İLETKEN POLİMERLER ÜZERİNE GLİKOZ OKSİDAZ TUTUKLANMASI

Bu çalışmada, biyosensör üretiminde kullanılmak amacıyla, Kritikötesi Akışkan Tutuklanması (SFI) ile iletken polimer matrisler üzerine glikoz oksidaz (GOX) tutuklanmıştır. Kritikötesi karbon dioksit ($scCO_2$) basıncının (80-150 bar, 40 °C) ve sıcaklığın (30-60 °C) enzim-tampon çözeltisi üzerindeki etkilerine bakılmış ve 24 saat $scCO_2$ 'te tutulmuş enzim-tampon çözeltisinin aktivitesini koruduğunu görülmüştür. Yirmidört saat boyunca 30-50 °C sıcaklıkta tutulmuş enzim-tampon çözeltilerinin aktivitelerinde azalma görülmezken, 55 ve 60 °C sıcaklıklarda tutulmuş enzim-tampon çözeltileri aktivitelerinin sırasıyla yüzde 30 ve 94 azaldığı gözlemlenmiştir. Enzim-tampon çözeltisi ve $scCO_2$ arasındaki ilişkiye açıklık getirebilmek için, enzim-tampon çözeltisindeki protein miktarı, çözelti 80, 100 ve 150 bar basınçta ve 30, 40 ve 50 °C sıcaklıkta, 24 saat $scCO_2$ 'te tutulmadan önce ve sonra ölçülmüştür. Sonuçlar her koşulda enzim-tampon çözeltisindeki protein miktarının $scCO_2$ 'te tutulduktan sonra azaldığını göstermiştir. $ScCO_2$ ortamında 100 bar basınçta, 30, 40 ve 50 °C'de tutulduktan sonra GOX-tampon çözeltisindeki GOX miktarı orjinal çözeltiye göre sırasıyla yüzde 18, 14.3 ve 49.5 azalmıştır. Üzerine GOX tutuklanmış polimer matrisler Çevresel Taramalı Elektron Mikroskobu (ESEM) ve enzimatik aktivite tayin yöntemleri kullanılarak analiz edilmiştir. Tutuklama deneylerinde önce elektrik geçirgen bir polimer olan polipirrol (PPY) filmlere GOX tutuklanmasında dört farklı tutuklama ortamı denenmiştir: Kuru GOX ve kuru PPY (Dry), su buharı ile birlikte kuru GOX ve kuru PPY (Semi-Dry), GOX ve PPY suyun içinde (Wet), GOX ve PPY tampon içinde (Wet-b). Tutuklama işlemi “Dry” ve “Semi-Dry” ortamlarda (150 bar, 40 °C ve 72 saate kadar) yapıldığı zaman GOX tutuklanmış PPY filmlerde aktivite gözlenmezken, “Wet” ve “Wet-b” ortamlarında GOX tutuklanmış PPY filmler enzimatik aktivite göstermişlerdir. “Wet” ortamda (150 bar, 40 °C) tutuklama süresi arttırıldıkça (5-72 saat) aktivitenin de arttığı görülmüştür. En yüksek aktivite “Wet-b” ortamda elde edilmiştir. Bu nedenle Poliüretan/Polipirrol (PU/PPY) matrise GOX

tutuklanırken, tutuklama ortamı olarak tampon çözeltisi kullanılmıştır. Tutuklama zamanının (4, 24 ve 72 saat), tutuklama için kullanılan çözeltinin konsantrasyonunun (6.25, 0.625 ve 0.0625 mg/ml), tutuklama öncesi ve sonrasında polimer örneklerin yıkanmasının, sıcaklığın (30, 40 ve 50 °C) ve basıncın (80, 100 ve 150 bar) GOX tutuklanan PU/PPY örneklerin aktivitesi üzerindeki etkilerine bakılmıştır. Polimer örnekler üzerine tutuklanan GOX miktarı da ölçülmüştür. Tutuklama işleminde kullanılan GOX-tampon çözeltisinin konsantrasyonun yüz kat azaltılması, GOX tutuklanmış PU/PPY örneğinin özgül aktivitesini 25 kat arttırmıştır. Tutuklama öncesi ve sonrası yıkama sürelerinin uzaması, polimer örneklerin aktivitelerinin artmasını sağlamıştır. Tutuklama öncesi scCO₂ ile yıkanan polimer örneklerin aktivitelerinin scCO₂ ile yıkanmayanlara göre daha yüksek olduğu gözlemlenmiştir. PU/PPY kompozit matrislerinde tutuklama süresinin 4 saatten 24 saate yükseltilmesi aktivitenin yüzde 71.8 artmasını sağlarken, tutuklama süresinin 24 saatten 72 saate çıkarılması aktiviteyi olumsuz etkileyerek yüzde 16.4 azalmasına sebep olmuştur. Bütün basınç değerlerinde PU/PPY matris üzerine tutuklanan GOX miktarı, sıcaklığın artırılmasıyla artmış, bütün sıcaklık değerlerinde ise basıncın artmasıyla azalmıştır. Özgül aktivite değerleri (U/mg) basıncın 80 bardan 100 bara yükselmesiyle artmış fakat basıncın 100 bardan 150 bara yükseltilmesi özgül aktivite değerlerinde herhangi bir artışa sebep olmamıştır. Sabit basınçta, sıcaklık arttıkça özgül aktivite değerleri düşmüştür. Kritikötesi ortamda yapılan tutuklama işlemlerinde en yüksek aktivite ve özgül aktivite 100 bar ve 30 °C’de sırasıyla 0.040 U/cm² ve 4.9 U/mg olarak ölçülmüştür. Tutuklama işlemi kritikötesi ortamda yapıldığı zaman elde edilen aktivite ve özgül aktivite değerleri, tutuklama atmosfer koşullarında CO₂’siz yapıldığında elde edilen aktivite ve özgül aktivite değerlerinin yaklaşık iki katıdır. Özgül aktivite değerlerinin yüksek olması, SFI işlemi ile daha az enzim kullanılarak atmosfer koşullarında yapılan tutuklama işlemi ile daha yüksek aktivite sağlandığını göstermekte, bu da üretilecek biyosensörün daha az enzimle daha ucuza üretilme olanağı olabileceğini göstermektedir.

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1. INTRODUCTION

A biosensor combines a biological recognition component and a transducer in one single device, which enables the measurement of target analytes without using reagents. Biosensors owe their specialty to their specificity, speed, simplicity in analysis, and continuous monitoring capability. In biosensor production, the immobilization method used for the biological recognition components, i.e. the enzyme, and the immobilization matrix of the biosensor affect the biosensor performance significantly. The main immobilization methods used for biosensors are adsorption (Yavuz *et al.*, 2002, Ding *et al.*, 2005), covalent bonding (Bautista *et al.*, 2001, Yang *et al.*, 2003), entrapment (Retama *et al.*, 2003, Lin *et al.*, 2007) and cross-linking (Zhou and Chen, 2001, Delvaux and Demoustier-Champagne, 2003).

Various matrices such as polymeric films, membranes, gels, carbon graphite, silica etc. have been used for the immobilization of enzymes in the preparation of biosensors. Beside the conventional immobilization matrices, conducting polymers have also found increasing interest as suitable matrices for the immobilization of enzymes in biosensors (Foulds and Lowe, 1988, Selampinar *et al.*, 1997, Cosnier, 1999, Chaubey *et al.*, 2000a, Yıldız *et al.*, 2005). Biological recognition components usually immobilized to the conducting polymer via entrapment during its electrochemical synthesis (Foulds and Lowe, 1988, Yıldız *et al.*, 2005). However, this is a one step procedure and conducting polymer synthesis and immobilization conditions cannot be separately optimized. Additionally, denaturation of the enzyme may be possible during the simultaneous polymerization and the immobilization processes. Besides one step immobilization (entrapment during electrochemical synthesis), two step procedures (first polymerization then immobilization by one of the main immobilization methods) such as adsorption (Sharma *et al.*, 2003), crosslinking (Chaubey *et al.*, 2000b, Gade *et al.*, 2006) and covalent bonding (Schuhmann *et al.*, 1990, Rahman *et al.*, 2004) are also used for immobilization of enzymes on conducting polymers. Various studies were conducted for immobilization of enzymes on conducting polymers. The common aim of these studies is to develop an immobilization technique, which combines the retention of high enzyme activity and long-term stability, as well as the ease in fabrication and miniaturization of the biosensors. Every immobilization

method has its own advantages and disadvantages; therefore a single immobilization method is not universally accepted. Investigations on new techniques and new materials are the subjects of various studies (Cosnier *et al.*, 1999, Liu *et al.*, 2004, Luo *et al.*, 2005, Ramanavicius *et al.*, 2006).

In this work, as an attempt to develop a new technique, immobilization of a biological component on a conducting polymer is studied in supercritical carbon dioxide (scCO₂) environment for further use in biosensor production. The high diffusivity, low surface tension, sterilization effect, the swelling and modification ability of scCO₂ are utilized to obtain stable immobilizations with low enzyme consumption.

There are some studies utilizing supercritical fluid technologies for incorporation of biological materials into a polymer matrix (Kazarian and Martirosyan, 2002, Sproule *et al.*, 2004) or microencapsulation of biological materials (Elvassore *et al.*, 2001, Dos Santos *et al.*, 2002, Caliceti *et al.*, 2004) by supercritical fluid technologies. In those studies, mostly protein particles are used as the biological material and mostly biodegradable polymers are used as the polymer matrix. These biological- material/polymer systems are studied for the purpose of developing new techniques of preparing drug delivery systems. We have not yet encountered an open literature source on biosensor production using supercritical fluid technologies.

The glucose biosensors have the largest commercial application, thus the glucose biosensors are the most extensively studied biosensor type in literature; hence, GOX is the most extensively studied enzyme. In addition to its metabolic and medical importance, it makes a good standard compound on which to try out possible new biosensor techniques. Among the conducting polymers, polypyrrole (PPY) has been investigated the most. Therefore, in this dissertation the investigation was started with the GOX and PPY system.

This study must be evaluated as an exploratory study on supercritical fluid immobilization (SFI) process since there was no similar study and data in open literature. As the studies proceeded, the SFI procedure evolved, and the evolved procedure was used for the next step. The studies started with PPY film, and as the studies progressed it was seen that by electropolymerization (at constant potential in our case) it was nearly

impossible to obtain homogeneous and reproducible polymer surfaces, thus the enzyme immobilization process over the polymer surface was not also homogeneous. Furthermore PPY films were brittle and cracked after the process. However, studies on PPY gave important information for the evolution of the rest of the studies, but it became evident that the studies must be continued with more flexible and homogeneous conducting polymers. Therefore, for the rest of the studies PU/PPY composite foams and films were synthesized to continue the investigation. For each polymer matrix, the studies were divided into two parts; first preliminary experiments that gave information on things to do for sample preparation, and the conditions of analytical procedures before deciding on the experimental procedure.

The background which initiated the idea for the subject of this dissertation is given in Chapter 2. Chapter 2 introduces the biosensor types and the conventional immobilization techniques. Supercritical fluid impregnation technology which is the base of Supercritical Fluid Immobilization (SFI) is also explained in that chapter. Chapter 3 presents the detailed description of the experimental set-up, materials used, the procedures applied, the detailed explanation of the analytical procedures and the experimental program. The experimental results are given and discussed in Chapters 4, 5 and 6. The effects of temperature and pressure on GOX-buffer solution were investigated in order to better understand the behavior of the system studied, before starting the immobilization process under scCO_2 . These results are presented and discussed in Chapter 4. Chapter 4 also includes the preliminary impregnation studies of GOX into non-conducting polymer matrix, i.e. polymethylmethacrylate. The results of application of SFI on PPY films were given in Chapter 5. The main contribution of the experiments done on PPY films on the whole study was the determination of the immobilization medium. Chapter 6 presents the results of application of SFI on PU/PPY composite foams and films. Mainly, the effects of immobilization pressure, temperature and time on the SFI process, and the long term stability of the immobilized enzyme are presented in Chapter 6, as well as the effects of various procedures in sample handling and preparation. Finally, concluding remarks with possible new research directions are provided in Chapter 7. In Appendix A, the properties of GOX are presented. Absorbance values obtained as results of enzymatic reactions and used for activity calculation of immobilization experiments are shown in Appendix B. The calibration charts used during the calculations of activity values, protein amount and

equivalent enzyme amounts are given in Appendix C, and also the methods and calculations followed for the determination of activity values and protein amounts are also explained.

2. BACKGROUND

Biosensors provide measuring the amount of a target analyte without using any reagents. In this section, initially the basic operational principles of biosensor are given. The difference of a biosensor from a sensor is that in biosensor a biological element is used for sensing. The biological element has to be immobilized on the sensor, and the immobilization methods used are also introduced along with some examples from literature. Then, the evolution of the biosensors and the importance of using conducting polymers in biosensor production are introduced. Finally, the principle of Supercritical Solution Impregnation (SSI) process and its application to drugs and proteins are given.

2.1. Operational Principles of Biosensors

A biosensor incorporates a biological sensing element which is connected to a transducer. The general arrangement of a biosensor can be seen in Figure 2.1. The biological sensing element on the surface of a transducer recognizes a specific chemical or biological analyte in a solution or in atmosphere. Cofactors, antibodies, enzymes, enzyme systems, receptors, organelles, cell tissues and organisms can be used as receptors in biosensors. A transducer converts the chemical or electrical response (physico-chemical change), occurring as a result of the analyte-receptor interaction, into a measurable output signal. Based on the transducer used, biosensors are divided into electrochemical, optical, piezoelectric and calorimetric biosensors. In this section, types of transducers will be briefly explained.

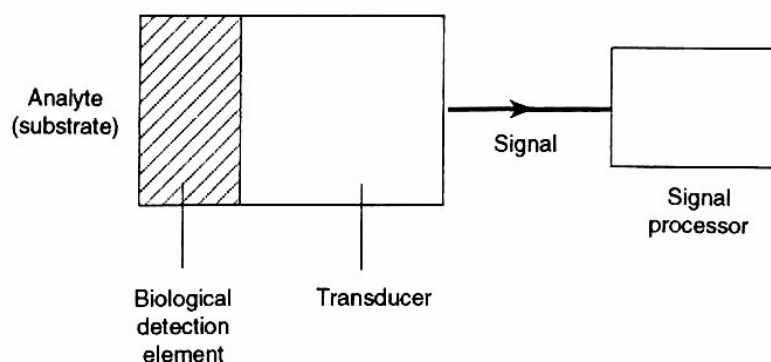


Figure 2.1. Schematic layout of a biosensor

There are some important performance factors for biosensors. A biosensor must have:

- a very small sensitivity range (needs to be sub-millimolar).
- a low detection limit.
- reproducible results, and
- must be highly selective,

It is also important to know the analyte concentration range covered by the biosensor, e.g: a glucose biosensor must have sensing ability at least in a range of 0.2-20 mM to cover the blood glucose levels encountered in normal and diabetic patients (Eggins, 1999). Additionally, the response time, the recovery time and the life time of a biosensor are important performance factors. The enzyme amount on the biosensor and immobilization method of the enzyme also affect the biosensor performance. The immobilization methods used in biosensor production will be discussed briefly in the following sections.

The transducer can take many forms depending upon the parameters being measured: electrochemical, optical, mass and thermal changes are the most common. The signal produced by a transducer after a change is measured may or may not need to be converted to an electrical signal, depending on the type and application of the biosensor. In the following subsections biosensors with electrochemical, optical, piezoelectric and calorimetric transducers will be introduced.

2.1.1. Electrochemical Biosensors

The name electrochemical biosensor is applied to a molecular sensing device which intimately couples a biological recognition element to an electrode transducer. The purpose of the transducer is to convert the biological recognition event into a useful electrical signal. The most common electrochemical transducers being utilized are amperometric (also called voltammetric) and potentiometric. Conductimetric transducers are also useful for biosensors which utilize the electrochemical processes.

An amperometric biosensor measures the current produced during the oxidation or reduction of a product or reactant, usually at a constant applied potential. Amperometric biosensors have fast response times and good sensitivity. However, the excellent specificity of the biological component can be compromised by the partial selectivity of the electrode. This lack of specificity sometimes requires sample preparation, separation or some compensation for interfacing signals (Gerard et. al, 2002). The most important factor affecting the functioning of amperometric biosensors is the electron transfer between catalytic molecule and the electrode surface.

A potentiometric biosensor measures the potential of a cell produced as a result of a redox reaction at zero current. Potentiometric biosensors relate electrical potentials to the concentration of analyte by using an ion-selective electrode or a gas-sensing electrode as the physical transducer. These are selective, have large dynamic ranges and are non-destructive. The most common example for potentiometric biosensor which uses ion-selective electrode is the pH meter. An illustration of a pH electrode is given in Figure 2.2.

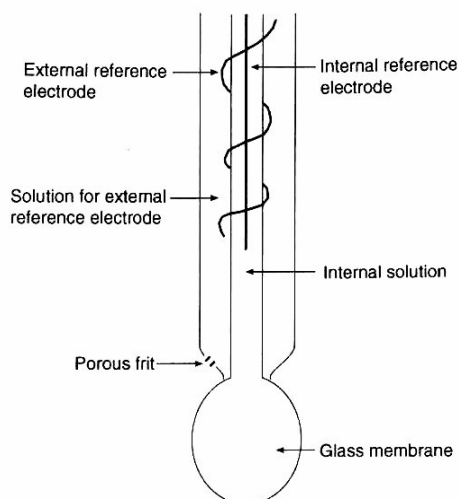


Figure 2.2. Schematic representation of the pH electrode (Eggins, 1999)

Conductimetric biosensors measure the change in conductivity, as a result of a chemical or biochemical reaction, arising between a pair of metal electrodes. A change in conductance can be used to follow any reaction that produces a change in the number of ions, the charge on ions, the dissociation of ions or the mobility of ions (Eggins, 1999).

Field effect transistors (FETs) are devices in which a transistor amplifier is adapted to a miniature transducer for the detection and measurement of potentiometric signals, produced by a potentiometric sensor process on the gate of the FET. The gate is parallel to the body of the FET, which forms a parallel plate capacitor with an oxide dielectric. The voltage on this capacitor defines the behavior of the FET device. Compared to other electrochemical transducers, circuit wiring is minimized which lead to miniaturization and decrease in electronic noise. The measured value can be the current at constant gate voltage or can be the changes in gate voltage at constant current.

2.1.2. Optical Biosensors

Optical biosensors are based on the measurement of the light absorbed or emitted as a consequence of a biochemical reaction. In such biosensors, light waves are guided by means of optical fibers to suitable detectors. These type of biosensors have been used for the detection of pH, O₂ and CO₂ etc. The main types of photometric behavior which have been exploited in biosensors are as follows: a) Ultraviolet-visible absorption, b) Fluorescence (and phosphorescence) emission, c) Bioluminescence, d) Chemiluminescence, e) Internal reflection spectroscopy (IRS) and finally f) laser light scattering methods.

2.1.3. Calorimetric biosensors

The basic principle of such biosensors is that all biochemical reactions involve a change in enthalpy. Such a change in enthalpy is detected by calorimetric biosensors. The technique requires a very sensitive electrical resistance thermometer, which will detect the temperature changes down to 0.001 °C.

2.1.4. Piezoelectric biosensors

These biosensors operate on the principle of generation of electric dipoles on subjecting an anisotropic natural crystal to mechanical stress. Due to the adsorption of an analyte, the mass of the crystal is increased resulting in altered frequency of oscillation. Such type of biosensor types have been utilized for the measurement of ammonia, hydrogen, methane, carbon monoxide, nitrous oxide and other organophosphorous compounds.

All transducer types have some drawbacks. For example, the optical biosensors, though very sensitive, however, cannot be used in turbid media. Thermal biosensors cannot be utilized with systems with very little heat change, moreover, they are not easy to handle. Electrochemical biosensors have been the most commonly used biosensors. They have been found to overcome most of the disadvantages, which inhibit the use of other types of biosensors. These biosensors are rapid, easy to handle and are of low cost.

2.2. Immobilization Techniques

Most of the biological components such as enzymes, antibodies, cells etc. have very short life times in the solution phase. Thus, in order to make a viable biosensor, the biological component has to be properly immobilized to the transducer directly or in a suitable matrix. There are five conventional enzyme immobilization methods: adsorption, microencapsulation, entrapment, cross-linking and covalent bonding and they will be briefly explained in the following sub-sections.

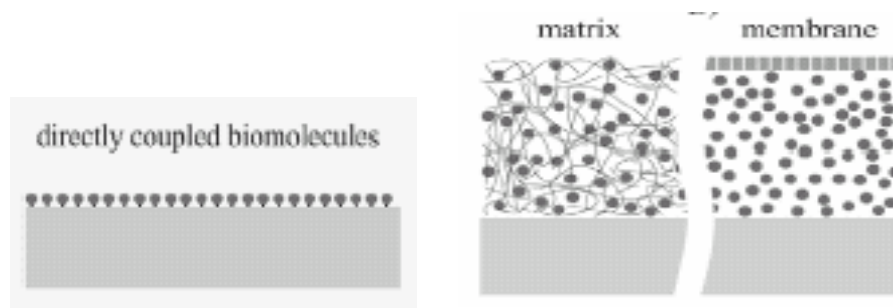


Figure 2.3. Schematic representation of basic immobilization principles of biological recognition component onto a transducer (Trau and Renneberg, 2003)

2.2.1. Adsorption

Physical adsorption based on van der Waals attractive forces is the oldest and simplest method and involves minimal preparation. In adsorption, a solution of enzyme, a suspension of cells or a slice of tissue is immobilized on an analyte permeable membrane covering the transducer. The adsorption method does not require chemical modification of the biological components and it is possible to regenerate the matrix membrane. The advantage of this method is the simplicity; however, loss of adsorbed enzymes is possible

if changes in pH, ionic strength or temperature occur during measurements (Wu *et al.*, 2004).

Kamyshny *et al.* (1999) investigated how the hydrophobization of GOX by palmitic chains influences its adsorption onto hydrophobic surfaces such as polystyrene and phosphatidyl choline and cholesterol-covered silica beads. The results of their study demonstrated the successful immobilization of GOX through adsorption and reported that modified GOX displayed a much higher adsorption affinity.

Yavuz *et al.* (2002) worked on the preparation of Congo Red (CR) attached and Fe^{+3} incorporated small sized and non-porous poly (HEMA-co-MMA) support in the microspheres form for the immobilization of a model enzyme, i.e. GOX, using adsorption. They claimed that, these support systems could provide a large specific surface area and therefore, the enzyme could be immobilized only on the surface, and would also provide less of a diffusion limitation problem than that of the porous supports. They characterized the resultant immobilized GOX systems and compared their activity retention, immobilization efficiency, catalytic properties, reusability and stability aspects. The authors (Yavuz *et al.*, 2002) concluded that, GOX was adsorbed with high affinity interaction on microspheres and added that, poly (HEMA-co-MMA) microspheres modified by the attachment of Congo Red (CR) and Fe^{+3} ions revealed good adsorption properties. The results indicated that non-porous poly (HEMA-co-MMA) microspheres could be used as support material in enzyme immobilization.

Urease was immobilized onto Procion Brown MX-5BR attached and Ni(II) incorporated microporous polyamide hollow-fibre membrane via adsorption in the study of Akgöl *et al.* (2002), and the adsorption conditions (initial urease concentration, pH of the medium and temperature) were investigated. They concluded that only 8 per cent of the initial activity was lost was through adsorption and added that a hollow fibre membrane could be used for biotechnological processes or biomedical purposes especially in artificial kidney devices for the removal of urea from blood.

Ding *et al.* (2005) introduced silica nanotubes as enzyme immobilization carriers and analyzed the properties of the immobilized lysozyme molecules. With the hollow structure,

silica nanotubes were proven to be efficient for lysozyme immobilization in the adsorption experiment. Lysozyme was supported to form monolayer adsorption on silica nanotubes which evolved into multi layer adsorption with an increasing lysozyme concentration.

2.2.2. Microencapsulation

Microencapsulation is the method used in the early biosensors. The biomaterial is held in place behind a membrane, giving close contact between the biomaterial and the transducer. Microencapsulation limits contamination and biodegradation. It is stable towards changes in temperature, pH, ionic strength and chemical composition. The biosensors produced by microencapsulation type immobilization can be permeable to some materials, e.g. small molecules, gas molecules and electrons.

Park and Chang (2000) studied microencapsulation of microbial cells. Their study indicated the advantages of the microencapsulation method over the other immobilization techniques by showing that microencapsulation satisfies the requirements of industrial applications of biotechnology which are immobilization in high density with low mass transfer resistance and stability.

A comparative study of different techniques (adsorption, entrapment and microencapsulation) for the immobilization of urease enzyme in tetramethylorthosilicate (TMOS) derived sol-gel matrix on the sensing surface of glass pH electrode was done by Sahney *et al.* (2006). The comparison indicated that microencapsulation technique with urease immobilization on the sensing surface of the glass pH electrode was better in terms of the more homogeneous enzymatic layer. The advantages of microencapsulated biosensor over physically entrapped sandwich configuration biosensor are higher sensitivity, lower detection limit, and larger linear range of urea determination. The authors (Sahney *et al.*, 2006) claimed that the entrapment technique offered faster response times but smaller life-times than microencapsulation of enzyme electrodes and showed that immobilization of urease onto TMOS films could not be performed using the simple physical adsorption method. They concluded that the advantage of microencapsulated biosensor over entrapped biosensor were of higher sensitivity, lower detection limit, larger linear range of urea determination and reasonably long stability. They also added that

microencapsulated enzyme electrode system gave more satisfactory results with the blood serum sample.

Trau and Renneberg (2003) report on an immobilization strategy utilizing layer-by-layer encapsulated microparticles of enzymes within a nanoscale polyelectrolyte film. Encapsulation of glucose oxidase (GOX) microparticles was achieved by the sequential adsorption of oppositely charged polyelectrolytes onto GOX biocrystal/microparticle surface. The polyelectrolyte system of polyallylamine/polystyrene sulfonate was used under high salt conditions to preserve the solid state of the highly water soluble GOX biocrystals during the encapsulation process. The resulting polymer multilayer capsule of about 15 nm wall thickness is permeable for small molecules (glucose), but non-permeable for macromolecules thus preventing the enzyme from leakage and at the same time shielding it from the outer environment e.g., from protease or microbial activity. Encapsulated GOX was subsequently used to construct a biosensor by nano-engineered immobilization of the capsules onto an electrode surface. The approach presented demonstrates a general method to encapsulate highly soluble solid biomaterials and an immobilization strategy with the potential to create highly active thin and stable films of biomaterial. A scheme of the layer-by-layer process for the encapsulation of microparticulate biomaterials (e.g. microcrystals of catalase or GOX) is given in Figure 2.4. Deposition of polyelectrolyte stops after the system reaches the state of the lowest surface energy and biggest entropy gain.

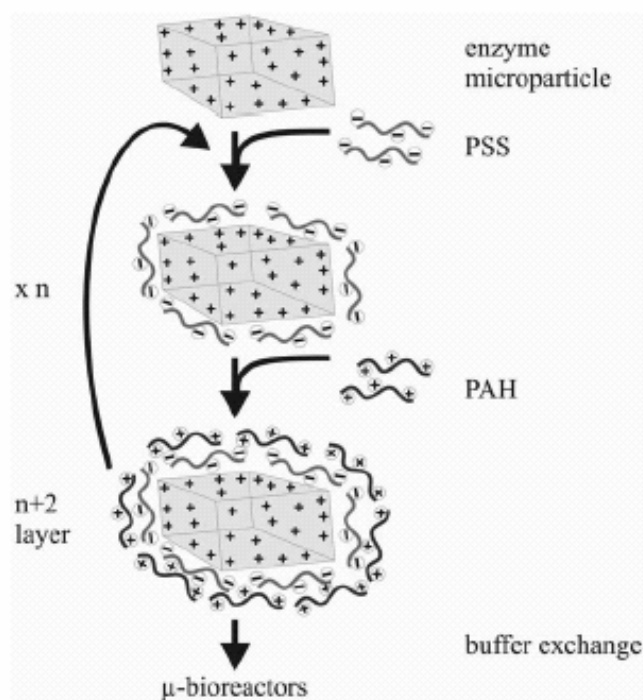


Figure 2.4. Schematic representation of layer-by-layer coating to encapsulate an enzyme (Trau and Renneberg, 2003)

2.2.3. Entrapment

In entrapment, the biomaterial is mixed with a chosen monomer solution, which is then polymerized to a gel, trapping the biomaterial. However, this can cause barriers to the diffusion of the substrate, thus slowing the reaction. It can also result in bioactivity loss through gel pores. On the other hand, by entrapment, it is possible to control the release of active ingredients, sustain the effectiveness of ingredients, protect reactive components until the time of use, and allow the safe handling of toxic materials (Ji *et al.*, 2005). When immobilization of an enzyme is done into a conducting polymer during its electrochemical polymerization, it is also called entrapment.

Retama *et al.* (2003) studied the immobilization of GOX onto cross-linked polyacrylamide (PAA) microgels through entrapment. Singh *et al.* (2004) worked on the technical development and optimization of an amperometric biosensor observed by immobilizing cholesterol esterase and cholesterol oxidase in polypyrrole films via the entrapment technique. The results of the study showed that the proposed biosensor could operate with high sensitivity. Lin *et al.* (2007) entrapped horseradish peroxidase (HRP)

into a biocompatible silica matrix via a one-pot process using protein friendly silica precursors and sugarsilane modifiers. The results showed that, the activity of HRP was enhanced in the presence of covalently coupled sugars to the protein where the specific activity was close to that obtained for aqueous HRP. Also, sensors prepared using HRP entrapped in materials were shown to be highly responsive to the concentration of the added peroxidase.

2.2.4. Cross-linking

In cross-linking, the biomaterial is chemically bonded to solid supports, or to another supporting material such as a gel. Bifunctional reagents such as glutaraldehyde are used as a cross-linking agent. In this method, there is some diffusion limitation and biomaterial can be damaged. Also, the mechanical strength is poor. However, it is a useful method to stabilize adsorbed biomaterials.

Zhou and Chen, (2001) studied the immobilization of β -galactosidase through cross-linking on graphite surface by glutaraldehyde. They showed that efficient enzyme immobilization was achieved by cross-linking and the specific activity of the immobilized enzyme was higher than that reported previously by other researchers between 17 per cent and 25 per cent, with respect to the free enzyme. The immobilized enzymes were stable and active in operational conditions, with a considerable lactose conversion. Also, they claimed that their work showed the potential of cross-linking in industrial applications. Delvaux and Champagne (2003) investigated the possibility of fabricating a highly sensitive amperometric biosensor through the immobilization of GOX by cross-linking within tubular nanostructures. The results of their study revealed that GOX can effectively be immobilized within nanotubes using coupling agents and the resulting nanotube-based biosensors showed remarkable sensitivity to glucose.

2.2.5. Covalent Bonding

Covalent bonding involves a carefully designed bond between a functional group in the biomaterial to the support matrix. Nucleophilic groups in the amino acids of the biomaterials, which are not essential for the catalytic action of an enzyme are suitable for

covalent bonding. Covalent immobilization on the external surface of a support material has also been used to decrease mass transfer limitations.

Bautista *et al.* (2001) studied the kinetic properties of GOX when immobilized on amorphous AlPO_4 support. The results revealed that not only little deactivation of enzyme along immobilization process was obtained, but also that the stability of the covalently bonded enzyme studied appeared to have increased over the soluble native enzyme. In the study of Vasileva and Godjevargova, (2004) accessible polyamide (PA-6) was used as the basic material for the preparation of membranes with bonded enzymes. They successfully immobilized GOX onto PA-6 membranes through covalent bonding after preliminary modification with hydrochloric acid and 1,2 diaminoethane (DAE) and investigated the behavior of the enzyme. A comparison of immobilization on different membranes were done. The highest relative activity was measured for GOX immobilized onto membrane modified with six weight per cent HCl and 10 weight per cent DAE.

Overall, the lifetime of the biosensor is greatly enhanced by proper immobilization. Typical lifetimes for the same biosensor, in which different methods of immobilization are used can be very different. For the same biosensor, the life time is one day with adsorption type immobilization, one week with the membrane entrapment, three to four weeks with physical entrapment, whereas it can be between four to fourteen months with covalent entrapment.

In summary, chemical methods result in longer lifetimes, but can limit the response, by blocking the mass transport processes, and vice versa for physical methods. The chemical methods may sometimes damage the enzyme and physical methods (entrapment or adsorption) lead to more rapid loss of enzyme because of weak bonds. In Table 2.1, the advantages and disadvantages of these techniques are summarized.

From the literature (covering the time beginning with the development of biosensors up to now) it is understood that there is not a unique technique, which combines the retention of the high enzyme activity and stability with the simplicity of fabrication and miniaturization of the biosensor. Studies are going on to investigate new techniques and new materials (Nguyen *et al.*, 2003; Trau and Renneberg, 2003; Wang *et al.*, 2003; Li *et*

al., 2004, Barton *et al.*, 2004). Therefore as an attempt to develop a new technique, the supercritical fluid technologies is utilized for the immobilization of GOX on a conducting polymer matrix for further use in biosensors.

Table 2.1. Advantages and disadvantages of conventional immobilization techniques
(Gerard *et al.*, 2002)

Method	Advantages	Disadvantages
Physical adsorption	• No modification of biocatalyst. • Matrix can be regenerated. • Low cost	• Binding forces are susceptible to change in pH, temperature and ionic strength
Entrapment	• Only physical confinement of biocatalyst near transducer. • Low cost	• High diffusion barrier
Cross-linking	• Loss of biocatalyst is minimum. • Moderate cost	• Harsh treatment of biocatalyst by toxic chemicals
Covalent bonding	• Low diffusional resistance • Stable under adverse conditions	• Harsh treatment by toxic chemicals. • Matrix not regenerable

2.3. Glucose Biosensors

Typical application areas of biosensors include environmental monitoring and control, and chemical measurements in the agriculture, food and health industries. Health care is the main area of application. Measurements of blood gases, ions and metabolites are often necessary to show the patient's metabolic state, especially for patients in hospitals. Some commonly required instant assays in diagnosis of patients are given in Table 2.2. Many of these substrates have been determined by samples of urine and blood being taken away to medical analytical laboratory for classical analysis, which may not be complete for hours or even days. The use of biosensors enables analytical results to be obtained within minutes.

Table 2.2. Commonly required instant assays in diagnosis of patients (Eggins, 1999)

Ions	Gases	Drugs	Substrates	Enzymes
Sodium	Oxygen	Paracetamol	Glucose	Creatine kinase
Potassium	Ammonia	Salicylate	Cholesterol	Amylase
Calcium	Carbon dioxide		Creatinine	Aspartate
pH				Aminotransferase

Of these assays mentioned above, glucose is the most popular one to be studied. Glucose biosensor is the most extensively studied biosensor in the literature, hence glucose oxidase (GOX) is the most extensively studied enzyme. It also has the greatest commercial application among biosensors. In addition to its metabolic and medical importance, GOX makes a good standard compound on which to try out possible new biosensor techniques. There are in fact a number of different ways of determining glucose just using electrochemical transducers.

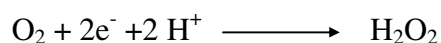
Sometimes, biosensors are classified as first, second and third generation biosensors. First generation biosensors are oxygen electrode-based sensors, the second generation biosensors are mediator-based sensors and the third generation biosensors are directly coupled enzyme-electrodes. Three generations of biosensors will be explained on the basis of the glucose biosensor.

2.3.1. First Generation Glucose Biosensors

The original glucose enzyme electrode used molecular oxygen as the oxidizing agent. The glucose oxidation reaction, catalyzed by glucose oxidase (GOX) is as follows:



At the electrode:



The reaction is followed by measuring the decrease in the oxygen concentration using a Clark oxygen electrode. The first oxygen electrode was developed by Clark in 1953 (Clark *et al.*, 1953). Then first biosensors, called enzyme electrodes, were first described by Clark and Lyons in 1962 (Clark and Lyons, 1962) for the determination of glucose. A glucose biosensor based on the Clark oxygen electrode is shown in Figure 2.5. The biosensor uses the voltammetric principle of electrochemically reducing the oxygen. The cell current is directly proportional to the oxygen concentration, the glucose concentration is then proportional to the decrease in current. The oxygen electrode has an oxygen-permeable membrane covering the electrodes. A layer of enzyme is placed over this membrane and held in place with a second membrane covering it.

These types of biosensors have some problems. In order to have a reliable electrode response (decrease in oxygen concentration must be proportional to the decrease in the glucose concentration), the ambient oxygen level must be controlled and be constant. Another problem was the fairly high reduction potential of the oxygen, i.e. -0.7 V. At this potential, other materials might interfere. This problem was solved by measuring the oxidation of hydrogen peroxide, which has an oxidizing potential of +0.65 V. But this time the problem was that, at this potential, ascorbic acid, commonly present in biological samples, is oxidized leading to an unreliable result.

As a result of these problems, replacing oxygen with other oxidizing agents (or electron transfer agents) which were reversible, had lower oxidation potentials and whose concentrations could be controlled was further considered. These agents are usually called mediators. The biosensors utilizing mediators (artificial electron acceptors having low oxidation potentials) are called second generation biosensors.

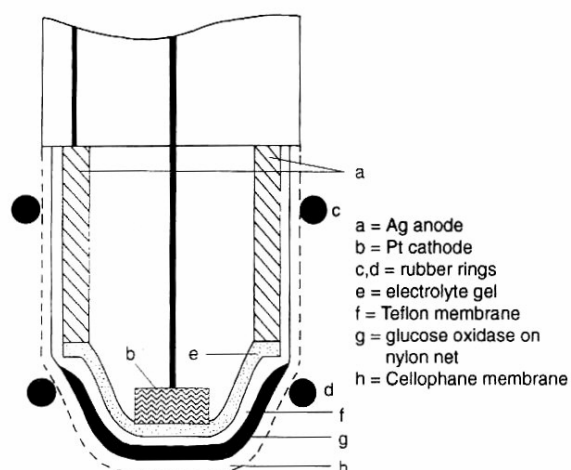
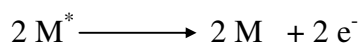
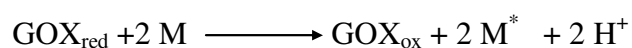
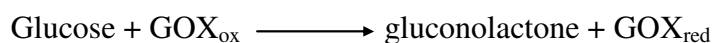


Figure 2.5. A glucose biosensor based on the Clark oxygen electrode (Eggins, 1999)

2.3.2. Second Generation Glucose Biosensors

The development of second generation biosensor (Cass *et al.*, 1984) involves a two step procedure, in which the enzyme takes part in the first redox reaction with the substrate, and is in turn reoxidized by a mediator. Finally the mediator is oxidized by the electrode. This can be seen schematically in Figure 2.6, and the reaction mechanism is as follows:



where M and M* are the oxidized and reduced the forms of the mediator. Commonly used mediators used with glucose oxidase and their redox potentials versus are given in Table 2.3.

The immobilization of the redox species can be carried out by adsorption, polymer coating and covalent attachment.

Table 2.3. Mediators used with glucose oxidase (Chaubey et. al, 2002)

Mediators	Redox potential (versus SCE)
1,1-dimethyl ferrocene	100
Ferrocene	165
Vinyl ferrocene	250
Ferrocene carboxylic acid	275
Hydroxy methyl ferrocene	185
$[\text{Ru}(\text{Cn})_6]^{4-}$	685
Benzoquinone	39
N-methyl phenazinium	-161
Tetrathiafulvalene	300
Tetracyanoquinodimethane	127
$[\text{Fe}(\text{Cn})_6]^{4-}$	180

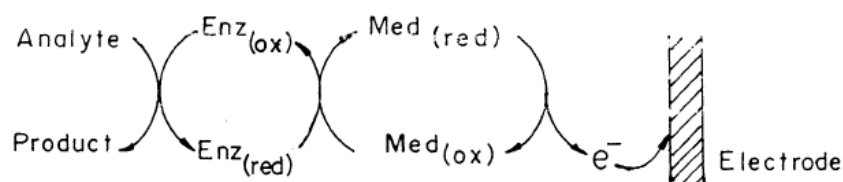


Figure 2.6. The schematic representation of mediated electron transfer in biosensors (Chaubey and Malhotra, 2002)

Conducting polymers have attracted much interest as a suitable matrix for the immobilization of enzymes. Conducting polymers have the ability to efficiently transfer electric charge produced by the biochemical reaction to the electronic circuit. The conducting polymer itself can be regarded as a mediator, and there are systems which combine conducting polymers and mediators. The electron transfer pathway in conducting polymer-based biosensors is shown in Figure 2.7.

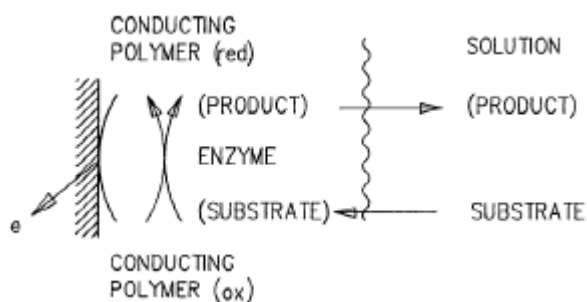


Figure 2.7. Suggested pathway for electron transfer in conducting polymer based biosensors (Gerard *et al.*, 2002)

2.3.3. Third Generation Glucose Biosensor

Some electrodes have been developed which can directly oxidize the reduced enzyme and do not require any mediator. These have been called ‘third generation biosensors’. However, there are also some problems encountered in this type of biosensors. Proteins tend to be denatured on the electrode surfaces and electron transfer may be slow and irreversible. The possible solution can be to use organic salt electrodes. Such enzyme electrodes can be prepared by the coating of the electronic conductors (conducting salts). Conducting salts such as *N*-methyl phenazinium-tetracyanoquinodimethane (NMP-TCNQ) could also be used as the electrode materials. But when these are used, the mechanism is like that of the mediators. These electrodes are often used for reactions involving NAD^+ dependent dehydrogenases as they allow the electrochemical oxidation of the reduced forms of these coenzymes. For example, the reduced enzyme GOX/ FADH_2 can be directly oxidized.

2.4. Supercritical Fluid Technologies

A supercritical fluid (SCF) is defined as a substance for which both pressure and temperature are above its critical values. The special combination of gas-like viscosity and liquid-like density of SCFs makes it attractive particular attention in various applications. The solubilizing power of SCFs is sensitive to small changes in operating conditions, and is possible to fine tune the pressure and temperature to tailor the physical properties of SCFs. Among supercritical fluids, carbon dioxide (CO_2) is the most widely used fluid in SCF processes. Supercritical carbon dioxide (scCO_2) offers tremendous potentials in

industrial applications, since it is safe, environmentally benign, inexpensive and non-toxic and has no odor. Its supercritical conditions are easily attained ($T_c = 31\text{ }^{\circ}\text{C}$; $P_c = 73.8\text{ bar}$) and it can be removed from a system by simple depressurization. Also, the low critical temperature of CO_2 makes it attractive for temperature labile materials such as pharmaceuticals. It has the capacity to swell polymers up to several volume percentages, especially polymers with an apolar character, since these have a better affinity with the apolar CO_2 . Besides swelling, scCO_2 can act as a plasticizer for a variety of polymers and causes a great increase in the mobility of amorphous polymer segments due its plasticizing effect (Gooijer *et al.*, 2004). Moreover, scCO_2 alters physical properties of polymers such as density, diffusivity and swollen volume (Nalawade *et al.*, 2006). Another advantage of utilizing scCO_2 is its sterilization effect. As it is known, sterilization plays a significant role in health applications and thus in biosensors. The main disadvantage to be dealt with biosensors is that heat sterilization is not possible due to the denaturation of the biological part of the biosensor. When scCO_2 is used for immobilization, there should be no need for additional sterilization since exposure to scCO_2 provides simultaneous sterilization (Sproule *et al.*, 2004).

Early examples of SCF applications include the extraction of caffeine from coffee beans, the extraction of oil from seeds and the extraction of organic compound from woods and coal. Many of these processes have reached commercial development. In more recent years, attention has turned to applications in pharmaceuticals, polymer processing, chromatography and environmental remediation and even dry cleaning (Zhang *et al.*, 1997). Also in the polymer area, supercritical fluids have been applied for polymerization, swelling, impregnation, fractionation, purification, and formation of powdered polymers (Kikic and Vecchione, 2003).

As cited in the study of Zhang *et al.*, (1997); Berens and Huvard (1989) showed that the diffusivity of a solute increases with CO_2 concentration and that liquid CO_2 is a swelling agent for a wide variety of polymers. The authors claimed that the increased diffusivity is due to the increased freedom of motion of the swollen polymer chains. During impregnation, the polymer matrix is relaxed by the scCO_2 which creates free space in the polymer matrix which is able to contain more of the protein than would otherwise be

possible. After depressurization, the polymer matrix shrinks and the biomaterials are trapped inside. Since CO₂ escapes as a gas, no toxic solvent residue is left behind.

A very wide range of physico-chemical operations from extraction/fractionation of natural products to reactions operating in supercritical media and material processing have been developed benefiting from the advantages offered by SCFs. Some of these SCF technologies developed may be useful in biosensor development. Production of solid particles of predetermined size and morphology, as well as impregnation of solids in polymer matrices are the SCF techniques that may be utilized to improve biosensor production. In this study, Supercritical Solution Impregnation (SSI) was taken as the basis for immobilization of GOX from solution to polymer matrix, therefore the details of SSI is summarized in the following sub-section.

2.4.1. Supercritical Solution Impregnation

The interest in the supercritical fluid impregnation of polymeric materials stems from the opportunity to utilize high diffusivity, low surface tension and the ease of solvent recovery for the preparation of new polymeric materials (Kikic and Vecchione, 2003). SSI process has a wide range of applications including the preparation of drug delivery systems via impregnation of an active component in a polymer matrix, dye and organic metallic complexes impregnation and preparation of polymer blends via impregnation of a monomer and an initiator in a swollen polymer matrix.

In Supercritical Solution Impregnation (SSI) process, there are three basic constituents: a solvent, a solute material to be impregnated and a polymer matrix. The solvent stretches the polymer matrix and enhances diffusion of the drug through the polymer matrix and helps place the solute material inside the polymer body.

In SSI, an increase in pressure leads to faster impregnation due to increased SCF density, and an increase in temperature leads to faster impregnation but lower swelling due to higher solubility of the material impregnated but lower SCF density (Perman, 1996). Additionally, higher solute concentrations leads to higher substrate loadings. Amorphous polymers and polymers with lower extent of cross linking are more suitable for SSI.

In the SSI technique the main limitation is related to the solubility of the solute material. However literature reports show that despite the low solubility of many organic compounds in the supercritical phase high concentrations in the polymeric phase is easily obtained (Condo *et al.*, 1996).

With SSI, impregnation can be done simply by placing a polymer and a solute material (generally a drug component) into a pressure vessel and simply pressurizing the vessel. The solubility of the polymer in SCF is not necessary; the only requirement is that it will expand or stretch in the scCO₂ atmosphere. If solubility of drug component is sufficient, SCF which has dissolved the drug material will diffuse into the polymer matrix carrying the drug molecules. After allowing a sufficient contact time before depressurizing the vessel at once, or at a suitable rate will be enough to entrap the drug molecules. This method may be done batch wise as well as continuous in terms of supercritical fluid flow. In the continuous case, a flow of drug saturated scCO₂ passes through the pressure vessel in which polymer is placed. Some works on impregnation imply the benefits of swelling the polymer by pure scCO₂ before contact with drug charged SCF (Güney and Akgerman, 2002).

Güney and Akgerman, (2002) studied impregnation of a biodegradable polymer matrix, poly-dl-lactide-co-glycolide (PLGA) with 5-fluorouracil and β -estradiol that are used in chemotherapy and estrogen hormone therapy respectively. They performed impregnation runs for 1.5 and 6 hr time periods at 35 and 55°C at a pressure of 207 bar and reported the loadings of 9-25 mg/g PLGA for β -estradiol and 0.5 mg/gPLGA for 5-fluorouracil depending on release studies of impregnated polymer. Their results show that increasing temperature increased the amount of loading. Also an increase in impregnation time from 1.5 to 6 hours increased the loading by 120 per cent.

Nikitin *et al.* (2003) studied the swelling and the impregnation of polystyrene (PS) using scCO₂ with ethyl-cyano-3(4'-dimethylaminophenyl) acrylate (ECDA). They performed swelling experiments at 38-65°C and 90-205 bar. Using a pressure cell with a 10 mm thick quartz glass and a digital camera pictures of samples were taken at fixed time intervals. Measuring from these digital images volume expansion was found to be ~3 per

cent. The impregnation of ECDA into a PS film of 40 μm thickness were performed at 40°C and 100 bar for 2 hr and the increase in PS sample weight owing to presence of ECDA was near 1 per cent of mass.

Berens *et al.* (1992), focused on both swelling and impregnation of films. Although amorphous polymers are said to be more suitable for SSI process, Berens *et al.* (1992) used scCO_2 for the impregnation of additives into a number of glassy polymers. In their experiments, dimethylphthalate (DMP) was used as the additive, and PVC films as the impregnation matrix. They concluded that the impregnation of glassy polymers with many additive compounds was dramatically accelerated by the presence of CO_2 at sufficiently high pressure and the partitioning of the additive between the CO_2 and polymer phase was important in CO_2 -assisted impregnation processes. Therefore; the major factor in producing a substantial concentration of absorbed additive was solubility of the additive in the polymer. Additionally, upon the release of pressure on a CO_2 /polymer/additive system, CO_2 was rapidly desorbed and the degree of plasticization was sharply reduced, leaving the absorbed additive trapped in the polymer to diffuse at the normal rate for the polymer/additive binary composition.

Cortesi *et al.* (2000) used the supercritical fluid chromatography technique for impregnation experiments. They impregnated ketoprofen into PMMA and Explotab, nimesulide into ethyl cellulose and polyvinylpyrrolidone, and megestrol acetate into polyvinylpyrrolidone. Considering their results, it was underlined that the pressure dependence of impregnated amount exhibited the same behavior as solubility vs. pressure. When solubility of drugs increased with pressure the impregnated amount increased, conversely when solubility of drugs decreased the impregnated amount decreased. They showed that the supercritical fluid chromatography could be used for screening purposes in the search of the proper operative conditions for impregnation studies and in the investigation of the suitability of some polymers to be impregnated from given solutes. Kazarian and Martirosyan (2002) studied the impregnation of ibuprofen into poly(vinylpyrrolidone) from scCO_2 solution.

US Pat 4,820,752 by Berens *et al.* (1989), describe a process for infusing an additive into a polymer using a compressed fluid that is normally gas at room temperature. Among

the variety of examples stated (sub, near, supercritical) in this patent, impregnation of progesterone into polyurethane at 45°C and 151 bar is at supercritical conditions for CO₂. Inventors state that load is 8 per cent mass for 41.5 h of impregnation with CO₂.

Üzer *et al.* (2006) studied the swelling of polymethylmetacrylate (PMMA) and SSI of naphthalene into PMMA using scCO₂. The results indicated that the extent of volume increase was between 9 and 25 per cent for swelling with pure CO₂. Naphthalene impregnated samples swelled two to four fold of the original size at the same conditions. The effect of pressure and temperature on the extent of volume increase was said to be directly related to the solvent density, for both swelling and impregnation. It was also claimed that, the extent of swelling and the amount of solute impregnated increased with increasing pressure and temperature.

In the above studies mentioned, the solutes to be impregnated into the polymer matrix are soluble in scCO₂ phase to an extent. However, most of the bioactive compounds of interest in drug applications are proteins that are typically insoluble or having very low solubility in SCFs, therefore it can be thought that SSI is not suitable for proteins. But, Perman *et al.* (1996) described a process in order to impregnate substantially insoluble additives into polymer matrices in supercritical fluid environment. They simultaneously contact the polymer matrix with the protein to be impregnated and a carrier liquid, such as water, in the presence of supercritical fluid, and achieved to impregnate substantially insoluble solutes into polymer matrices in scCO₂ environment.

Additionally, Sproule *et al.* (2004), concentrated on the swelling of polymethylmetacrylate (PMMA) and impregnation of a fluorescently-labeled protein into these PMMA matrices. They used distilled water and a protein solution (rhodamine labeled antibody to immunoglobulin) as impregnation materials. They performed swelling and impregnation runs at 40°C and 140 bar for 2.5 hr. The extent of swelling was 10 to 21 per cent of the original volume. They also accomplished the impregnation of a large protein into CO₂-swollen PMMA indicating the efficacy of producing protein-polymer biomedical materials using SCF technologies. Although they emphasized on the swelling and impregnation of polymers, the main aim was to investigate surface characteristics of bioactive polymers and they determined that the inner and the outer characteristics of

polymer samples after depressurization depends on the pressure release rate as well as upon the presence of moisture.

There are also some studies which incorporate protein particles into polymer matrices by infusion. Luzzi *et al.* (2001) describes a method of incorporating proteins or peptides into a matrix. They provide an intimate mixture of therapeutic agent (protein or peptide) and matrix material (microparticles) in a pressure vessel and contact this mixture with a compressed solvent such as scCO₂. The contact of protein-polymer mixture with compressed solvent permits sorption of at least a portion of the protein into the matrix, and separating the solvent from the matrix provides entrapment of the protein or peptide.

Howdle *et al.* (2001) described supercritical mixing process in which scCO₂ plasticised and substantially lowering the viscosity and allowing efficient incorporation of insoluble guest particles into the polymer. Efficient agitation of the scCO₂ swollen polymer led to homogeneous distribution of the guest particles. In that process, neither the bioactive guest nor the polymer dissolves in scCO₂. They demonstrated the validity of the method by incorporating enzymes ribonuclease A, catalase, and d-galactosidase in poly(lactic acid) poly(DL-lactide) (PLA) and poly(lactide-co-glycolide) (PLGA) using scCO₂, with retained enzyme activity.

As it is mentioned, despite the low solubilities of many organic compounds in the supercritical phase, high concentrations in the polymeric phase is easily obtained (Condo *et al.*, 1996). Also, from the studies reported in open literature and summarized above, it is known that even if the solute has very low solubility or is not soluble in scCO₂, it can still be impregnated to the polymer matrix from a solution (i.e. protein-water solution) or infused/mixed with polymer matrix in supercritical environment. Since there is no open literature data on GOX interaction with scCO₂ in terms of solubility and no open literature data on impregnating GOX into a polymer matrix in scCO₂ environment, in our study we first investigated to determine a reasonable immobilization medium for impregnation. The immobilization mediums used are mainly dry (as in the case of conventional SSI or supercritical infusion process) and liquid (i.e. water, ethanol, and buffer solution) mediums.

3. EXPERIMENTAL WORK

The main purpose of this work is to immobilize a biological recognition element into several conducting polymer matrices in supercritical carbon dioxide environment for further use in biosensor preparation. For this purpose, glucose oxidase (GOX) was used as the biological recognition element and immobilized on the following conducting matrices using supercritical immobilization method: PPY film, PU/PPY foam and PU/PPY film composites. First of all, the effect of supercritical carbon dioxide (scCO₂) on the free GOX-buffer solution activity was determined since there is no open literature data is available on the subject. Then, the supercritical immobilization of GOX on conducting polymers were conducted in a exploratory way of study. GOX immobilized conducting polymer matrices were analyzed by enzymatic activity assay and characterized by Environmental Scanning Electron Microscopy (Philips XL30 ESEM-FEG). Section 3.1 represents the materials used in the experiments. In Section 3.2, the experimental set-up used in this study is described and depicted. The experimental procedure consisting preparation of polymer matrices, procedure of free-enzyme experiments, supercritical immobilization procedure is discussed in Section 3.3. The analyses performed in this study are explained in detail in Section 3.4, and the program of the experimental work is given Section 3.5.

3.1. Materials Used

In this study, glucose oxidase from *Aspergillus Niger* (GOX, 200 U/mg, Fluka) was used as the biological recognition element and immobilized on several conducting polymers. Pyrrole (98 per cent purity), used for polypyrrole (PPY) film and polyurethane/polypyrrole (PU/PPY) composite formation, and *p*-Toluenesulfonic acid monohydrate (PTSA, min 98.5 per cent), used as dopant in film formation, were obtained from Aldrich. Pyrrole was used without further purification. The other dopant used in PPY film formation was Sodium dodecyl sulfate (SDS, min 98.5 per cent) and was purchased from Sigma-Aldrich. Iodine (I₂, Riedel de Haen, pure) was used as initiator and oxidant in the polymerization of PPY for composite formation. The polyurethane (PU) foam, with a density of 0.32 g/cm³, was obtained from Rogers Corporation, (Rogers, CT, USA) and

polyurethane dispersion in water (Baybond® PU 130) was supplied by Bayer Material Science AG. Both PU foam and dispersion were used for composite preparation. Potassium phosphate monobasic (Sigma, 98 per cent) and potassium phosphate dibasic (Sigma, min 99 per cent) were used for buffer solution preparation. β -D-glucose (Sigma, min 97 per cent), peroxidase (POD) from Horseradish (Sigma, Type II, 181 U/mg), *o*-dianisidine (Sigma), sulphuric acid (Fluka, 95-97 per cent), hydrogen peroxide (Riedel de Haen, 30 per cent solution) were used throughout the enzyme activity assay. Sulphuric acid was diluted to 5 M before use. Carbon dioxide (99.7 per cent pure), in cylinders equipped with dip tubes, was supplied by HABAS A.Ş. (İstanbul, Turkey). Coomassie Brilliant Blue G (Sigma) and perchloric acid (Fluka, 70 per cent) were used for Bradford reagent preparation. Bradford reagent (Sigma) was also used for protein determination. BSA standard solution (1 mg/ml, Sigma) was used for the preparation of calibration chart of the protein determination method.

3.2. The Experimental Set-up

Figure 3.1 shows the experimental set-up used for the Supercritical Fluid Immobilization (SFI) process. Liquid CO₂, which is supplied in cylinders with dip tubes at a pressure of 60 bar and 15 °C, was passed through a coiled, two meter long, 1/4" O.D. high pressure stainless steel tubing immersed in a cooling bath, CB, (Heto D.T.). The cooling bath was filled with water ethylene-glycol mixture and its temperature was set at -5 °C to keep the vapor pressure of the CO₂ below the atmospheric pressure. In this way CO₂ was kept in the liquid phase and probable gasification during pump suction was prevented. A 40-micron filter was placed between the cooling bath and the pump inlet to prevent the pump from being damaged by the possible presence of suspended particles in the commercial grade CO₂ used.

The solvent, CO₂, was delivered to the system at the desired pressure by the diaphragm pump type EK (Lewa). During operation, the pump head was continuously cooled by the water ethylene-glycol mixture supplied from the cooling bath by the help of a circulation pump. Since the compressibility of the CO₂ increases dramatically at the critical point and reduces the pump efficiency, cooling of the pump head is necessary.

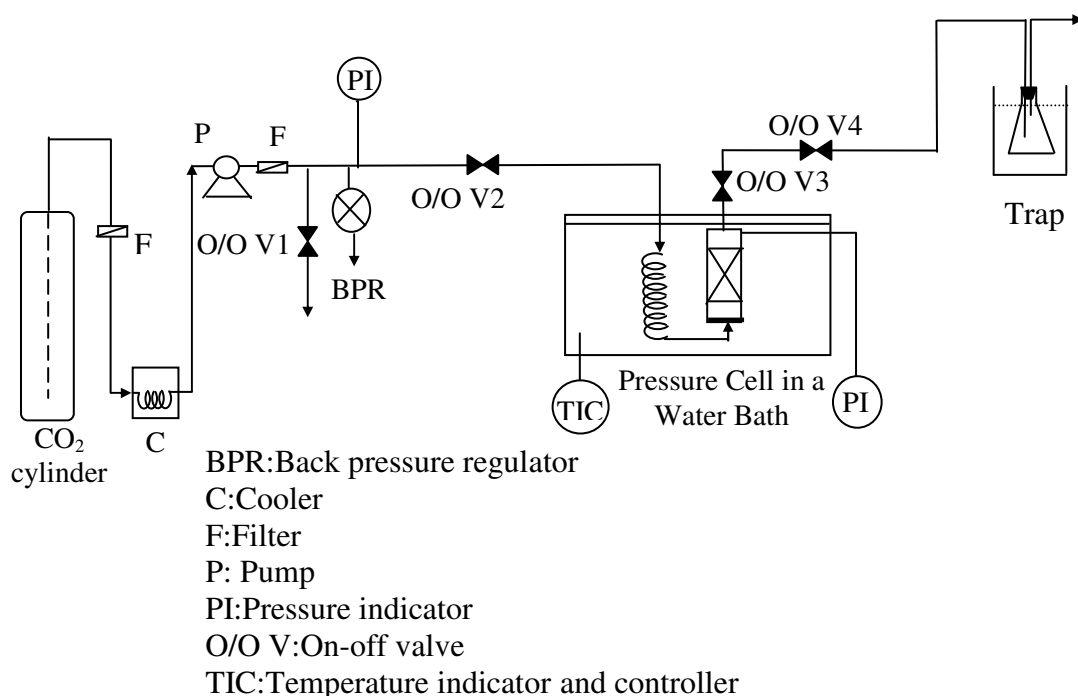


Figure 3.1. The experimental set-up

A back pressure regulator (Tescom Corp.), which is serviceable up to 172 bar, was installed after the pump exit. A second filter (40 μm) was placed between the pump outlet and the back pressure regulator inlet to prevent the back pressure regulator from any contamination. The back pressure regulator was heated by a jacket type electrical resistance to prevent freezing. The pressure of the system was manually controlled (± 1 bar) by the use of this regulator. The on-off valve V1 was placed before the back pressure regulator in order to depressurize the system in an emergency.

As shown in Figure 3.1, after the CO₂ passes through back pressure regulator, on-off valve V2 directs the CO₂ through the preheating coil, 1/4 in. O.D. high pressure stainless steel tubing, immersed in the water bath. After passing through the preheating coil CO₂ enters the pressure cell. The pressure cell was immersed in the constant temperature water bath. The water bath and the cell were manufactured by Erikman Kardeşler Makina Sanayi (İstanbul, Turkey). The water bath can be lifted up or brought down with a hand operated gear mechanism. The constant bath temperature was maintained with a thermostat with three heating resistances that are electrically connected to the thermostat and placed to the bottom of the water bath. The cell is suitable for use up to 250 bar. It has an internal volume of 318 ml, with an internal diameter of 45 cm and a depth of 200 cm. The cell

contains three main parts: cap, cover and body. Each of these parts is shown in Figure 3.2. The cover is placed on the body. A suitable Teflon gasket is used to ensure proper seating of the cover on the body. The threaded cap is screwed onto this assembly with the use of two bars, which were inserted into the mounting holes present on the sides of the cap. The cover includes three male connectors and a thermowell that extends two-thirds of the inside depth of the body. One of the fittings is connected to a pressure gauge (Pakkens Instrument, İstanbul, Turkey) of 0–250 bar range, and the other two fitting are closed with a plug nut. Inside the body, there is a cylindrical wire netting, which was used to hold the test tubes.

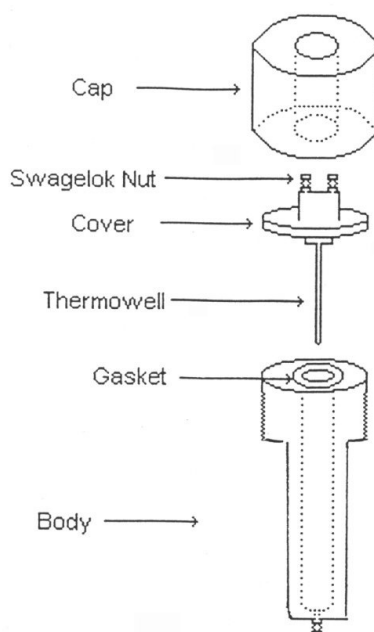


Figure 3.2. Parts of the pressure cell (Köse, 1998)

3.3. Experimental Procedure

This section includes the preparation procedure of conducting polymer matrices, the procedure for free enzyme experiments and the experimental procedure developed for supercritical immobilization.

3.3.1. Preparation of Polymer Matrices

Three different polymer matrices were prepared: PPY film, PU/PPY composite foam and PU/PPY composite film. The methods of preparation are given in the following sections.

3.3.1.1. Preparation of PPY Film Samples. PPY films of nearly 50 μm thickness, were synthesized by electropolymerization on platinum electrodes in Middle East Technical University, Ankara, Turkey in laboratory of Prof. Levent Toppare. PPY films were synthesized by electropolymerization on three different electrode sizes: 1.25, 2.5 and 100 cm^2 . Two different electrolytes were used during polymerization: SDS and PTSA.

For preliminary studies, PPY film was synthesized on 100 cm^2 stainless steel electrode. The electropolymerization solution (800 ml) contains 0.05 M PTSA, 0.05 M NaOH, and 10 ml of pyrrole. The electropolymerization was performed at constant potential, and the potential applied was 1.1 V. The polymerization period was 2 h. After polymerization, the PPY films were washed and peeled from the electrode.

For the rest of the study, platinum electrodes were prepared and PPY was synthesized on them and directly used for immobilization process. Electropolymerization was performed in a two compartment cell using platinum working and counter electrodes. The reference electrode was silver. The 0.05 M PTSA and SDS solutions, prepared by distilled water, were used as electrolyte solutions. The electrolyte solution was poured into the cell and then 100 μl of pyrrole was injected to the working electrode compartment. The potential applied for electropolymerization was 1.1 V and the polymerization time was 30 min.

3.3.1.2. Preparation of PU/PPY Foam Samples. PU/PPY composites were prepared by chemical polymerization using I_2 as both oxidant and initiator according to the method given by Shenoy *et al.* (2002). 0.9794 g of I_2 was put in a beaker and the PU foam or film in predetermined shape and dimensions was placed in the beaker above the I_2 to allow for I_2 penetration into the PU foam/film. However, PU foam/film was placed in the beaker in such a way that it was not allowed for PU and I_2 crystals to have direct contact. The beaker

was sealed with parafilm and put in a convection oven (Nüve, EN 025) at 43 °C for 24 hours. At the end of soaking time of 24 °C, I₂ soaked PU film or foam was immediately transferred to another beaker containing 5 ml of pyrrole. The beaker was again sealed with parafilm and placed in a convection oven to allow for pyrrole polymerization at 25 °C for 48 °C. At the end of 48 h, following the composite formation PU/PPY, the samples were dried in a fume hood for 7 days until the excess monomer/oxidant evaporated from the sample. However, as composites were immersed in GOX solution for immobilization, the color of solution became brown-black even at atmospheric pressure (without CO₂) during immobilization. Therefore, it was thought that the monomers, dimers, trimers, or I₂, which cannot diffuse out of the composite when exposed to air, come out of the composite as it is immersed in the solution. Such chemicals can reduce the enzyme activity of the GOX solution. Furthermore, due to these additional chemicals in the immobilization solution, protein determination was impossible. Therefore, PU/PPY composites had to be washed thoroughly before immobilization. Two different washing procedures were tried for this purpose. In the first method, the samples were put in 3 ml buffer solution, which were kept for 24 hours at 50 °C. In the second way, the samples were exposed to scCO₂ at 150 bar and 50 °C in again 3 ml buffer solution for 24 h. In both methods PU/PPY foam samples were immersed in buffer solution, using a stainless steel wire to hold them submerged. After the samples were taken out of the washing solutions, they were washed via pipetting 3 ml of buffer solution on them to get rid of the residues which might be on them. The polymer samples washed with both methods were used for SFI, and the effect of washing on the immobilization process thus the activity of the GOX immobilized samples are give in Section 6.

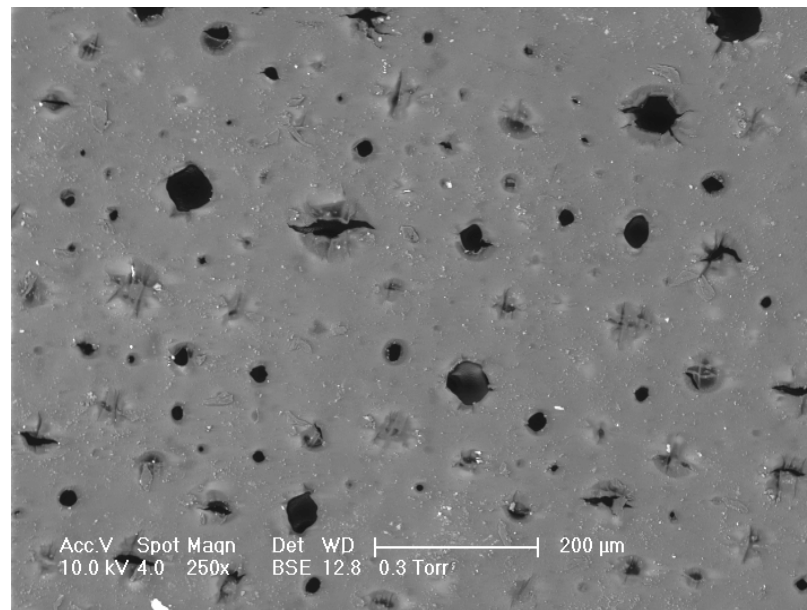
The PU/PPY composites were synthesized starting from two different shapes and dimensions of PU foam. The preliminary and exploratory study on GOX immobilization on PU/PPY foam was made with PU/PPY samples of 9×6 mm which were cut from ready made composites of 25.4×25.4 mm size, given by Prof. Can Erkey (Koç University). However, our doubts about the concentration homogeneity of the composite forming chemicals at the center, side and corner pieces obtained from these square pieces lead us to choose the strip geometry in the further study.

PU/PPY conducting composites were prepared in our laboratory from PU strips of 80×5 mm, each strip containing 10 pieces of 8×5×3.2 mm size PU foam. This strip geometry was chosen in an effort to obtain more uniform exposure to iodine and pyrrole vapors during composite formation. Each strip was premarked with indentations. The indentations were made by cutting the strip partially widthwise before the composite formation. The strip was then curled and two samples on each edge of the strip were punched in order to form a circular shape. These two samples on the edges were used for immobilization. The circular shape of the strip designed to obtain homogeneous polymerization for each PU/PPY sample used for immobilization is shown in Figure 3.3. After the PU/PPY composite was formed, the strip was cut at the indentations to obtain the individual samples for GOX immobilization. The PU foam got larger after polymerization of pyrrole and the PU/PPY composite samples used for immobilization attained approximately 10×6×3.6 mm, the actual sample dimensions were measured before the immobilization experiments.

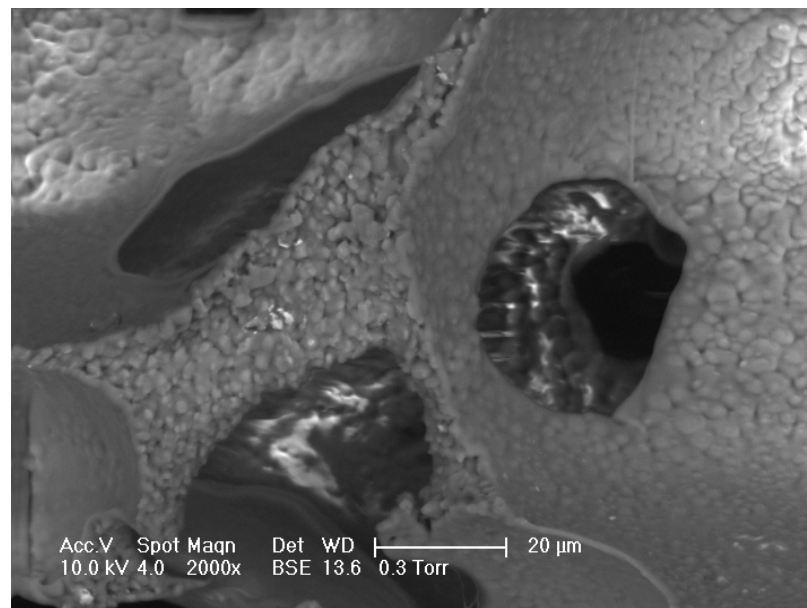


Figure 3.3. The image showing the circular strip geometry of PU/PPY foam used during pyrrole formation

PU foam and PU/PPY composite foams were visually investigated by ESEM. The outer and inner surfaces images are presented on Figure 3.4 and 3.5. PU foam prior to composite formation was sensitive to heat, at high magnifications when the electron beam stroke the sample, the surface of PU foam tended to melt, and it was difficult to investigate its surface. However PU/PPY foam did not cause such problems leading to easier investigation. Additionally, PU foam had a soft and a puffy image, whereas PU/PPY foam had rigid look.

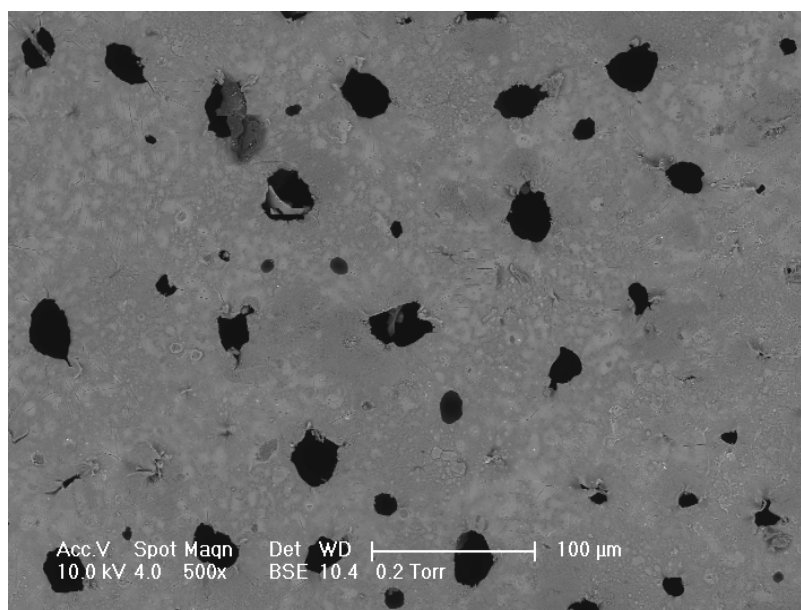


(a)

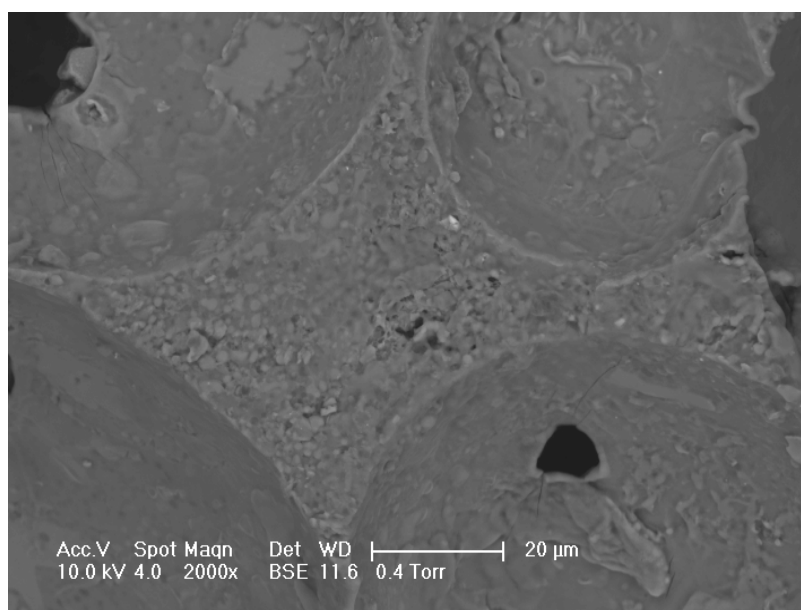


(b)

Figure 3.4. ESEM images of PU foam prior to composite formation, (a) outer surface, (b) cross-section



(a)



(b)

Figure 3.5. ESEM images of PU/PPY foam (a) outer surface, (b) cross-section

3.3.1.3. Preparation of PU/PPY Film Samples. Five milliliter of PU 130 was poured on to flat, stainless steel plates having a length of 5cm; a width of 5cm and placed in an autoclave at 50 °C for 24 hours. Due to the physical properties of PU 130, it is known that, when PU 130 is exposed to 50 °C, water present (70 per cent by mass) evaporates and film becomes transparent. After the PU film was formed, the PU/PPY composite was prepared according to the method given in the previous subsection. The PU/PPY film samples were

cut from the whole sample just after the polymerization period, and then they were dried in a fume hood. PU/PPY samples were cut in round disc shape, the diameter of the samples were approximately 10 mm, the thickness were approximately 1 mm. The actual dimensions of each sample were measured prior to experiment. These PU/PPY film samples were also washed in buffer prior to immobilization process.

3.3.2. Free-Enzyme Experiment Procedure

The set-up shown in Figure 3.1 was used for free-enzyme experiments. In order to investigate the effect of pressure on the free-enzyme solution, 0.0125 g GOX was dissolved in 2 ml of pH 5.7 phosphate buffer in a test tube and put in the pressure vessel. Prior to beginning a run, the refrigerated bath, which is used to cool the CO₂ before entering the diaphragm pump and to provide pump head cooling, and the constant temperature water bath, which includes the CO₂ preheating coils and the pressure cell were brought to their respective run temperatures. The operating temperature of the refrigerated bath was always -5 °C. The pump head was cooled with water-ethylene glycol mixture supplied from the -5 °C cooling bath. The jacket type electrical heater of the back pressure regulator was also turned on two hours before a run. After obtaining the desired temperatures throughout the system, the pressure vessel was filled with CO₂ through the on-off valve V2. The diaphragm pump was then turned on to bring the system to the desired pressure. After the desired pressure was achieved in the vessel, the diaphragm pump was turned off and the on-off valve V2 was closed. GOX-buffer solution was exposed to scCO₂ in the pressure vessel at the desired temperature and pressure for 24 h. After the desired exposure period was over, the pressure vessel was depressurized through the on-off valves V3 and V4. At the end of the experiment, after the GOX-buffer solution was taken out of the pressure vessel, the enzymatic activity of the solution was immediately measured. For every solution, the enzymatic analysis was repeated three times and the average of activities measured was reported.

The above procedure was also used to determine the amount of protein in GOX-buffer solution before and after exposure to scCO₂. The differences with above procedure are the concentration of the GOX-buffer solution which is exposed to scCO₂ and the analysis method. Since the amount of possible GOX extract are in µg scale, it was

impossible to determine the extracted amount correctly from a solution of 6.25 mg GOX/ml buffer (6250 $\mu\text{g/ml}$). This concentration was too concentrated; therefore the 2 ml of 0.0625 mg/ml GOX-buffer solution (62.5 $\mu\text{g/ml}$) was exposed to CO_2 in this case, which was obtained by 1/100 diluting the 0.00625 g/ml GOX-buffer solution. The protein amounts in the solution before and after exposure to scCO_2 were determined by Bradford method, and the difference between these two values gave the amount of GOX extracted by scCO_2 . The Bradford analysis was repeated three times for each sample.

The effect of temperature on free-enzyme solution was also investigated. For this purpose, 0.0125 g GOX was dissolved in 2 ml of pH 5.7 phosphate buffer in a test tube, covered with a parafilm, and placed in a water bath, which was set at the desired temperature. GOX-buffer solution was exposed to temperature for 24 h. At the end of 24 h, the enzymatic activity of the solution was immediately measured. For every solution, the enzymatic analysis was repeated three times and the average activity was reported.

3.3.3. Procedure of the Immobilization Experiments

The aim of this study was to develop a supercritical immobilization procedure (SFI) for enzyme immobilization on conducting polymers. Since different conducting polymer matrices were used and as the study progressed, new treating procedures were developed for polymer matrices to get the best result in terms of enzyme activity. There are some additional treatments within the immobilization procedure specific to the polymer matrices, such as pre-washing and after washing. These concepts are explained in detail and discussed in the corresponding chapters.

Immobilization experiments started with the PPY films. To immobilize GOX on PPY films, four different experimental mediums were used and the experiments were named according to the mediums used, namely: “Dry”, “Semi-Dry”, “Wet” and “Wet-b”. In “Dry” and “Semi-Dry” experiments, a pre-determined amount of GOX was weighed (the amount for each experimental run is given in tables of Section 3.5) and put in a test tube and the polymer matrix was placed in the same test tube. Then, the test tube was placed in the pressure vessel. In the “Semi-Dry” experiments an additional test tube containing 10 ml de-ionized water (dH_2O) was also put in the pressure vessel. In the case

of the “Wet” experiments, a pre-determined amount of GOX was dissolved in a pre-determined amount of dH₂O, or pH 5.7 phosphate buffer depending on the case. Then, the polymer matrix was submerged in that solution and positioned in the pressure vessel. Buffer medium was used in further studies, for immobilization of GOX into PU/PPY composites, since the buffer medium was the best medium among all the immobilization mediums that leading to higher activities for the GOX immobilized samples. Whatever the immobilization medium was, prior to beginning a run, the refrigerated bath, which is used to cool the CO₂ before entering the diaphragm pump and to provide pump head cooling, and the constant temperature water bath, which includes the CO₂ preheating coils and the pressure cell were brought to their respective run temperatures. The operating temperature of the refrigerated bath was always -5 °C. The pump head was cooled with water-ethylene glycol mixture supplied from the -5 °C cooling bath. The jacket type electrical heater of the back pressure regulator was also turned on two hours before a run. After obtaining the desired temperatures throughout the system, the pressure vessel was filled with CO₂ through the on-off valve V2. The diaphragm pump was then turned on to bring the system to the desired pressure. After the desired pressure was achieved in the vessel, the diaphragm pump was turned off and the on-off valve V2 was closed. The system in the pressure vessel was kept at the desired temperature and pressure for different time periods changing between 4-72 h. After the desired immobilization period was over, the pressure vessel was depressurized through the on-off valves V3 and V4. After the polymer matrices were taken out of the pressure vessel and out of the enzyme solution in the test tube, the GOX solution remaining on them were washed with dH₂O or buffer and put in a test tube containing 2 ml pH 5.7 phosphate buffer solution and kept in a constant temperature water bath equipped with a shaking apparatus at 30 °C for various washing periods (10 min-10 h). After removing the sample from the buffer solution, the enzymatic analysis was performed on the GOX immobilized samples within an hour. The dry GOX or the GOX solution taken out of the pressure vessel were kept at -18 °C. Immobilization experiments were also performed at atmospheric pressure to compare with SFI process. At atmospheric pressure experiments, no CO₂ was involved and they were all done in buffer medium. The polymer matrix was submerged in 2 ml of GOX-buffer solution in a test tube, covered with a parafilm and put in a constant temperature water bath at the desired temperature for a predetermined period. After immobilization period was over, the rest of the procedure was the same as it was done in the SFI procedure.

Although the general SFI procedure was given above, it should be noted that each of the polymer matrices has its own special treatment in putting them to the buffer solution or in “washing before immobilization” and “washing after immobilization” steps. These differences of treatment among the polymer matrices are explained in their corresponding chapters (Chapters 5 and 6).

3.4. Analysis

GOX immobilized conducting polymer matrices were analyzed by enzymatic activity assay and characterized by Environmental Scanning Electron Microscopy (Philips XL30 ESEM-FEG). The immobilized GOX amount and the amount of GOX extracted by scCO_2 from the solution were determined by the Bradford method (Bradford, 1976). The conductivities of conducting polymers were measured with a voltmeter. In this section, the analysis methods such as activity assay used and the Bradford method are explained.

If there were loosely bounded or not fully immobilized particles on the polymer matrix, they should be washed off before the enzymatic analysis; thus the washing step after immobilization was crucial. After the immobilization process, as indicated in the previous section, the GOX immobilized polymer samples were washed for different washing periods between 10 min and 10 h. Since the time of washing was important, preliminary tests were performed to determine the optimum washing time before enzymatic activity analyses. For this purpose, GOX immobilized PU/PPY foam composite samples were taken out of the enzyme solution and the GOX solution remained on it was washed away by 6 ml of buffer solution, at the end of immobilization period. Then the sample was put in a test tube containing 2 ml buffer and kept at that buffer solution in a constant temperature water bath equipped with a shaking apparatus at 30 °C for 10 min. At the end of 10 minutes, the sample was taken out of that buffer solution, and put in 2 ml new (unused) buffer solution for 20 minutes at 30 °C constant temperature water bath with a shaking apparatus. At the end of 20 min washing time, the sample was taken out of the buffer solution and put in a new (unused) 2 ml buffer solution for additional 30 minutes. The washing procedure continued in increased time intervals (1st washing: 10 min, 2nd washing: 20 min, 3rd washing: 30 min, 4th washing: 1 h, 5th washing: 2 h, etc.) until no

activity was observed in the washing solution (Figure 3.5). As it is seen in Figure 3.5, there were no observed activities in washing solutions after 4 h, but the washing procedure was extended to 10 h to ascertain the immobilization procedure, in that no more enzyme was lost from the sample(s). After the washing was complete, the GOX immobilized polymer samples still had enzymatic activities. As it is seen from Figure 3.6, the major release of unimmobilized GOX particles (90 per cent of total amount released- calculated via “free enzyme amount vs. activity calibration chart” that was prepared) from the polymer support took place in first 30 min. The enzymatic analysis could be performed at the end of 30 min washing, since 90 per cent of the unimmobilized enzyme was removed from the polymer support at the end of 30 min, and the possibility for the remaining 10 per cent unimmobilized enzyme to fall into the analysis solution in very short reaction times (2, 4 and 6 min) compared to washing times (30, 60, 120 min) during activity analysis of polymer sample was negligible. Thus, to economize the experiment duration without harming the quality, 30 min washing time was chosen. These initial experiments to determine the optimum washing time were done with PU/PPY foam composites because washing is more important for PU/PPY foam composite than PPY film since it is much thicker (~3.6 mm) than PPY film (~50 μm) and its foamy structure allows more surface area for immobilization. In the experiments in which the aim was to optimize the “washing after immobilization”, the concentration of immobilization GOX-buffer solution was 6.25 mg/ml (the highest concentration used in the immobilization experiments). The washing steps of the GOX immobilized PU/PPY composite shown in Figure 3.6 was immobilized from that solution concentration. When the concentration was diluted to 0.0625 mg/ml, no activity was observed in the washing solutions in any step of the washing period (10 min-10 h). Although, no detectable activity was obtained at this lower immobilization solution concentration, PU/PPY composite foam samples were still washed for long “washing after immobilization” periods such as 4 or 10 h. The reason for this situation will be explained in the corresponding section with the graphs.

Since PPY films are very thin (~50 μm), 30 min was enough for “washing after immobilization” period. For PU/PPY film composite samples, preliminary tests were also done for “washing after immobilization” period and it was seen that no activities were observed in washing solution when immobilization was done from 0.0625 GOX mg/ml solution as in the case of PU/PPY foam. Contrary to PU/PPY foam, also no activity was

observed in the washing solutions when immobilization was done even from 6.25 GOX mg/ml solution. The effect of washing period after immobilization on the enzymatic activity of GOX immobilized polymer samples are given in Section 6.3.

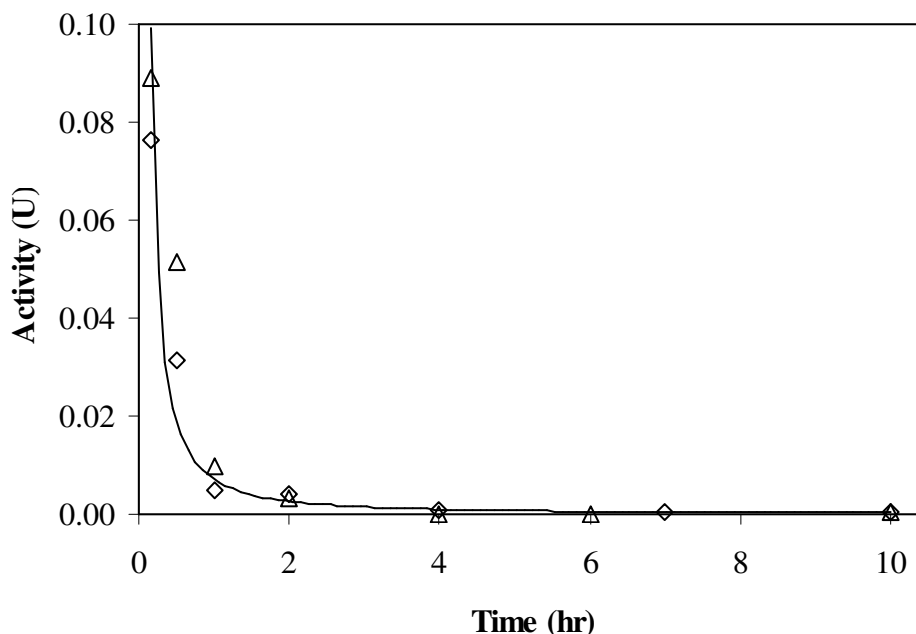


Figure 3.6. Activities of solutions used to wash the GOX immobilized samples (◇ and Δ are the duplicate runs at 105 bar, 40 °C and for 24 h).

GOX immobilized polymer matrices were analyzed by enzymatic activity method developed according to Sigma Technical Bulletin, No 510. Four different β -D-glucose solutions (5, 10, 50, 100 mM), prepared by 0.1M pH 5.7 phosphate buffer, were used for GOX immobilized PPY film enzymatic analysis. For enzymatic analysis of GOX immobilized on PU/PPY composites, again four different β -D-glucose solutions (5, 10, 20, 100 mM), prepared by pH 5.7 phosphate buffer, were used. 2.9 ml of β -D-glucose solution and 0.1 ml buffer solution were put in a test tube and placed in the water bath. Enough time (30 min) was given to glucose solutions to come to the reaction temperature (30 °C). The reaction temperature was set after making enzymatic analysis at different reaction temperatures with free enzyme. The activities of free-enzyme solution at different reaction temperatures are shown in Figure 3.7. Figure 3.7 reveals that 30 °C is a suitable temperature at which the enzymatic analysis can be performed. After temperature equilibrium was established, polymer matrix was placed in the glucose solution and

incubated at 30 °C for different reaction times (2, 4 and 6 minutes) while the tubes were shaken. During these reaction times β -D-glucose undergoes a chemical reaction with GOX being the catalyst and releases H_2O_2 as a product.



When the reaction time was over, 1 ml of the reaction solution was taken and mixed with 0.1 ml POD (1 mg POD/ml buffer) -to catalyze the reaction- and 2.4 ml o-dianisidine (0.21mM) -as the reducing agent- immediately. At this point, o-dianisidine reacts with the H_2O_2 produced chemically under the catalyst of POD and finally o-dianisidine is oxidized.

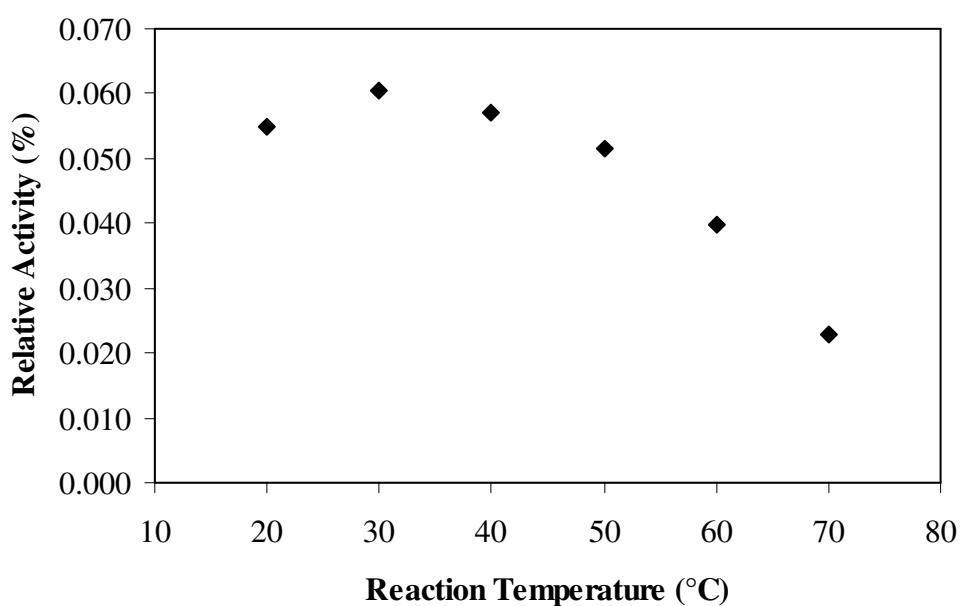
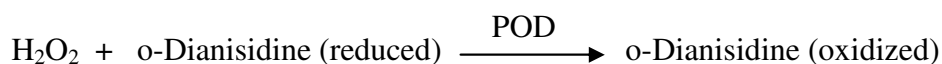


Figure 3.7. Free-enzyme solution activities evaluated at different reaction temperatures

The existence of oxidized o-dianisidine causes formation of an orange solution. The resultant orange solution was not stable. 5 ml of 5M H_2SO_4 was added for stabilization. The absorbance of the resultant pink solution was read at 530 nm by double-beam spectrophotometer (Analytic Jena, Specord 200) after 20 min waiting time. The quantity

and the concentration of the H_2SO_4 added to the reaction mixture were also optimized. 0.5, 1, 2, 3, 4, 5 ml H_2SO_4 at different concentrations (2.5, 5, 10 M and concentrated (97-98 per cent)) were added to the reaction solution. Then the absorbances were scanned between 380 and 1000 nm. Then the absorbances were again read at the wavelength giving the highest absorbance of the corresponding solution and at 530 nm at every 5 sec for 2 min. The results showed that 2.5 M H_2SO_4 was not enough for stabilization (the absorbance value decreased rapidly) even when 5 ml of it was added. 1 and 2 ml of 10 M H_2SO_4 did not stabilize the reaction solution, however 3 ml and higher amounts stabilized the reaction solution but the temperature of the solution rose significantly. The reaction was terminated with concentrated H_2SO_4 , but the resultant solution got very hot which could crack the test tube. 5 ml of 5 M H_2SO_4 could terminate the reaction and thus stabilize the absorbance read without heating the solution, therefore they were chosen as the optimum H_2SO_4 concentration and amount to use in enzyme assay.

In free enzyme assay, at the beginning of the procedure, 2.9 ml of β -D-glucose solution was kept long enough in water bath to bring it to the reaction temperature (30°C) and then 0.1 ml of enzyme solution (0.2 mg GOX/ml), diluted 50 times prior to analysis, was added into the glucose solution to start the reaction. The reaction times and the rest of the procedure was the same as that given above for immobilized enzyme.

Before deciding on the above enzymatic analysis method, two more methods were also investigated. In both methods, the change in the glucose concentration were measured. Dinitro-salicylic acid (DNSA) method was the first method used, and then glucose amount in solution was also determined by HPLC. The details, and the difficulties encountered during the application of these methods with the used polymer matrices are given in Section 5.2.

The absorbance values obtained were interpolated to determine the amount of H_2O_2 produced as a result of chemical reaction by using a calibration curve, given in Appendix C (Figure C.1) prepared with known amounts of H_2O_2 between molarities of 0.01 to 0.2 mM. One unit of GOX activity is defined as 1 μmol of β -D-glucose oxidized to gluconic acid with simultaneous production of H_2O_2 per minute at pH 5.7 and 30°C .

The kinetic parameters K_m (Michaelis-Menten constant) and v_m (maximum velocity) of free and immobilized GOX were determined. The kinetic parameters of Michaelis-Menten equation (Equation 3.1) were found by nonlinear curve fitting without transformation of the data. Maximum velocity (v_m) which corresponds to the highest velocity achieved during the reaction catalyzed by the enzyme. K_m is a kinetic parameter, shows the possibility of the encounter of the enzyme and the substrate and also defines the possibility of the formation of substrate-enzyme complex. This value which corresponds to the amount of substrate concentration at half of the maximum velocity.

$$v = \frac{v_m S}{K_m + S} \quad (3.1)$$

The protein amount immobilized on the polymer support was determined from the protein difference of the immobilization solution before and after the immobilization process. If the washing solutions had enzymatic activity, then the protein amount in the washing solutions were also taken into account. The protein amounts in the solution before and after exposure to $scCO_2$ were determined by the Bradford method. Bradford reagent was used without further dilution by mixing 1 ml of protein solution with 1 ml of Bradford reagent. The calibration chart was prepared with BSA standard solution, is given in Appendix C (Figure C.2), with a linear range between 1-10 μg in 1 ml solution.

3.5. Experimental Program

The outline and the main points of the experimental study of this work is given in the below list:

- Solubility studies of GOX in $scCO_2$,
- Preliminary impregnation studies of GOX in non-conducting polymers (i.e. polymethylmethacrylate),
- Studies to find the suitable enzyme assay to determine the activity of GOX immobilized polymer samples and optimization of the enzyme assay,
- Studies investigating the effect of $scCO_2$ on GOX-buffer solution,
- Studies investigating the effect of temperature on GOX-buffer solution,
- SFI of GOX on PPY films,

- Studies to find the best SFI medium,
- SFI of GOX on PU/PPY composite foams,
- SFI of GOX on PU/PPY composite films.

The details of the experimental program are given in the following sections.

3.5.1. Free-Enzyme Experiments

Although the possibility to observe a detectable solubility of free GOX in scCO₂ was very low, several methods were used to investigate it. For this purpose, both commercial GOX obtained from Delta Gıda A.Ş. (10000 Units) and original GOX (Fluka, 200 U/mg) were used. Two types of methods were followed. In the first type, only free GOX was exposed to scCO₂, but in the other method other than GOX, there was also a polymer matrix in the supercritical environment. In this second method, the aim was both to investigate the solubility of GOX in scCO₂ and also to perform preliminary impregnation studies with non-conducting polymers. In Table 3.1, experiments performed in order to investigate the solubility of GOX in scCO₂. For all of the runs, incorporating a polymer matrix, shown in Table 3.1, the gravimetric analysis method was used. In Table 3.1, if asterisk “*” was used with the enzyme amount, it means that in that run original GOX (Fluka, 200 U/mg) was used.

Table 3.1. Experiments done with free GOX

Run No.	Pressure (bar)	Temperature (°C)	Enzyme amount (g)	Polymer used	Exposure to scCO ₂ (h)
FE1	180	40	1.0	-	5
FE2	100	40	0.1*	-	24
FE3	150	40	4	PMMA	5
FE3R	150	40	4	PMMA	5

One of the immobilization mediums investigated in the SFI process was the buffer medium; in fact the best one in terms of activity obtained. Therefore, the effect of temperature and scCO₂ on GOX-buffer solution was investigated to better explain the immobilization results. Table 3.2 shows the experiments done for this investigation.

Additionally, the effect of scCO₂ on GOX-buffer solution was investigated by determining the protein amount in the solution before and after exposure to scCO₂. The experimental runs and their respective experimental conditions are given in Table 3.3.

Table 3.2. Experiments investigating the effect of temperature and pressure on GOX-buffer solution activity

Run No.	Pressure (bar)	Temperature (°C)	Enzyme concentration (mg/ml)	Duration (h)
FES1	1	30	6.25	24
FES2	1	40	6.25	24
FES2 ^R	1	40	6.25	24
FES3	1	50	6.25	24
FES3 ^R	1	50	6.25	24
FES4	1	55	6.25	24
FES5	1	60	6.25	24
FES6	80	40	6.25	24
FES6 ^R	80	40	6.25	24
FES7	100	40	6.25	24
FES8	150	40	6.25	24

Table 3.3. Experiments investigating the protein amount in GOX-buffer solution before and after scCO₂ expansion

Run No.	Pressure (bar)	Temperature (°C)	Enzyme concentration (mg/ml)	Duration (h)
FES9	80	30	0.0625	24
FES10	100	30	0.0625	24
FES11	150	30	0.0625	24
FES12	80	40	0.0625	24
FES13	100	40	0.0625	24
FES13 ^R	100	40	0.0625	24
FES14	150	40	0.0625	24
FES15	100	50	0.0625	24

3.5.2. SFI Experiments with PPY Film

The experiments performed with PPY films were divided in two parts. The first part was performed mostly with commercial GOX obtained from Delta Gıda A.Ş. (10000 Units) and PPY films synthesized on the 100 cm² electrode. The experiments performed in the first part are named as the “preliminary experiments”. The experiments conducted in the first part are given in Table 3.4. The types of experiments are explained in Section 3.3.3. (Immobilization Experiments Procedure). The only unusual type of experiment appear in Table 4 is “Semi-Dry-E”, which was the same as “Semi-Dry” type of experiment mentioned in Section 3.3.3 in principle, the only difference was that instead of water, 10 ml of ethanol was put in an additional test tube, which was also placed in the pressure vessel.

Table 3.4. Preliminary SFI experiments performed on PPY films

Run No.	Pressure (bar)	Temperature (°C)	Type of experiment	Enzyme amount	Imm. period (h)	Type of electrolyte used
PPY1 ^{PR}	150	40	Dry	1 g	5	PTSA
PPY2 ^{PR}	150	40	Dry	1 g	24	PTSA
PPY3 ^{PR}	150	40	Dry	1 g	48	PTSA
PPY4 ^{PR}	150	40	Dry	1 g	72	PTSA
PPY5 ^{PR}	150	40	Wet	0.2 g/6 ml H ₂ O	48	PTSA
PPY6 ^{PR}	1	40	Wet	0.2 g/6 ml H ₂ O	48	PTSA
PPY7 ^{PR}	150	40	Wet	0.2 g/6 ml H ₂ O	72	PTSA
PPY8 ^{PR}	150	40	Wet-E	0.2 g/6 ml EtOH	24	PTSA
PPY9 ^{PR}	150	40	Semi-Dry-E	1 g	24	PTSA
PPY10 ^{PR}	150	40	Wet	0.01 g*/6 ml H ₂ O	24	PTSA
PPY11 ^{PR}	150	40	Wet	0.01 g*/6 ml H ₂ O	24	PTSA

The GOX donated by Delta Gıda A.Ş. (10000 Units) was not pure and expensive as the GOX (200 U/mg, Fluka) used in further studies. Therefore, in preliminary experiments high amount of enzyme such as 1 g could be used. In most of preliminary experiments

GOX taken from Delta Gıda A.Ş. were used, only in experiments PPY10^{PR} and PPY11^{PR} original GOX from Fluka were used. In Table 3.4., in the column representing the enzyme amount, if “*” is used after the enzyme amount, it means that the original GOX (Fluka) was used in that experiment. Immobilization of experiments PPY10^{PR} and PPY11^{PR} were done at the same conditions, but their enzymatic analyses were done by different methods. Therefore PPY10^{PR} and PPY11^{PR} are not shown as replicates.

Table 3.5. SFI experiments performed on PPY films

Run No.	P (bar)	T (°C)	Type of experiment	Enzyme amount	Imm. period (h)	Type of electrolyte used	Electrode area (cm ²)
PPY1	150	40	Dry	0.0050 g	24	SDS	1.25
PPY2	150	40	Dry	0.0050 g	72	SDS	1.25
PPY3	150	40	Dry	0.0100 g	24	SDS	1.25
PPY4	150	40	Dry	0.0100 g	24	PTSA	1.25
PPY5	150	40	Semi-Dry	0.0100 g + 10 ml H ₂ O	24	SDS	2.5
PPY6	150	40	Wet	0.0250 g/4 ml H ₂ O	5	SDS	2.5
PPY7	150	40	Wet	0.0250 g/4 ml H ₂ O	24	SDS	2.5
PPY8	150	40	Wet	0.0125 g/2 ml H ₂ O	72	SDS	1.25
PPY9	150	40	Wet-b	0.0250 g/4 ml buffer	24	SDS	2.5
PPY10	150	40	Wet-b	0.0250 g/4 ml buffer	24	PTSA	2.5
PPY10 ^R	150	40	Wet-b	0.0250 g/4 ml buffer	24	PTSA	2.5
PPY11	1	40	Wet-b	0.0250 g/4 ml buffer	24	SDS	2.5
PPY12	1	40	Wet-b	0.0250 g/4 ml buffer	24	PTSA	2.5
PPY12 ^R	1	40	Wet-b	0.0250 g/4 ml buffer	24	PTSA	2.5

After performing preliminary experiments with PPY films, the second part of the experiments performed with PPY films took place. In the second part, only original Fluka enzyme was used and the PPY films were introduced to the immobilization experiments on

the electrode they were synthesized. The experimental conditions of the second and the actual part of experiments on PPY films are given in Table 3.5.

3.5.3. SFI Experiments with PU/PPY Composites

The experiments performed with PU/PPY foams were also divided in two parts: preliminary experiments performed with ready made polymers supplied by Prof. Can Erkey (Koç University) and main experiments performed with home made polymers. PU/PPY samples of 9×6 mm were cut from ready made composites of 25.4×25.4 mm size for preliminary experiments. For the rest of the study, approximately 10×6×3.6 mm samples prepared by strip geometry were used with no further cutting. The details of strip geometry is given in Section 3.3.1.2. In all the experiments performed with PU/PPY composites, GOX (Fluka) with 200 U/g activity was used. Based on experience from PPY films, only buffer medium was utilized in SFI experiments with PU/PPY composites and the concentration of the GOX-buffer solution was 6.25 mg/ml, prepared by dissolving 0.0125 g GOX in 2 ml pH 5.7 phosphate buffer.

In the “preliminary experiments”, as it is mentioned, the samples were cut from a larger sample and the position of the sample relative to the large sample was also important. Therefore, the position of the sample (9×6 mm) from where it was cut is also given in Table 3.6. Figure 3.5 represents what corner, inner and side positions mean in the ready made PU/PPY composite foam samples. The corner samples were exposed to I₂ and pyrrole vapor more than the side and inner samples, thus the PPY formation on that samples were higher. The effect of position of the sample and the effect of extent of PPY formation on the immobilization process were also investigated in the experiments depicted in Table 3.6.

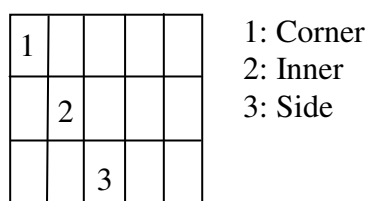


Figure 3.8. Schematic representation of ready made PU/PPY composite foam

Table 3.6. Preliminary SFI experiments performed on PU/PPY composite foams

Run No.	P (bar)	T (°C)	Type of experiment	Enzyme amount	Imm. period (h)	The position of the sample
PU/PPY1 ^{PR}	150	40	Wet-b	0.0125 g/2 ml buffer	24	Inner
PU/PPY2 ^{PR}	150	40	Wet-b	0.0125 g/2 ml buffer	24	Side
PU/PPY3 ^{PR}	1	40	Wet-b	0.0125 g/2 ml buffer	24	Side
PU/PPY4 ^{PR}	105	40	Wet-b	0.0125 g/2 ml buffer	24	Side
PU/PPY5 ^{PR}	105	40	Wet-b	0.0125 g/2 ml buffer	24	Corner
PU/PPY6 ^{PR}	75	40	Wet-b	0.0125 g/2 ml buffer	24	Corner
PU/PPY7 ^{PR}	75	40	Wet-b	0.0125 g/2 ml buffer	24	Inner
PU/PPY8 ^{PR}	105	40	Wet	0.0125 g/2 ml buffer	24	Inner
PU/PPY9 ^{PR}	150	40	Wet-b	0.0125 g/2 ml buffer	24	Corner
PU/PPY5 ^{PR} -2 nd	105	40	Wet-b	0.0125 g/2 ml buffer	24	Corner
PU/PPY6 ^{PR} -2 nd	75	40	Wet-b	0.0125 g/2 ml buffer	24	Corner
PU/PPY9 ^{PR} -2 nd	150	40	Wet-b	0.0125 g/2 ml buffer	24	Corner

Runs PU/PPY1^{PR} and PU/PPY2^{PR} are seem to be replicates but they are not since the depressurization of PU/PPY2^{PR} was 10 times faster (30 bar/min) than the depressurization step of PU/PPY1^{PR} (3 bar/min). The GOX immobilized polymer samples obtained from the experiments given in Table 3.6 were washed in 2 ml buffer after immobilization, before enzymatic analysis.

In the “preliminary experiments”, the immobilized GOX amounts were not determined since the immobilization solution became brown-black after the immobilization period. Therefore, it was decided to wash the polymer samples prior to immobilization process. As a first attempt, they were washed with water at 40 °C for 30 h, and immobilization was performed at different GOX-buffer solution concentrations to optimize the immobilization solution concentration.

Table 3.7. SFI experiments performed on PU/PPY composite foams to optimize the immobilization concentration

Run No.	P (bar)	T (°C)	Type of experiment	Imm. solution concentration (mg/ml)	Imm. period (h)	Wash. period after imm. (h)
PU/PPY1	100	40	Wet-b	6.25	24	10
PU/PPY1 ^R	100	40	Wet-b	6.25	24	10
PU/PPY2	100	40	Wet-b	0.625	24	10
PU/PPY3	100	40	Wet-b	0.0625	24	10

Table 3.8. SFI experiments performed on PU/PPY composite foams to optimize the washing step before immobilization, 100 bar, 40 °C

Run No.	Imm. solution concentration (mg/ml)	Imm. period (h)	Washing before imm.	Wash. period after imm. (h)
PU/PPY1	6.25	24	Water, 30 h	10
PU/PPY1 ^R	6.25	24	Water, 30 h	10
PU/PPY4	6.25	24	-	10
PU/PPY4 ^R	6.25	24	-	10
PU/PPY5	6.25	24	Buffer, 30 h	10
PU/PPY5 ^R	6.25	24	Buffer, 30 h	10
PU/PPY6	6.25	24	Buffer, 30 h + 24 h CO ₂ +buffer	10
PU/PPY6 ^R	6.25	24	Buffer, 30 h + 24 h CO ₂ +buffer	10

The next attempt was to optimize the washing step before the immobilization process. The experiments performed on PU/PPY composite foams to optimize the washing type are represented in Table 3.8. Since the rest of the experiments were done in buffer medium, the type of the experiment is not stated in the tables. The PU/PPY samples used in Runs PU/PPY1 and PU/PPY1^R were washed in 3 ml of water at 40 °C for 30 h. The PU/PPY samples used in Runs PU/PPY5 and PU/PPY5^R were washed in 3 ml of buffer at 40 °C for 30 h. The PU/PPY samples used in Runs PU/PPY6 and PU/PPY6^R were first washed in 3 ml of buffer at 40 °C for 30 h, then they were put in new unused buffer solution (3 ml) and then exposed to scCO₂ at 100 bar and 40 °C for 24 h.

Table 3.9. SFI experiments performed on PU/PPY composite foams to optimize the washing step after immobilization, 100 bar, 40 °C

Run No.	Imm. solution concentration (mg/ml)	Imm. period (h)	Washing before imm.	Wash. period after imm. (h)
PU/PPY7	0.0625	24	Buffer, 30 h	4
PU/PPY7 ^R	0.0625	24	Buffer, 30 h	4
PU/PPY8	0.0625	24	Buffer, 30 h	10
PU/PPY8 ^R	0.0625	24	Buffer, 30 h	10

Table 3.10. SFI experiments performed on PU/PPY composite foams to optimize the immobilization period, 100 bar, 40 °C

Run No.	Imm. solution concentration (mg/ml)	Imm. period (h)	Washing before imm.	Wash. period after imm. (h)
PU/PPY8	0.0625	24	Buffer, 30 h	10
PU/PPY8 ^R	0.0625	24	Buffer, 30 h	10
PU/PPY9	0.0625	4	Buffer, 30 h	10
PU/PPY9 ^R	0.0625	4	Buffer, 30 h	10
PU/PPY10	0.0625	72	Buffer, 30 h	10
PU/PPY10 ^R	0.0625	72	Buffer, 30 h	10

After the experiments in Table 3.8 were performed, it was seen that washing the samples first with buffer and then with buffer+scCO₂ gave better results than other washing methods, but the total time of washing a sample took more than two days. Therefore in order to optimize the experimental time, in Runs between PU/PPY7-10 the PU/PPY samples were washed with buffer only (Tables 3.9 and 3.10). However, some problems were still encountered in Bradford analysis, thus two more experiments with their replicates were performed in order to change the washing step before immobilization. These experiments are represented in Table 3.11. In these experiments the PU/PPY samples are washed in only 3 ml of buffer at 50 °C for 30 h or in 3 ml of buffer under scCO₂ environment at 150 bar and 50 °C (the highest pressure and temperature used in immobilization experiments).

Table 3.11. SFI experiments performed on PU/PPY composite foams to further optimize the washing step before immobilization, 100 bar, 40 °C

Run No.	Imm. solution concentration (mg/ml)	Imm. period (h)	Washing before imm.	Wash. period after imm. (h)
PU/PPY11	0.0625	24	Buffer	10
PU/PPY11 ^R	0.0625	24	Buffer	10
PU/PPY12	0.0625	24	CO ₂ +buffer	10
PU/PPY12 ^R	0.0625	24	CO ₂ +buffer	10

Table 3.12. SFI experiments performed on PU/PPY composite foams to investigate the effect of temperature and pressure, Imm. period: 24 h

Run No.	P (bar)	T (°C)	Imm. solution concentration (mg/ml)	Washing before imm.	Wash. period after imm. (h)
PU/PPY13	80	40	0.0625	CO ₂ +buffer	4
PU/PPY13 ^R	80	40	0.0625	CO ₂ +buffer	4
PU/PPY14	100	40	0.0625	CO ₂ +buffer	4
PU/PPY14 ^R	100	40	0.0625	CO ₂ +buffer	4
PU/PPY15	150	40	0.0625	CO ₂ +buffer	4
PU/PPY15 ^R	150	40	0.0625	CO ₂ +buffer	4
PU/PPY16	1	40	0.0625	CO ₂ +buffer	4
PU/PPY16 ^R	1	40	0.0625	CO ₂ +buffer	4
PU/PPY17	80	30	0.0625	CO ₂ +buffer	4
PU/PPY17 ^R	80	30	0.0625	CO ₂ +buffer	4
PU/PPY18	100	30	0.0625	CO ₂ +buffer	4
PU/PPY18 ^R	100	30	0.0625	CO ₂ +buffer	4
PU/PPY19	150	30	0.0625	CO ₂ +buffer	4
PU/PPY19 ^R	150	30	0.0625	CO ₂ +buffer	4
PU/PPY20	1	30	0.0625	CO ₂ +buffer	4
PU/PPY20 ^R	1	30	0.0625	CO ₂ +buffer	4
PU/PPY21	100	50	0.0625	CO ₂ +buffer	4
PU/PPY21 ^R	100	50	0.0625	CO ₂ +buffer	4

The experiments performed with PU/PPY films are given in Table 3.12. Since no or very low activity was observed from these experiments, further runs to investigate the effect of process parameters was not proceed.

Table 3.13. SFI experiments performed on PU/PPY composite films, Imm. period: 24 h

Run No.	P (bar)	T (°C)	Imm. solution concentration (mg/ml)	Washing before imm.	Wash. period after imm. (h)
PU/PPYF1	100	40	6.25	Buffer	2
PU/PPYF2	100	40	0.0625	Buffer	2
PU/PPYF3	100	40	6.25	Buffer, then CO ₂ +buffer	0.5
PU/PPYF3 ^R	100	40	6.25	Buffer, then CO ₂ +buffer	0.5
PU/PPYF4	100	40	6.25	-	0.5
PU/PPYF4 ^R	100	40	6.25	-	0.5

4. FREE ENZYME EXPERIMENTS

In order to understand the interaction between scCO₂ and GOX, first the solubility of GOX in scCO₂ was investigated. Additionally, the effects of temperature (30-60 °C) and pressure (scCO₂) (80, 100 and 150 bar) on the activity of GOX-buffer solution were studied. It was observed that the GOX amount in GOX-buffer solution decreased when the solution exposed to scCO₂. The effect of scCO₂ on the decrease in GOX amount in GOX-buffer solution was also determined at 30, 40 and 50 °C and at 80, 100, and 150 bar.

4.1. Preliminary Studies on Solubility Investigation

In spite of very low possibility to observe a detectable solubility of free GOX in scCO₂, several methods were used to investigate it. In the first method, only free GOX were exposed to scCO₂, but in the second method there was not only GOX, but also a polymer matrix existed in the supercritical environment. In this second method, the aim was both to investigate the solubility of GOX in scCO₂, and also perform preliminary impregnation studies with non-conducting polymers. In Run FE1, the Rapid Expansion of Supercritical Solutions (RESS) apparatus (details can be found in Kayrak, 2001, Kayrak *et al.*, 2003) was utilized. The general process in RESS is that, a known amount of solute is placed in an extraction cell and the cell is pressurized with scCO₂ at the desired temperature, after solute is dissolved in scCO₂, the supercritical solution (solute-scCO₂) obtained is expanded through a nozzle. The rapid expansion of supercritical solution causes a decrease in solvent power of CO₂ and the solute dissolved in the supercritical solution can be collected as solid particles after the nozzle expansion. The particle collection was done at the extraction pressure by supplying CO₂ during expansion. Particles were collected for the desired time period on a glass slide placed in the precipitation unit. The extraction part of the RESS apparatus is same as the one shown in Figure 3.1. In Run FE1 1.0 g of GOX (Delta) was put in a bag made of filter paper and placed in the extraction cell and kept in the extraction cell with scCO₂ at 180 bar 40 °C for 5 h. If GOX was dissolved in scCO₂, then it was assumed that it would be collected in the precipitation cell after expansion of the solution through the nozzle. After keeping the supercritical solution for 5 h in the extraction cell, the solution was then expanded through

expansion nozzle for 30 min at 180 bar. During the collection time, there was no clear evidence of collection of GOX particles, there were only brown particles at the tip of the nozzle. The expansion apparatus was heated to 100 °C in order to avoid it from freezing and clogging. Therefore the particles seen at the tip of the nozzle can be GOX particles which turned out to be brown because of heat supplied to the nozzle. Additionally, if GOX was dissolved in scCO₂ with very low solubility, then the GOX could precipitate along the tubings of the apparatus before arriving to the nozzle, thus no GOX particles would be collected. As a result, there was no clear evidence of GOX solubility in scCO₂ at the end of Run FE1 performed by the RESS apparatus.

In Run FE2, 0.1 g of GOX (Fluka) was put in a filter paper bag, weighed and placed in the pressure cell. Prior to Run FE2, the filter paper bag used in the experiment was also subjected to scCO₂ at 40 °C and 150 bar for 24 h in order to clean the filter paper. Any residue and extractable component in the filter paper will be misleading during the gravimetric analysis. The GOX in the filter paper was kept at the pressure vessel at 100 bar 40 °C for 24 h. After depressurization, the GOX in the filter paper bag was taken out of the pressure cell and weighed immediately. If scCO₂ dissolved a detectable amount of GOX, then there would be a decrease in GOX amount. This was investigated by gravimetric analysis. The weight of the GOX+filter paper was prior to scCO₂ exposure was 0.1679 g and after the run GOX+filter was weighed as 0.1677 g. The result was that there was 200 µg decrease (0.12 per cent) in GOX amount showing the possible solubility of GOX in scCO₂.

In Run FE3 and FE3^R, 4 g of GOX (Delta) was put in a filter paper and placed in the pressure cell. A non-conducting polymer matrix, which was known to swell in scCO₂, was also placed in the pressure vessel, i. e. polymethylmethacrylate (PMMA). PMMA rods with 3.5×3.5×20 mm dimensions were used. The weight of PMMA rods were measured before runs. While keeping the GOX+filter paper and PMMA rod in the pressure cell at 150 bar, 40 °C for 5 h, the PMMA rod swollen and if GOX would dissolve in scCO₂, then it should diffuse into the polymer matrix with CO₂ and then upon the depressurization, solvent power of CO₂ disappeared and the GOX particles would impregnated into the polymer matrix. After depressurization, the PMMA rod was taken out of the pressure cell and its weight was recorded during one month. At the end of the month, if the weight of the PMMA rod was higher than its initial weight prior to the run, then the difference

between the initial and the final weight of the PMMA rod gave the impregnated amount of GOX.

The results of Run FE3 is given in Table 4.1 and Figure 4.1. As the results indicate, the final weight of PMMA rod was 0.2 mg higher than its initial value, which was the amount of GOX impregnated into the polymer matrix. When this data was converted into the amount of solute (mg) per amount of polymer matrix (g), it was found that the mass gain of PMMA was 0.87 mg/g. At the same experimental conditions, if the experiment was done with a more soluble solute, i.e. naphthalene, it was found that the mass gain of PMMA rod was nearly 210 mg/g (Üzer, 2004). In Run FE3^R, no mass gain was measured at the end of one month, but for both FE3 and FE3^R, it was visually observed that, the color of the PMMA rod became light yellow in color. Knowing that the color of GOX was yellow, it was shown that GOX can be transferred to the polymer matrix when it was kept in a filter paper, even its solubility in scCO₂ is extremely low.

Table 4.1. The weight change of GOX impregnated PMMA rod

Measuring time	Weight of PMMA rod (g)
Prior to run	0.2296
Immediately after the run	0.2674
3 rd day after the run	0.2394
4 th day after the run	0.2372
7 th day after the run	0.2344
11 th day after the run	0.2328
12 th day after the run	0.2327
14 th day after the run	0.2319
17 th day after the run	0.2313
20 th day after the run	0.2308
21 th day after the run	0.2307
29 th day after the run	0.2299
32 th day after the run	0.2298

GOX particles were also visualized by ESEM. Figure 4.2 shows the ESEM images of the GOX obtained from Delta and Fluka.

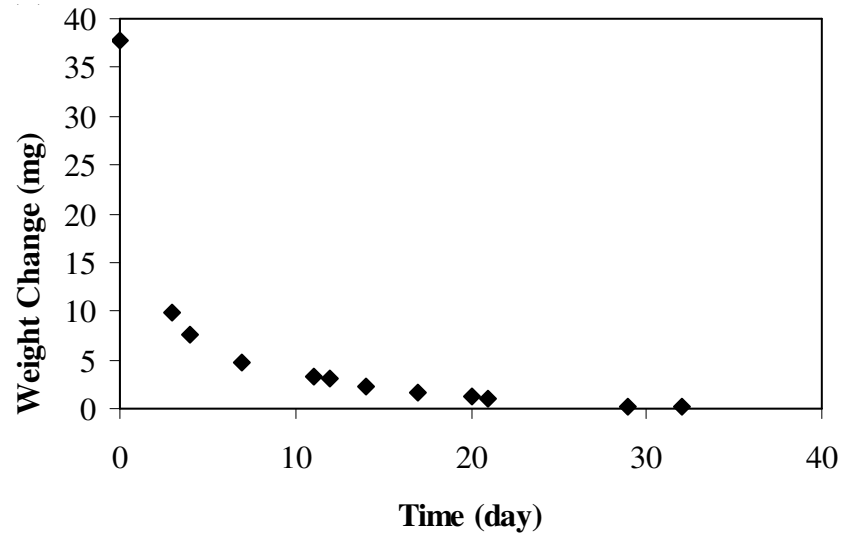


Figure 4.1. Weight change observed for GOX impregnated PMMA matrix following the impregnation process

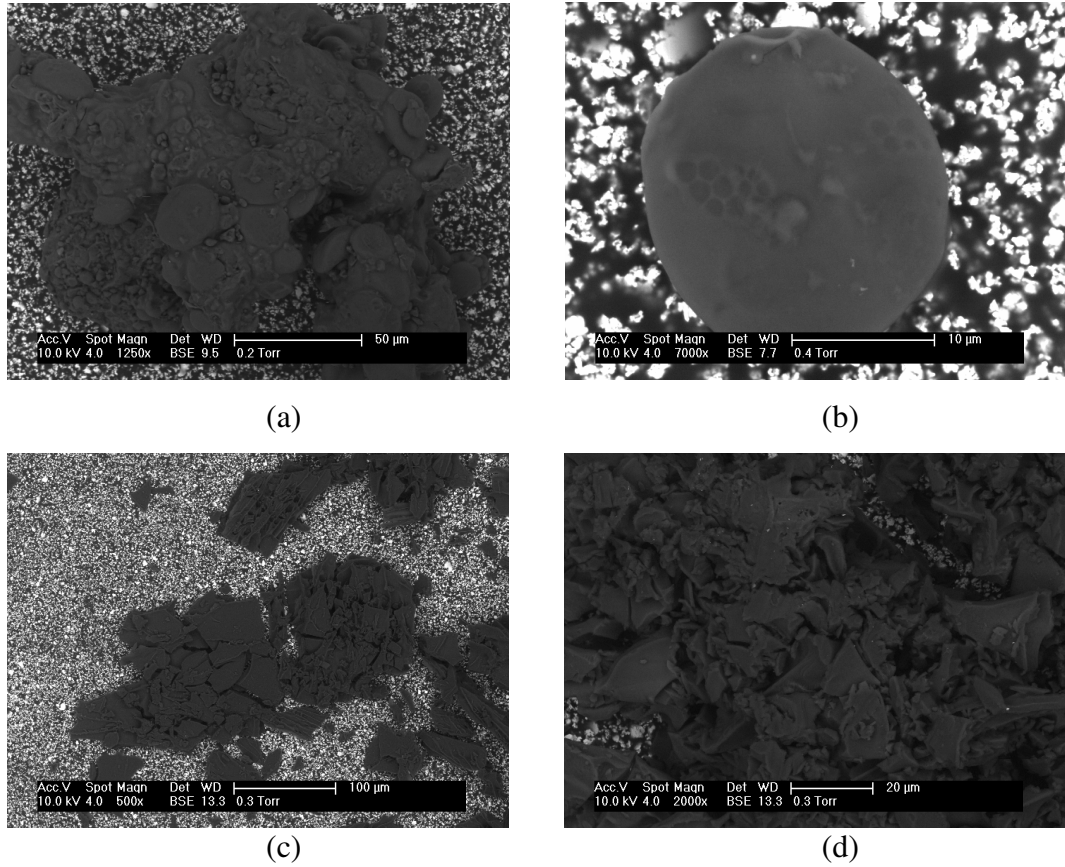


Figure 4.2. ESEM images of GOX powder, (a) and (b) are images of GOX obtained from Delta Gıda A. Ş. and (c) and (d) are images of GOX from Fluka

4.2. Effect of Temperature on GOX-buffer Solution

The effects of temperature and pressure on GOX-buffer solution were investigated in order to better understand the behavior of the system studied, before starting the immobilization process under scCO_2 . GOX-buffer (pH 5.7 potassium phosphate buffer) solution was left in a water bath at different temperatures between 30-60 °C for 24 h and then the activity of the solution was measured. Figure 4.3 reveals the effect of temperature on GOX-buffer solution. The data shown on Figure 4.3 are the averages of three enzymatic analyses. For the averaged data at 30, 40, and 50 °C, the standard deviations range between 1.42-2.59, 2.42-3.91, and 1.78-4.85 U/mg at 5, 10, and 20 mM glucose concentrations, respectively. The results are discussed on the basis of activity values evaluated at 20 mM glucose solution, because at lower glucose concentrations the difference between activity values were not distinguishable. When only average values are taken into account, as it is seen on Figure 4.3, there is a slight increase in the GOX-buffer solution activity when the solution was subject to 30 and 40 °C. The increases in activity were 8.2 and 2.4 per cent for 30 and 40 °C, respectively. The activity values at 20 mM glucose concentration are shown in Figure 4.4 with standard deviations. The line at 100 per cent indicates the activity of original GOX-buffer solution which was not subject to external possible denaturing effects such as heat and pressure. The dashed lines show the standard deviation from activity of original enzyme. Figure 4.4 depicts that keeping GOX-buffer solution at 40 and 50 °C for 24 h did not lead to activity decrease. Only keeping GOX-buffer solution at 30 °C for 24 h caused a slight increase in activity even when the standard deviations are taken into account. The activity of GOX basically depends on its structure, conformation and flexibility, but also on the environment around cofactor Flavin Adenine Dinucleotide (FAD) (Gouda *et al.*, 2003), and the hydration of the enzyme (Yang *et al.*, 2004). The hydration shell around a dissolved protein is depicted in Figure 4.5. Water does not act only as a solvent for reactant and products, but also takes part in the reaction. The slight enhancement in activity after kept at 30 °C for 24 h may be attributed to the increase of hydration shell around the enzyme, which may facilitate the reaction.

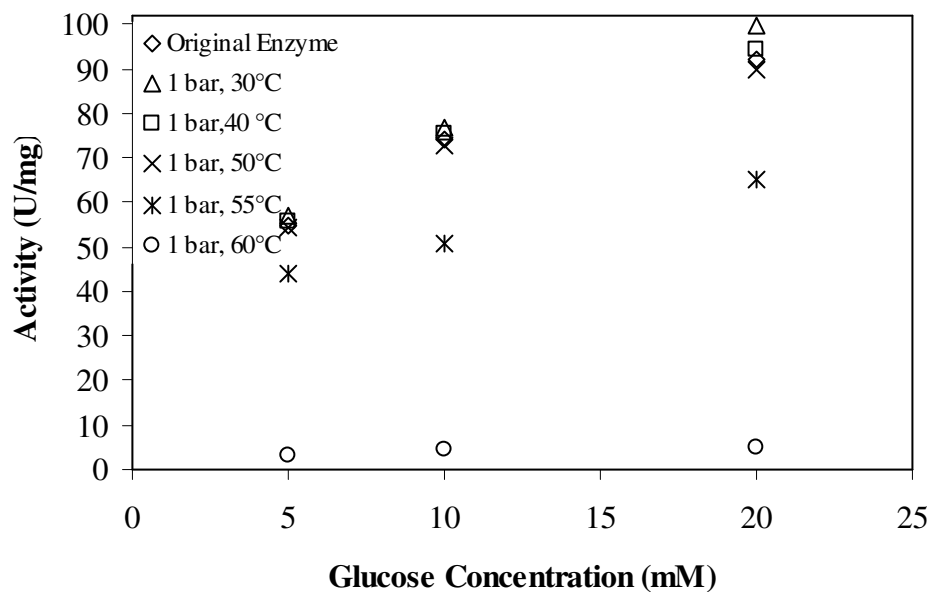


Figure 4.3. Activities of GOX-buffer solution which were exposed to different temperatures

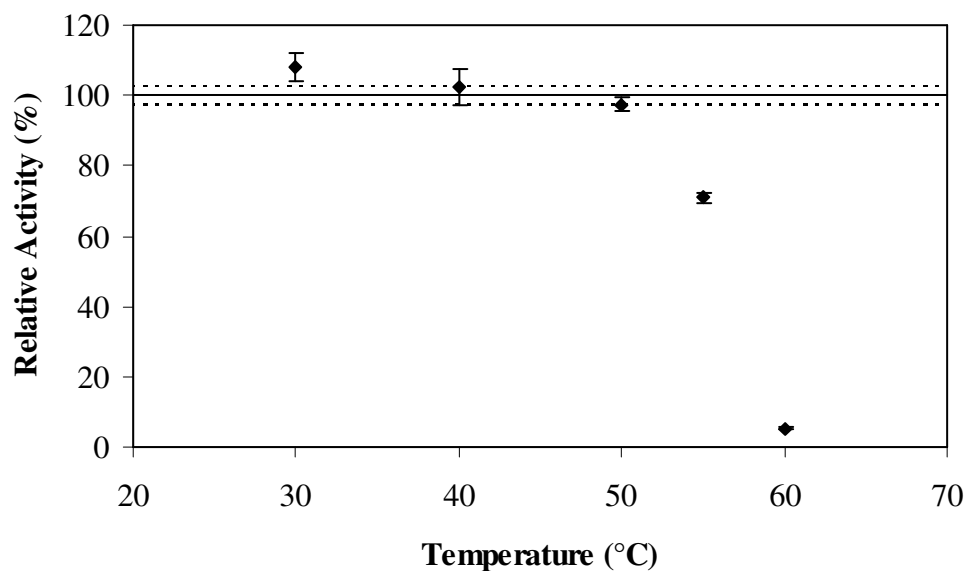


Figure 4.4. Activities, evaluated at 20 mM glucose concentration, of GOX-buffer solutions exposed to different temperatures are given relative to the original GOX-buffer solution, duration of exposure: 24 h, concentration of GOX solution: 6.25 mg/ml

However, as compared to the original GOX-buffer solution, the activities of the solutions kept at 55 and 60 °C decreased by 29 and 95 per cent respectively. The thermal

inactivation of GOX was mainly attributed to the dissociation of cofactor FAD, followed by conformational changes, unfolding and nonspecific aggregation of the enzyme (Gouda *et al.*, 2003). Additionally, the original clear yellow GOX-buffer solution became slightly cloudy after exposure to 55 °C and cloudy at 60 °C. Heat denaturated proteins are prone to aggregation (Heremans and Smeller, 1998) and aggregation can cause gelation. The cloudiness in GOX-buffer solutions, which were subjected to 55 and 60 °C for 24 h, can be attributed to aggregation and possible gelation.

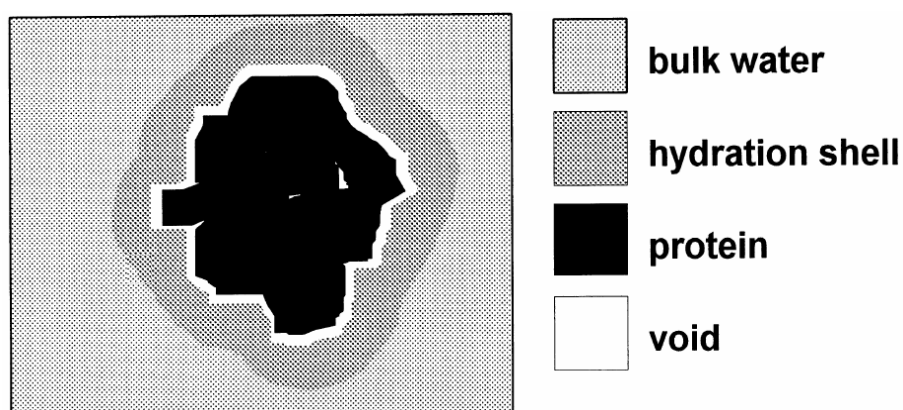


Figure 4.5. Schematic representation of the volumes associated with the dissolved protein (Heremans and Smeller, 1998)

4.3. Effect of Pressure on GOX-buffer Solution

Although the title of this section indicates that the concern of the section is on the effect of pressure on GOX-buffer solution, the pressure term also includes the effect of CO₂ on GOX-buffer solution. The effect of pressure formed by CO₂, and CO₂ itself can have separate effects on GOX-buffer solution. scCO₂ may change the micro environment of the enzyme and also may interact with the amino acid residues of the enzyme. Figure 4.6 shows the possible interactions between the components of the system.

The effect of scCO₂ on the activity of free dry GOX was investigated by Tanugichi *et al.* (1987). They exposed the non-aqueous GOX and some other enzymes to scCO₂ at 20 MPa and 35°C for one hour. The authors also added three per cent ethanol to the non-aqueous enzyme mixture. In both cases (non-aqueous and non-aqueous-3 per cent ethanol) the residual activities after scCO₂ treatment were measured. They reported that the residual activities were 97 and 93 per cent for non-aqueous and non-aqueous+3 per cent ethanol

mixture of GOX, respectively. In preliminary SFI studies on PPY films, buffer medium was found to be the best medium. Thus the effect of scCO₂ on GOX-buffer solution had to be examined, which was performed in this study since there was no open literature data that elucidates the effect of scCO₂ on GOX-buffer solution. The effect of scCO₂ was investigated by determining the enzymatic activity and the protein amount of the GOX-buffer solution after exposure. Two sets of experiments were performed, one set for activity determination and then one set for protein determination (Tables 3.2 and 3.3). The GOX-buffer solution concentrations were different for these two set of runs.

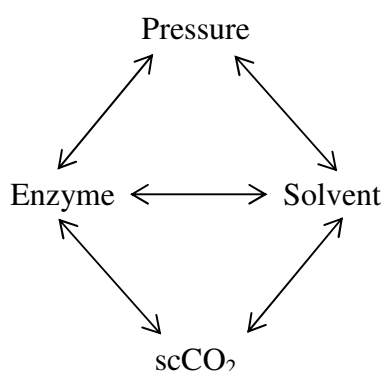


Figure 4.6. Schematic representation of the possible interactions

The effect of scCO₂ and pressure on the activity of the solution were investigated after the solution was subjected to scCO₂. All the solutions were subjected to scCO₂ for 24 h at 40 °C with GOX concentration of 6.25 mg/ml at different pressures (80, 100 and 150 bar). The concentration of GOX-buffer solution was chosen as 6.25 mg/ml, because initial SFI experiments started using GOX-buffer solution of 6.25 mg/ml based on the literature values. Figure 4.7 shows the effect of scCO₂ on the activity of free-enzyme buffer solution. The data shown are the averages of three enzymatic analyses. For these averaged data, the standard deviations range between 0.56-3.24, 1.30- 4.09, and 2.28-11.53 U/mg at 5, 10, and 20 mM glucose concentrations, respectively. As it seen from Figure 4.7, the difference between the activity values became more pronounced at high substrate concentrations, i. e. at 100 mM glucose concentration. Therefore the data at 100 mM glucose concentration, which is shown on Figure 4.7, is presented in detail with error bars in Figure 4.8. The line at 100 per cent indicates the activity of original GOX-buffer solution which was not subject to possible external denaturing effects such as heat and pressure. The dashed lines show the standard deviation from activity of original enzyme. Figure 4.8 depicts that the

residual activities of the GOX-buffer solutions which were exposed to 1, 80 and 150 bar, did not decrease, and even increased. Based on the average values, the increase in activity compared to the original GOX-buffer solution were 1.6, 6.6, and 6.2 per cent for 1, 80 and 150 bar, respectively. Only at 100 bar, there was a slight decrease in residual activity (4.3 per cent based on average values), but it was still in the confidence interval of the original solution. Lozano *et al.* (1996) reported that for immobilized α -chymotrypsin, at 40 °C, the residual activity after it was exposed to scCO₂ was increased as the pressure increased from 80 to 150 bar. In our case, the residual activities at 80 and 150 bar are nearly the same but the lower residual activity at 100 bar was an exception, therefore residual activity and density of scCO₂ cannot be related. Based on the average values and standard deviations of data points, it can be said that at 40 °C, for 24 h up to 150 bar, scCO₂ had no negative effect on the original enzyme activity.

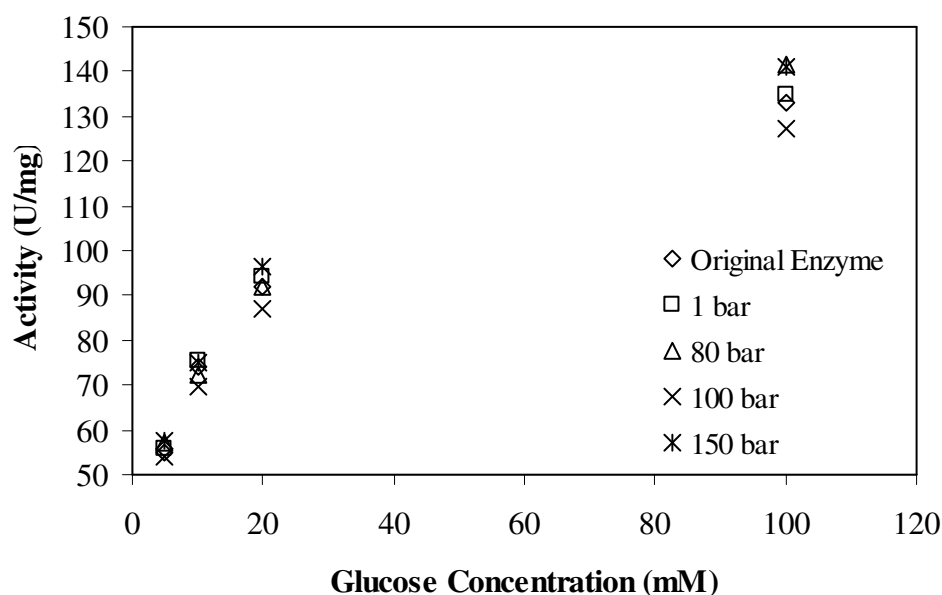


Figure. 4.7. Activities of GOX-buffer solutions which were exposed to different pressures, 40 °C, duration of exposure: 24 h, concentration of GOX solution: 6.25 mg/ml

Pressure is known to have a denaturizing effect on enzyme activity, and there are several review papers discussing the effect of high pressure on intra and intermolecular interactions, structure and functionality of proteins (Messens *et al.*, 1997, Heremans and Smeller, 1998, Boonyaratanakornkit *et al.*, 2002). However, in these studies, the pressure values that is said to cause changes in protein structure are much higher than the values

applied in typical scCO₂ studies. In this study, the maximum pressure applied was 150 bar, but Messens *et al.* (1997) stated that the tertiary and secondary structures of proteins can be significantly modified at pressures greater than 2000 bar.

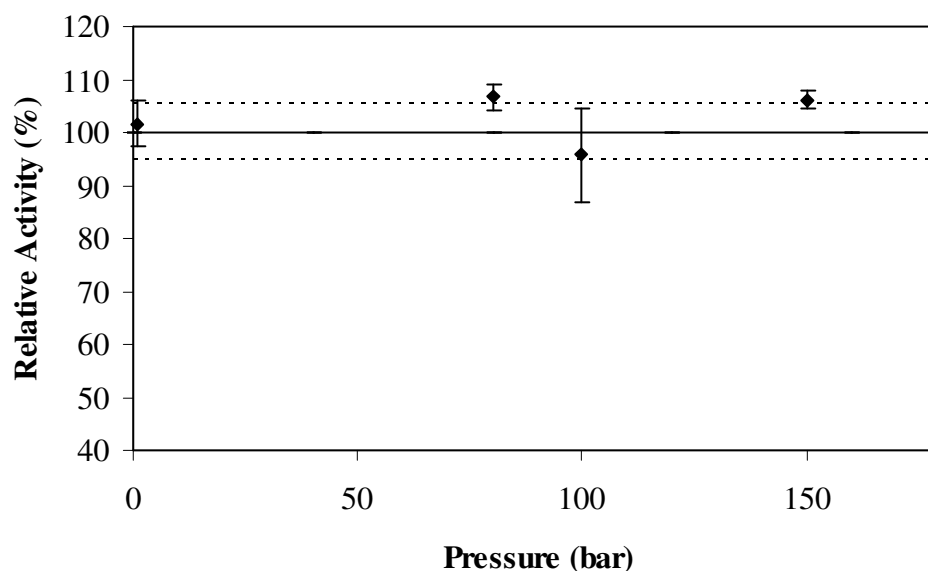


Figure. 4.8. Pressure exposed GOX-buffer solution activities, evaluated at 100 mM glucose concentration, are given relative to the original GOX-buffer solution, T: 40 °C, duration of exposure: 24 h, concentration of GOX solution: 6.25 mg/ml

There are many studies, which use the supercritical fluids as reaction medium and immobilized enzymes such as lipase (Celia *et al.*, 2005) and cutinase (Garcia *et al.*, 2005) as catalyst. In these studies the activity and performance of the enzymes under supercritical conditions were measured. In some studies, immobilized enzymes are exposed to supercritical fluids and then their activities after exposure were reported (Lozano *et al.*, 1996, Celia *et al.*, 2005, Oliveira *et al.*, 2006a, Oliveira *et al.*, 2006b). Investigations were concentrated on the effect of supercritical fluids on the activity immobilized enzymes, but the effect of supercritical fluids on free enzymes were also studied. The effect of supercritical fluids on free lipase and proteinase were represented by some authors (Giessauf and Gamse, 2000, Habulin *et al.*, 2000, Habulin *et al.*, 2005) as it is done in our case. Giessauf and Gamse (2000) exposed crude pancreatic lipase preparations to scCO₂ at 75 °C and 150 bar. The results showed that as time increased enzyme activity also increased. After 24 h of scCO₂ treatment, they observed a maximum relative activity of 860 per cent. Habulin *et al.* (2000) exposed lipases from different sources to scCO₂ and

near-critical propane at the pressure of 300 bar and 40 °C temperature for 24 h. Their results showed that there were no activity changes for any of the lipases when exposed to scCO₂ and near-critical propane. Habulin *et al.* (2005) reported that in scCO₂ at 300 bar, thermal activation of the examined proteinase was enhanced compared to the ones conducted at atmospheric pressure. However, in propane and dimethyl-ether at 300 bar pressure the activity of the proteinase decreased.

A few researchers studied the conformation of several enzymes in supercritical solvents (Randolph *et al.*, 1988, Kasche *et al.*, 1988, Zagrobelny and Bright, 1992). Randolph *et al.* (1988) studied the conformational changes of cholesterol oxidase in scCO₂ using electron paramagnetic resonance spectroscopy. They found that there was no significant conformational changes in the enzyme from *Gloeocysticum chrysocreas* as a function of CO₂ pressure or cosolvent addition. However, Kasche *et al.* (1988) reported that monomeric enzymes α -chymotrypsin, trypsin and oligomeric enzyme penicillin amidase were found to be partly denatured during the depressurization step. The degree of denaturation was larger in humid CO₂ than in dry CO₂, and denaturation was less for α -chymotrypsin and trypsin (enzymes with S-S bridges) than penicillin amidase (the enzyme without cysteine). They stated that the denaturation was caused by partial unfolding during the depressurization step depending on their results and also by indications of electrophoretic and spectroscopic analysis. In both of these studies, the enzymes were analyzed after scCO₂ exposure. Zagrobelny and Bright (1992) studied the in situ conformation of trypsin as it was in scCO₂ by steady-state fluorescence spectroscopy. Their results indicated that decompression has a very little effect on the conformation which was contrary the results of Kasche *et al.* (1988). According to Zagrobelny and Bright (1992), most of the conformational change in trypsin occurred during pressurization step and the enzyme segments that undergo significant environment changes may not be associated with the active site. From these studies, it is understood that there is no general statement which elucidates the effect of CO₂ on enzyme structure and function. But, in our study, no adverse effect of pressure was noticed, and even a slight increase in residual activity was observed at 80 and 100 bars. Additionally, it should be noted that in most of the work done on enzyme-scCO₂, there was no or only a trace amount of water, but in our work the effect of scCO₂ on GOX-buffer solution was investigated where high concentration of water existed.

Beside the enzymatic activity analysis of the GOX-buffer solution, the protein amount in the solution was also measured. For this purpose, 2 ml of original GOX-buffer solutions (0.0625 mg/ml) were subjected to scCO₂ at 30, 40 and 50 °C and 80, 100 and 150 bar for 24 h. The protein amount of GOX-buffer solutions were determined before and after exposure to scCO₂ by the Bradford method and the difference between these protein amounts gave us the decrease in the amount of GOX. Regardless of the temperature and pressure, it was observed that the protein amount always decreased after exposure to scCO₂. The decrease in GOX amount at different temperature and pressures are given in Figure 4.9. For simplicity, in the text, the decrease in GOX amount is called “extracted GOX amount”, although it may not be really “extracted”. As it is seen from Figure 4.9, at constant temperature (30 and 40 °C) as the pressure increased from 80 to 100 bar, extracted GOX amount also increased, whereas further increase in pressure to 150 bar caused decreases in extracted GOX amount by scCO₂. At constant pressures of 100 and 150 bar, as the temperature increased from 30 to 40 °C, the extracted amounts of GOX decreased, possibly due to the decrease in CO₂ density. However, the extracted amount of GOX enhanced significantly at 100 bar when the temperature was increased from 40 to 50 °C, although the density of scCO₂ decreased. At 80 bar, as the temperature increased from 30 to 40 °C, the extracted amount of GOX also increased, showing that the CO₂ density was not the dominant effect on “extracted” GOX amount.

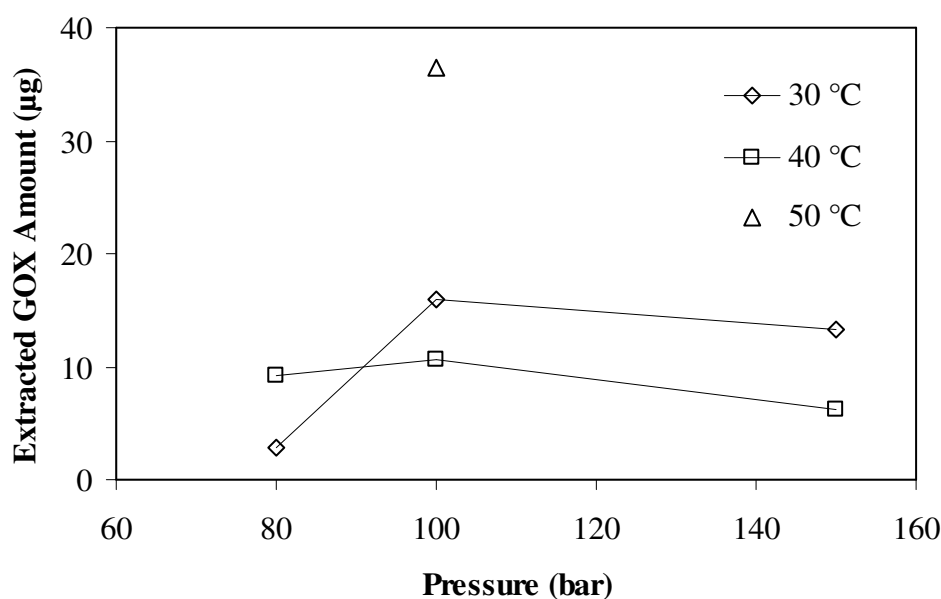


Figure 4.9. The decrease measured in protein amount after scCO₂ exposure at different temperatures and pressures

There may be three independent or combined possible reasons for the observed decreases in GOX amounts. Although, the proteins are nearly insoluble in scCO_2 , the possibility that scCO_2 can extract GOX still exists and then it means that the decrease in protein amount of solution is equivalent to the amount extracted by scCO_2 . The extraction or the modification of some part of the enzyme may also be responsible for the decrease in protein amount. GOX from *Aspergillus Niger* has 10 to 16 per cent (w/w) of carbohydrate moiety (Pazur *et al.*, 1965, Hayashi and Nakamura, 1981). The carbohydrate content of the enzyme may be extracted by scCO_2 , but this cannot be directly related to the protein amount found in the solution if the carbohydrate content directly takes place in protein determination step. In the Bradford protein determination method, Coomassie dye primarily binds with arginine residues and binds less with histidine, lysine, tyrosine, tryptophan, and phenylalanine residues. The interaction of scCO_2 with the mentioned amino acid residues which may cause irreversible changes and the possible extraction of carbohydrate content may also lead to unfavorable conformation of GOX for dye binding.

Giessauf and Gamse (2000) observed a weight loss (4.6 per cent) from the enzyme preparation after it was subjected to scCO_2 , but a slight decrease (from 15.3 to 14.9 per cent) in protein amount which they did not consider as a significant change. They suggested that the weight loss was because of the extraction of fatty acids in the crude porcine pancreatic lipase powder preparation. In this study, the decrease in protein amount was between 5 to 50 per cent compared to the original protein amount.

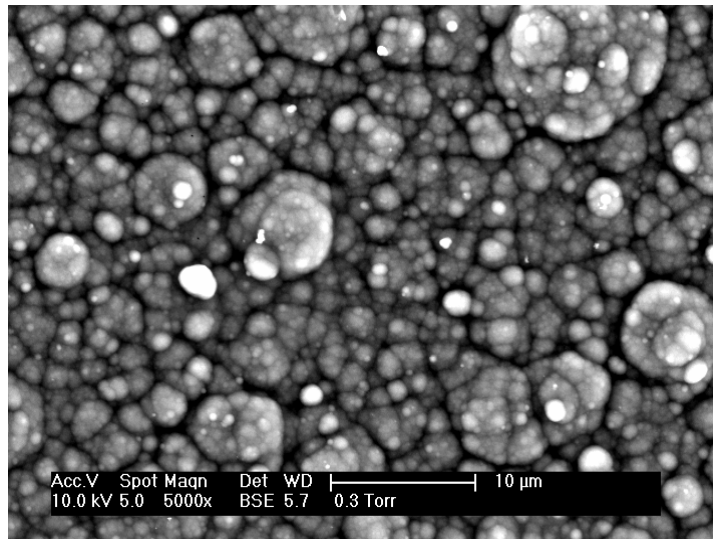
5. IMMOBILIZATION OF GOX ON PPY FILM

The most extensively studied conducting polymer is PPY. The SFI studies were started electrochemically synthesized PPY films. The effect of scCO_2 on PPY film was observed with ESEM. The preliminary and main SFI experiments were performed with PPY films. The preliminary experiments provided the determination of enzymatic activity method. Four different immobilization mediums were tested with PPY films since it was not known which immobilization medium would be appropriate for SFI. In the following sub-sections, the results of these three experiment groups will be covered.

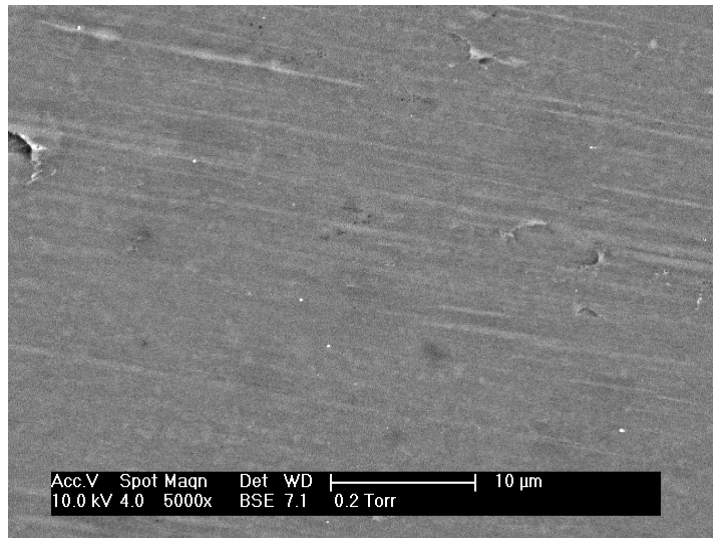
5.1. Effect of scCO_2 on PPY Film

In open literature, there is no previous study which investigated the relation between GOX-PPY- scCO_2 . In the previous section, the effect of scCO_2 on GOX was presented. In this section, before going in to the details of SFI of GOX on PPY film, initially, the effect of scCO_2 on PPY was investigated. The surface structures of PPY films were determined by ESEM before and after scCO_2 exposure. Figure 5.1 shows the solution and electrode side of the PPY film. The electrode side was a flat surface, but the solution side was just like cauliflower as it can be seen from Figure 5.1.

After exposure to scCO_2 , when PPY films were put in water, there were bubbles coming out of the PPY films, which were thought to be CO_2 bubbles. ESEM pictures indicated possible relaxations on the PPY surfaces after exposure to scCO_2 . The problem in working with the PPY film and scCO_2 was that if the PPY film was exposed to scCO_2 on the platinum electrode on which it was synthesized, the PPY film cracked during or after the experiment. Even though, no clear evidence of swelling of PPY by scCO_2 was noticed after the experiment, cracking may also reveal the possibility of swelling of PPY by scCO_2 .



(a)



(b)

Figure 5.1. ESEM images of a PPY film (a) solution side, (b) electrode side

5.2. The Results of Preliminary Experiments

PPY films were synthesized by electropolymerization on three different electrode sizes: 1.25, 2.5 and 100 cm². The experiments performed with PPY experiments were divided in two parts as indicated in Section 3.5 presenting the experimental program. The first part was performed mostly with commercial GOX obtained from Delta Gıda A.Ş. and PPY films synthesized on 100 cm² electrode. The immobilization procedure was examined by three different enzymatic analysis methods and ESEM images. The experiments in the first part were named as preliminary experiments and denoted as PPY^{PR}. In the second

part, experiments were performed with PPY films synthesized on 1.25 and 2.5 cm² electrodes. In the experiments of the second part, only pure GOX from *Aspergillus Niger* (200 U/mg, Fluka) was used and the enzymatic activity method developed according to Sigma Technical Bulletin, No 510 was utilized. The results of the experiments of the second part are denoted as PPY and are discussed in the following sections.

The preliminary experiments were mostly performed in order to investigate interaction between GOX-PPY-scCO₂, but the most important outcome of these experiments was the determination of the enzymatic analysis method that should be used in the evaluation of GOX immobilized conducting polymers. In preliminary experiments, as it is mentioned in the previous paragraph, PPY films which were synthesized on 100 cm² electrode were used. After film formation, the film was peeled off from the electrode and were cut into pieces of 2×2 cm to be used in experiments. Before use in immobilization experiments, they were washed in water for 24 h and then exposed to scCO₂ at 150 bar, 40 °C for 48 h. Then, the corresponding immobilization procedure, depending on the immobilization medium explained in Section 3.3.3, was followed. The amounts of GOX, water, buffer or ethanol used for the experiments were shown in Table 3.4.

The immobilization procedure was examined by enzymatic analysis, ESEM images, and gravimetric analysis. The enzymatic assay first used in preliminary experiments was dinitro-salicylic acid (DNSA) method (Miller, 1959). The aim was to react the GOX immobilized on the PPY films with a known amount of glucose solution and then to measure the glucose solution left in the solution after reaction. In DNSA method, 0.2 ml (2.5 g/l) of glucose solution, 0.6 ml of H₂O and 1.2 ml of buffer solution (either pH 5.6 potassium or citrate phosphate buffer solutions were used) were mixed. The PPY film was placed in this mixture and incubated for 30 min. Then 3 ml DNSA reagent were added and the solution was boiled for 10 min followed by cooling to room temperature in an ice bath. After the solution cooled down to room temperature, it was diluted by 8 ml of distilled water and the absorbance of the solution was read at 540 nm. A standard curve was prepared using known concentrations of glucose (0.4–2.0 g/l: in linear range). From the standard curve the concentration of glucose was determined.

In every analysis, the original glucose solution was analysed without enzyme and then original solution was reacted with GOX immobilized PPY films. The expected result was a decrease in glucose amount when GOX immobilized PPY films reacted with a known amount of glucose. However, when GOX immobilized PPY films were incubated with glucose and the resultant glucose amount was measured with DNSA method, it was found that the amount of glucose in the solution increased, which should not be the case. The same DNSA procedure was also applied for PPY films without GOX, the increase in glucose amount was also observed in that case. The reason for the increase in glucose amount may be the boiling process involved in the DNSA analysis. During boiling the PPY films were still in the reaction solution and they gave off their color and darken the solution. The PPY films were supposed to be taken out of the reaction solution before boiling process but it was impossible to remove the PPY films from the solution without causing any change in the reaction volume. Therefore it was decided to use another method for glucose determination. By the way, there was no pseudo increase in glucose amount when free enzyme was used.

The glucose amount in the solution was determined by HPLC (column: Bio-Rad Aminex[®] HPX-87P, 300 mm×7.8 mm, injection volume: 20 µl, column temperature: 80 °C) as an alternative method. Different reaction volumes (1 ml and 2 ml) and different free enzyme concentrations (1 mg/ml, 0.1 mg/ml and 0.01 mg/ml) were first tested. The glucose depletion at all enzyme concentrations were found to be detectable by HPLC. The glucose solution and the PPY film was incubated at 30 °C for 30 min. Then the resultant solution was filtered (Schleicher and Schuell, 0.45 µm, 30 mm) before injection. The results were not satisfactory with PPY films. The glucose amount again found to increase when PPY film with and without GOX were incubated with glucose solution. Therefore, it was decided to use an enzymatic analysis method which measures the activity of GOX immobilized polymer samples from the amount of components produced during the reaction between GOX and glucose, not from the depleted amount of the reactant, i.e. glucose. The enzyme assay given in Section 3.4 was utilized for the rest of the study which measures the amount of H₂O₂ produced as a result of reaction between glucose and glucose oxidase.

The enzymatic activity method carried out after immobilization for the preliminary experiments are given in Table 5.1. In first four experiments, “Dry” medium was used. In Runs PPY1^{PR} and PPY2^{PR}, DNSA method was used for enzymatic investigation, but in both runs, the glucose amount seemed to increase which should not be the case. Therefore, for the next two runs (Runs PPY3^{PR} and PPY4^{PR}) which were again performed in “Dry” medium but only their immobilization periods were higher, GOX immobilized PPY films were incubated in glucose solution and the remaining glucose amount in the incubation solution was measured both by the DNSA method and via HPLC. In both methods, unexpected increase in glucose amount was again observed. In Runs PPY5^{PR} and PPY6^{PR}, the immobilization medium was changed from “Dry” to “Wet”, and immobilization was conducted at supercritical and atmospheric conditions, respectively. In both cases, increases were observed in glucose amounts in the solutions.

Table 5.1. The results of preliminary SFI experiments performed on PPY films in terms of their enzymatic activity

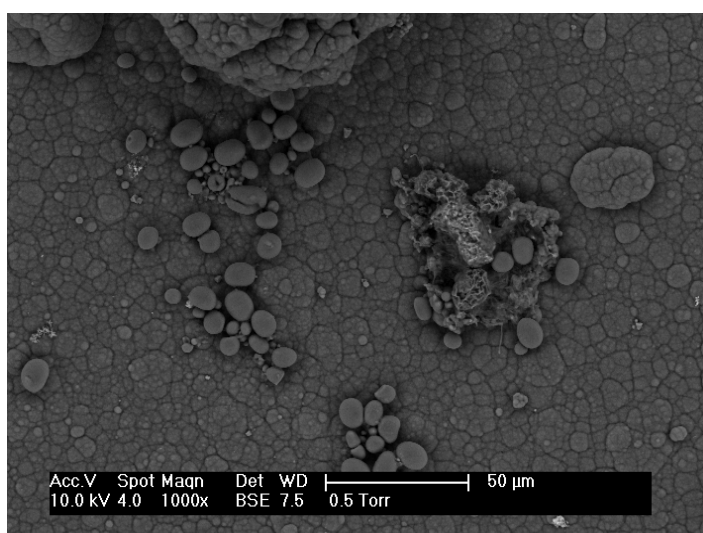
Run No.	Enzymatic analysis used	Result
PPY1 ^{PR}	DNSA	Pseudo-increase in glucose amount
PPY2 ^{PR}	DNSA	Pseudo-increase in glucose amount
PPY3 ^{PR}	DNSA, HPLC	Pseudo-increase in glucose amount in both methods
PPY4 ^{PR}	DNSA, HPLC	Pseudo-increase in glucose amount in both methods
PPY5 ^{PR}	DNSA	Pseudo-increase in glucose amount
PPY6 ^{PR}	DNSA	Pseudo-increase in glucose amount
PPY7 ^{PR}	Sigma Bulletin	No activity observed
PPY8 ^{PR}	Sigma Bulletin	No activity observed
PPY9 ^{PR}	Sigma Bulletin	No activity observed
PPY10 ^{PR}	DNSA	Pseudo-increase in glucose amount
PPY11 ^{PR}	Sigma Bulletin	Activity observed

In the DNSA method, as mentioned earlier the increase in glucose amount was related with the boiling step of the analysis. However, while determining the glucose with

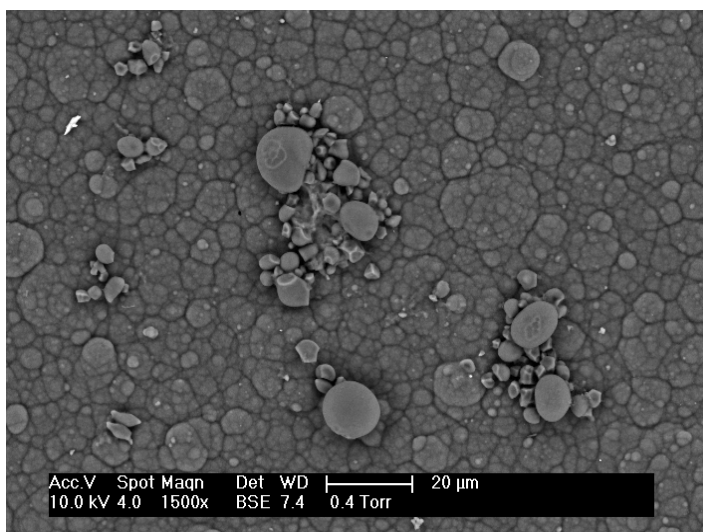
HPLC, there was no procedure like boiling, but there was still an increase in the glucose amount. Then, the question was that: Was it the polymer used or the commercial enzyme (Delta) which may have impurities in it caused this unusual increase in glucose amount after the reaction? To answer this question, PPY film without immobilized enzyme and PPY film immobilized with original GOX (Fluka) (PPY10^{PR}) were also incubated in glucose solution and the resultant glucose amounts were analyzed by the DNSA method. Additionally, the glucose amount left after incubation was also analyzed by HPLC. In both cases and methods, the glucose amounts were measured to increase. Therefore, it was concluded that the unusual situation was related with the polymer not the enzyme type. Since, the glucose amount was measured to increase after encountering with PPY film, the enzymatic activity of the GOX immobilized PPY samples could not be measured through the glucose amount. Therefore, a new enzyme assay which measures the enzymatic activity from the product of the reaction between GOX and glucose was established. This enzyme assay measures the amount of H₂O₂ produced and the details are given in Section 3.4. The method is denoted as “Sigma Bulletin” in Table 5.1. The samples from Runs PPY7^{PR}, PPY8^{PR}, PPY9^{PR} and PPY11^{PR} were analyzed according to the enzyme assay given in Sigma Bulletin 510. Runs PPY7^{PR}, PPY8^{PR}, and PPY9^{PR} were conducted with commercial GOX (Delta), only Run PPY11^{PR} was performed with original enzyme (Fluka). In Runs PPY8^{PR}, and PPY9^{PR} where ethanol were involved in the reaction, no enzymatic activity was observed with the PPY samples. The immobilization pressure (150 bar), temperature (40 °C), immobilization medium (“Wet”) of Runs PPY7^{PR} and PPY11^{PR} were the same. The enzyme type and amount, and immobilization period were the differences in the experimental conditions of both runs. An activity was observed for the sample obtained from Run PPY11^{PR}, but no activity was observed for the PPY sample from Run PPY7^{PR}, although the immobilization time and enzyme concentration of the immobilization solution of Run PPY7^{PR} were three and 20 times higher than the immobilization time and enzyme concentration of the immobilization solution of Run PPY11^{PR}, respectively. The only superiority for Run PPY11^{PR} over Run PPY7^{PR} was the original enzyme (Fluka) used in that run. In remaining part of the study pure and high activity enzyme (Fluka) was used.

Although the enzyme activity measurements were in the development stage in preliminary experiments and no exact activity was measured for PPY samples, the visual investigation by ESEM showed that there were GOX particles immobilized on PPY. First

samples from Runs PPY1^{PR} and PPY2^{PR} were investigated by ESEM, and GOX particles were seen on the polymer. Then, it was decided to examine the PPY film that would be used for immobilization, before and after the immobilization procedure for the rest of the study. The center of the PPY film was marked with a needle point hole, so that the changes after immobilization could be noticed easily. This was a long procedure, but as a result of careful and sensitive investigation, it was seen that there were GOX particles immobilized on PPY surface as a result of immobilization in both “Dry” and “Wet” mediums. The ESEM images of PPY samples from experiments PPY4^{PR} (“Dry”) and PPY4^{PR} (“Wet”) are presented in Figures 5.2 and 5.3, respectively.



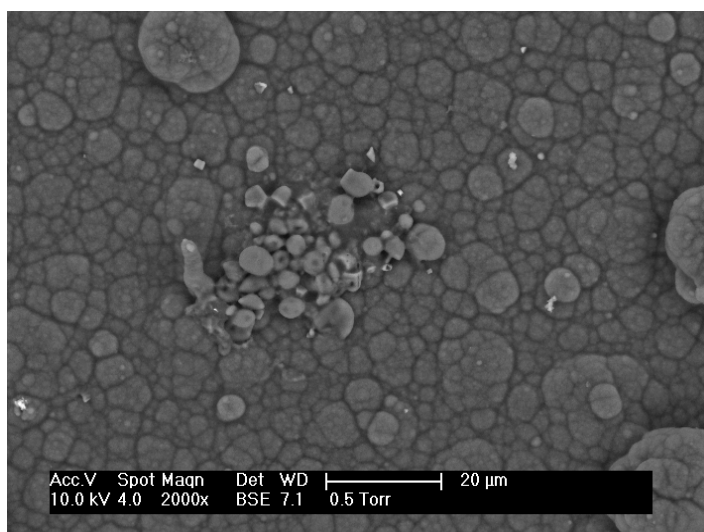
(a)



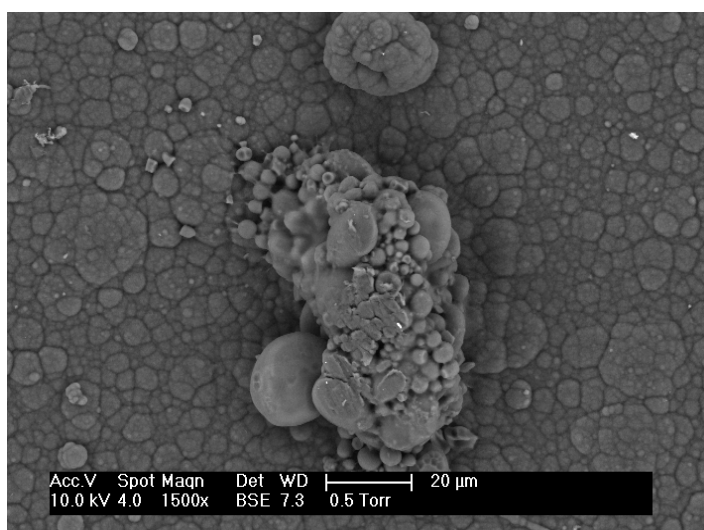
(b)

Figure 5.2. ESEM images from PPY4^{PR} sample

The visual inspections of the samples by ESEM were done after the samples were washed and dried. Once the GOX particles were observed in ESEM, the sample was taken out of the ESEM system and the sample were exposed to pressurized air and again visualized. It was seen that after pressurized air treatment, the GOX particles were exactly at the same position on PPY film as they originally were, indicating the stability of the immobilization. Increasing the immobilization time did not cause any change in the activity



(a)



(b)

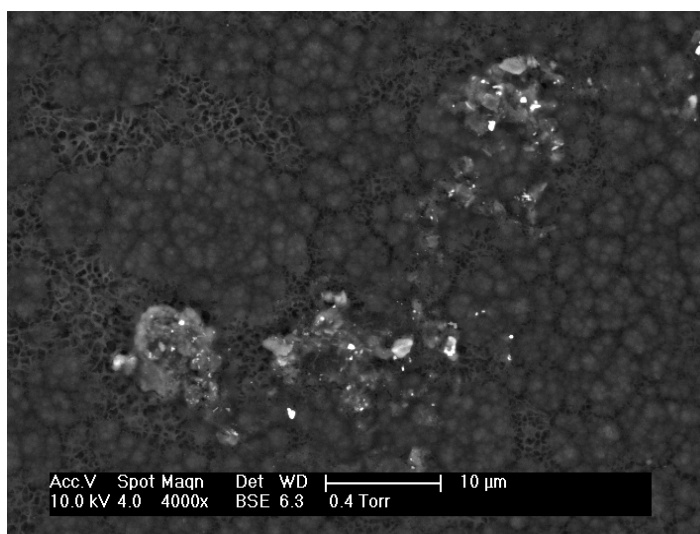
Figure 5.3. ESEM images from PPY7^{PR} sample

GOX immobilized PPY samples were also analyzed by gravimetric analysis. The weight of PPY films were determined before and after immobilization. Although the GOX particles were observed in ESEM images, the gravimetric analysis was not sensitive enough to determine the immobilized amount of GOX in each run. Only in Runs PPY4^{PR} and PPY9^{PR}, 0.1 mg increase in the weight of PPY film was recorded. In the rest of the runs, no change in PPY film weight was observed. Therefore it was decided that the gravimetric analysis was not a good choice for evaluation of the immobilization procedure.

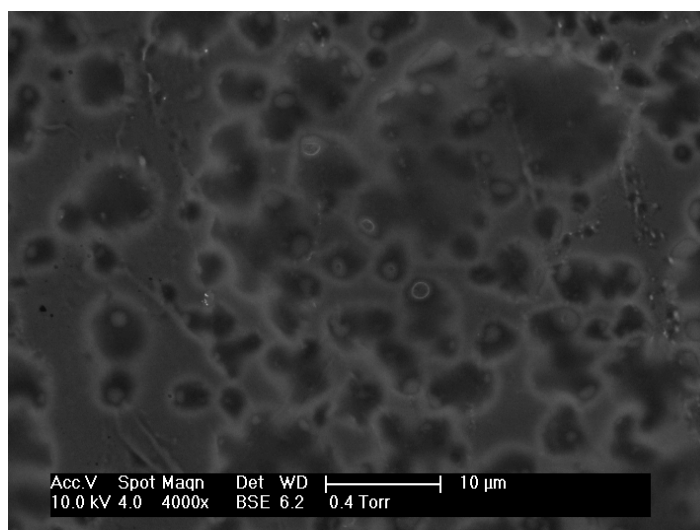
As a result of preliminary experiments, the evidences of GOX immobilization in supercritical fluid environment were observed and the enzymatic analysis method was determined. Then the main SFI experiments were performed with the PPY films. In the following sections, the results of these experiments are presented.

5.3. Effect of Immobilization Medium and Time on the Activity of GOX Immobilized PPY Films via SFI

The effect of immobilization medium on SFI of GOX on PPY films was investigated with four different experimental mediums; the experiments were named according to the mediums used, namely: “Dry”, “Semi-Dry”, “Wet” and “Wet-b”. As indicated in Section 3, in “Dry” and “Semi-Dry” experiments, pre-determined amount of GOX was weighed and put in a test tube and the polymer matrix was placed in the same test tube. Then, the test tube was placed in the pressure vessel. In “Semi-Dry” experiments an additional test tube containing 10 ml de-ionized water (dH₂O) was also put in the pressure vessel. In the case of “Wet” experiments, a pre-determined amount of GOX was dissolved in a pre-determined amount of dH₂O. In “Wet-b” experiments a pre-determined amount of GOX was dissolved in a pre-determined amount of pH 5.7 phosphate buffer. Then the polymer matrix was placed in that GOX-dH₂O or GOX-buffer solution depending on the case. In preliminary experiments, the PPY films used for SFI were synthesized on 100 cm² electrode, then peeled off from the electrode and cut into pieces to be used. In this part, the PPY films were synthesized on 1.25 and 2.5 cm² electrodes and they were used as they were on the electrodes for immobilization.



(a)



(b)

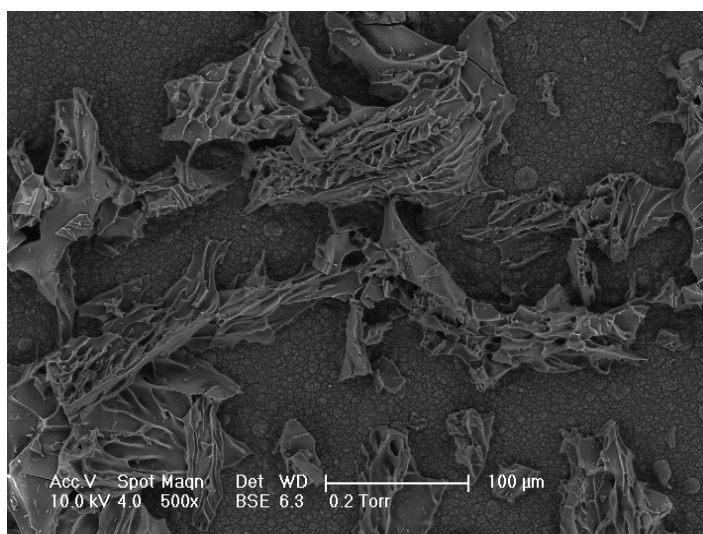
Figure 5.4. ESEM images of GOX immobilized PPY films from “Dry” experiments, (a) solution side of PPY film from Run PPY1, (b) electrode side of PPY film from Run PPY4

Four experiments were conducted in “Dry” medium (Table 3.5) at 150 bar and 40 °C. In “Dry” experiments no enzymatic activity of processed PPY films were observed up to 150 bar, 40 °C and 72 h immobilization time. Increasing both the amount of GOX used from 0.0050 g to 0.0100 g and immobilization time from 24 h to 72 h was not useful to detect activity for samples from “Dry” experiments. In ESEM, few GOX particles were seen on the PPY films, but enzymatic activity was not observed. The enzymatic activity of these enzyme particles may not be observed due to hindrance of the active sites or due to the small amount of GOX which may be lower than the detectable limit. The PPY film from

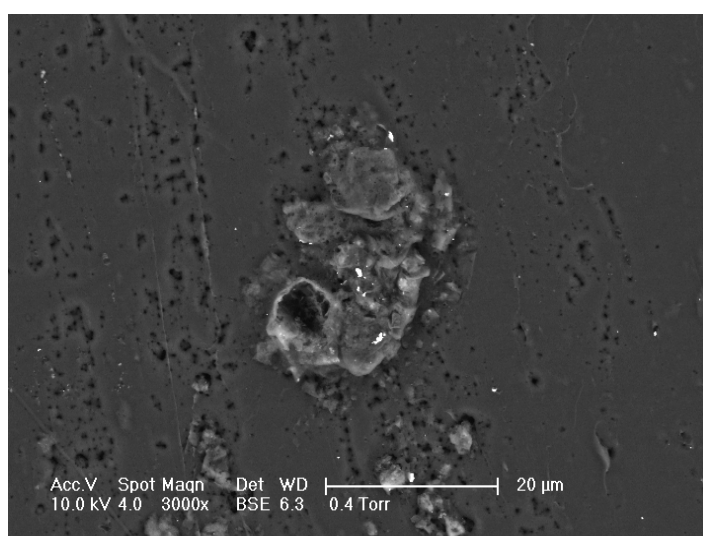
Run PPY3 of the “Dry” experiments showed a very low activity at 100 mM glucose solution after 60 min incubation time. This may show the existence of a diffusion barrier. But in other samples of “Dry” experiments, no activity was observed even at 100 mM glucose concentration for 60 min incubation time. In “Dry” experiments, after immobilization, it was observed that most of the time PPY film on one side or on both sides of the electrode cracked. This may also indicate the processibility of PPY film with $scCO_2$. Only in Run PPY4, the films on both side of the platinum electrode did not crack. In that case, since PPY films did not cracked, only the solution side of the film was subject to GOX. However, ESEM images reveals that there were also GOX particles embedded in the electrode side of the PPY film in Run PPY4 (Figure 5.4. (b)).

In “Dry” experiments, since no activity was observed with the GOX immobilized PPY films, the effect of water as an entrainer in the immobilization medium was investigated. Water may act as an entrainer and help CO_2 to transfer more GOX particles to the polymer film. In “Semi-Dry” medium, two test tubes, one containing the PPY film and 0.0100 g free GOX and other containing 10 ml dH_2O , were put in the pressure vessel. The immobilization pressure and temperature were 150 bar and 40 °C. At the end of the experiment, when the PPY film was taken out of the pressure cell, washed and dried, it was observed that there was white powder-like layer covering it (Figure 5.5).

As it is seen from Figure 5.5, the shape of the GOX particles were different from the ones observed in “Dry” experiments. They were more like as if they were sprinkled over the PPY film, and gave a visual impression that the particles were not really immobilized. However, even after repeated washing and drying cycles, the particles still on the surface of the PPY film. In “Semi-Dry” experiment, although, the surface of the PPY film was covered with GOX, again no enzymatic activity was observed. Not observing an activity in “Dry” and “Semi-Dry” experiments indicated the possible changes in enzyme structure, thus denaturation that eliminates GOX activity when SFI was done at these two mediums.



(a)



(b)

Figure 5.5. ESEM images of GOX immobilized PPY films from “Semi-Dry” experiments, (a) solution side of PPY film from Run PPY9, (b) electrode side of PPY film from Run PPY9

In “Wet” and “Wet-b” experiments, enzymatic activity was observed for all the GOX immobilized PPY films. The GOX immobilized PPY film samples in “Wet” medium from Runs PPY6 and PPY8 had a gel-like layer on them when they were wet. After the films were washed and dried, the gel-like layers became powder-like layers. When wetted, the powder again turned out to be a gel. However, the sample from Run7 (also a “Wet” experiment) did not have a gel-like cover on it like Runs PPY6 and PPY8, but the PPY

film from Run7 also showed an enzymatic activity. The ESEM photographs showed that PPY film from Run7 had a very thin coverage on it that cannot be seen by the naked eye (Figure 5.6). The PPY films processed in “Wet-b” medium did not have a gel-like coverage on them but had enzymatic activities. ESEM images showed that GOX immobilized PPY film from “Wet-b” experiments had a cloud of small particles bounded to their surface (Figure 5.7).

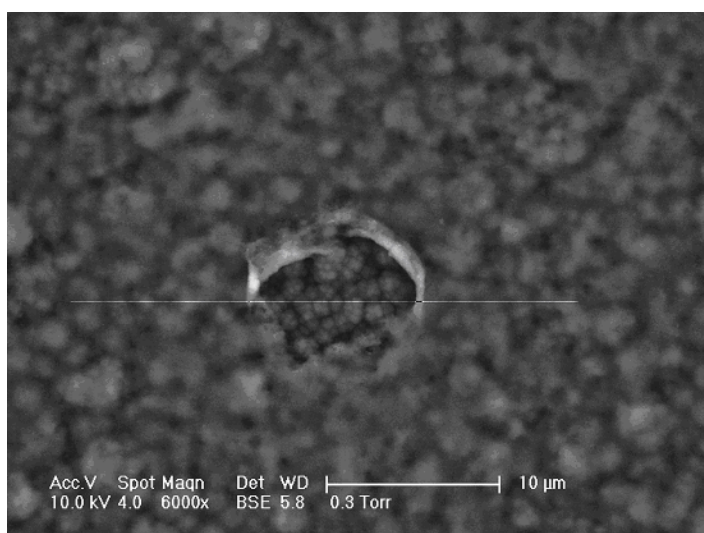
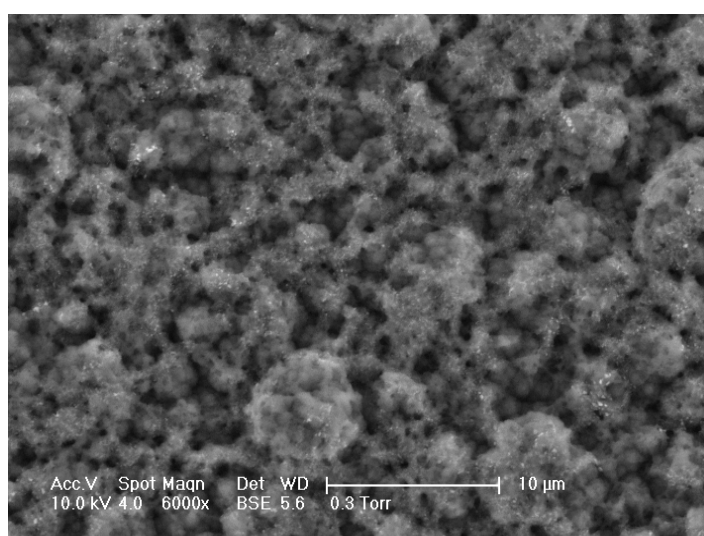


Figure 5.6. ESEM images of GOX immobilized PPY films from “Wet” experiments, solution side of PPY film from Run PPY7

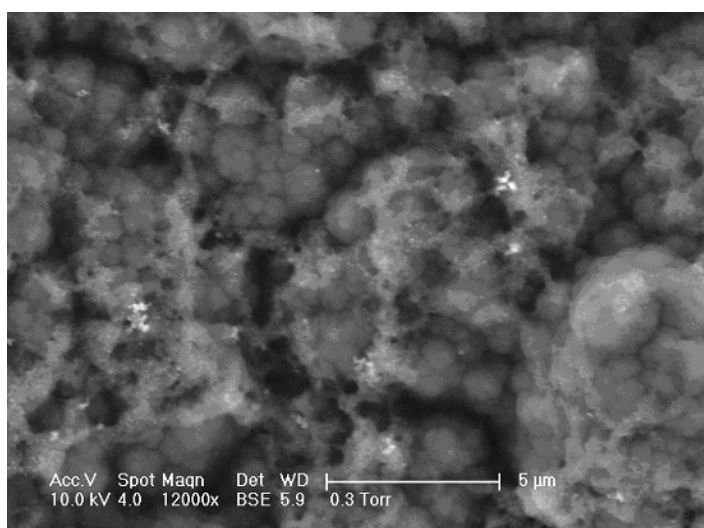
The activities of GOX immobilized PPY films from Runs PPY6-9 at 5-100 mM glucose concentration range are given in Figure 5.8. All of the experiments shown on Figure 5.8 were done at 150 bar and 40 °C, SDS was used as electrolyte for their PPY film synthesis. In all experiments, the amount of GOX was 6.25 mg/ml dH₂O for Runs PPY6-8, and 6.25 mg/ml buffer for Run PPY9. The experimental data shown in Figure 5.8 was also fit to the single substrate form of Michaelis-Menten equation without transformation of data, the kinetic parameters (K_m and v_m) were found and the results of the model were also given on the figure. The kinetic parameters are presented in Table 5.1. The K_m value of free enzyme was found as 9.12 mM. When the K_m value of free enzyme and immobilized enzymes were compared, it was seen that K_m values of immobilized enzymes were lower (Table 5.1) which indicated an increase in affinity between enzyme and substrate.

Table 5.1. Kinetic parameters of immobilized GOX on PPY film, electrolyte: SDS

Imm. Medium	Pressure (bar)	Temperature (°C)	Imm. time (h)	V_m $\mu\text{mol}/(\text{min}.\text{cm}^2)$	K_m (mM)
Wet	150	40	5	0.007	3.63
Wet	150	40	24	0.010	6.17
Wet	150	40	72	0.012	4.30
Wet-b	150	40	24	0.019	4.49
Wet-b	1	40	24	0.008	5.00



(a)



(b)

Figure 5.7. ESEM images of GOX immobilized PPY films from “Wet-b” experiments, solution side of PPY film from Run PPY9

As it is seen from Figure 5.8, when the immobilization medium is dH_2O , an increase in the supercritical immobilization period caused an increase in the activity of GOX immobilized on PPY film. This can be attributed to the possibility of an increase in the amount of the enzyme immobilized on the polymer film as immobilization time increased. In “Wet” medium, as the immobilization time increased from 5 h to 24 h, the increase in activity was 38 per cent, further increase in immobilization time from 24 h to 72 h caused a further 24 per cent increase in activity. All these comparisons were made according to the activity values taken from the model at 100 mM glucose concentration. Although the highest activity was obtained at 72 h immobilization time, as a result of trade off between time and activity, it was decided to make further studies at 24 h of immobilization time.

Figure 5.8 also reveals that pH 5.7 phosphate buffer is a better immobilization medium than dH_2O with scCO_2 . The activity of the GOX immobilized PPY sample in “Wet-b” medium was 96 per cent higher than the one processed in “Wet” medium. This may due to the carbonic acid formation and dissociation when scCO_2 mix with dH_2O , and the medium may become more acidic, which may lower the activity of GOX. Phosphate buffer was seen to have a stabilizing effect so that the maximum activity was obtained in the buffer medium. The rest of the SFI experiments were done in “Wet-b” medium.

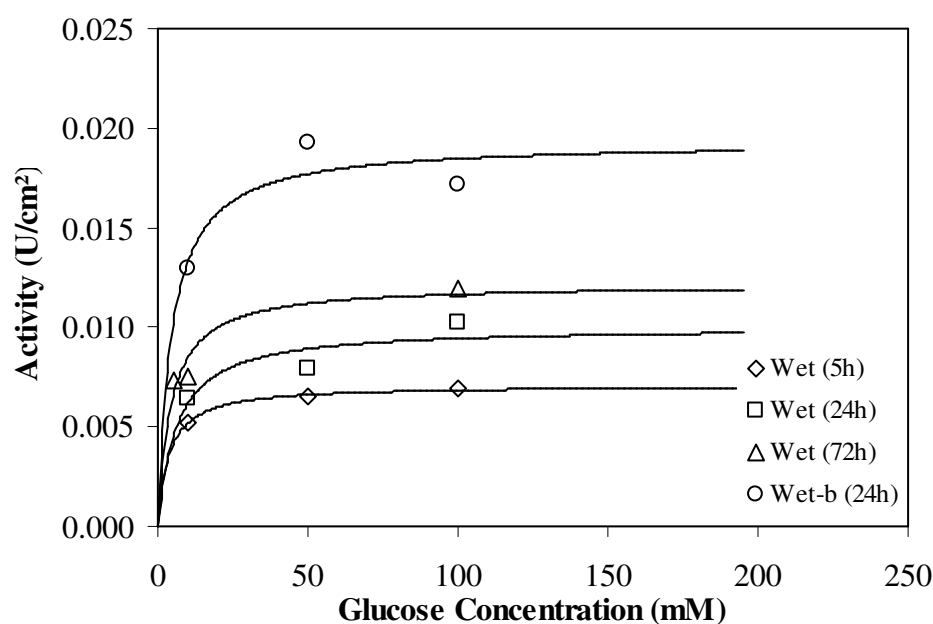


Figure 5.8. Effect of immobilization medium and time on the activity of GOX immobilized PPY films at 150 bar, 40 °C.

5.4. Comparison of SFI and Immobilization at Atmospheric Conditions

PPY has the ability to adsorb proteins. In order to assess the advantage of SFI on PPY films, immobilization was also performed at atmospheric conditions and the results compared with the SFI process. Normally, in such enzyme studies only the activity values at high substrate concentrations, quite high above K_m value, are presented. In this study, although the numerical comparison will be made according to the activity values taken from the model at 100 mM glucose concentration, the activity values measured at lower glucose concentrations for each sample are also presented. In order to compare the SFI process and the immobilization performed at atmospheric conditions six experiments were performed (Runs 9, 10, 10^R, 11, 12, 12^R). The activities of the PPY film samples obtained from these experiments are depicted in Figure 5.9. All the experiments were conducted at 40 °C immobilization temperature for 24 h, and the averages of the results from replicate runs are shown on the figure. As it is seen from Figure 5.9, when PPY films, synthesized with PTSA electrolyte, were used for immobilization, the activity of the PPY film immobilized by GOX via SFI process was slightly (19 per cent) higher than the activity of the PPY film immobilized by GOX at atmospheric conditions. However, when SDS was used as the electrolyte, the enhancement of activity with SFI process compared to atmospheric immobilization was more pronounced. The activity of PPY film from SFI process was 136 per cent higher than the one used in atmospheric immobilization. In literature, Tirkeş *et al.* (2002) compared the usage of PTSA and SDS as an electrolyte in PPY synthesis. They stated that if PTSA was used as the electrolyte in electropolymerization of PPY and graft polymers of PPY, then longer times (>2 h) were needed to obtain the same film thickness as the film prepared where SDS was used as the electrolyte. But they did not observe a higher enzyme activity when SDS was used instead of PTSA.

As it is seen from Figure 5.9, at 150 bar, PPY film synthesized with SDS electrolyte was a better immobilization matrix than PPY film synthesized with PTSA electrolyte, because PPY film synthesized with SDS electrolyte showed higher enzymatic activity, but for immobilization at 1 bar, it was vice versa.

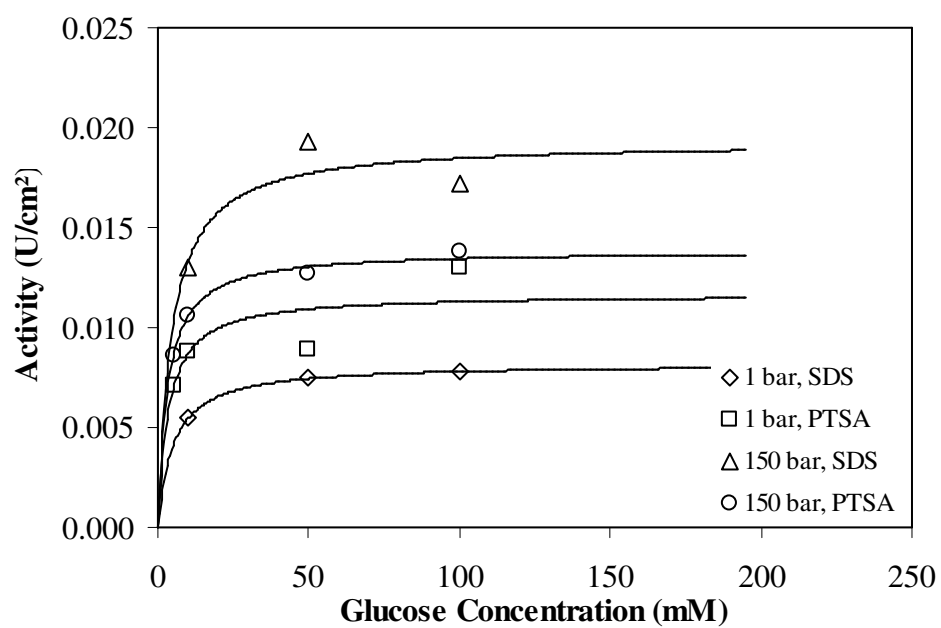


Figure 5.9. Comparison of the SFI with immobilization performed at atmospheric conditions

6. IMMOBILIZATION OF GOX ON PU/PPY FOAM AND FILM

It was nearly impossible to obtain homogeneous and reproducible polymer surfaces by electropolymerization, at constant potential in our case. Thus the enzyme immobilization process over the polymer surface was not also homogeneous. Studies on PPY gave important information for the evolution of the rest of the studies, but it became evident that the studies must be continued with more flexible and homogeneous conducting polymers. Therefore PU/PPY composite matrices were chosen in the continuation of the study.

6.1. Preliminary Studies with PU/PPY Composite Foam

Depending on the experience on PPY films, GOX was immobilized on PU/PPY composite foams only in the “Wet-b” medium. The preliminary PU/PPY experiments were performed with samples (9×6 mm, which were cut from ready made larger PU/PPY foam sample (25.4×25.4 mm) as it was earlier mentioned in Sections 3.3.1 and 3.5.3. The outer part of the foam sample, which was directly exposed to pyrrole vapor was harder than the inner parts. Therefore, the location of the PU/PPY samples cut from the bigger sample was also taken into account. The samples were named as corner, inner and side samples, and their schematic representation is given in Figure 3.5. In preliminary experiments, the PU/PPY samples were used in immobilization experiments without further processing like washing. The SFI immobilization was performed at 40 °C and at three different pressures (75, 105, and 150 bar) for 24 h. The rate of depressurization was 3 bar per minute, and after depressurization of every ten bars, depressurization was stopped for 3 min to avoid the freezing of the immobilization solution. Only at Run PU/PPY2^{PR}, the depressurization rate was 30 bar per minute. In this subsection, the results are given according to the location of the PU/PPY sample on the larger sample from which it was cut. As it was noted in Section 3.3.1, to circumvent the problems arising from sample location, a different geometry was used for the remainder of the experiments. The results of these experiments are given in the next sub-sections.

The data seen in Figure 6.1 show the pressure dependence of the activity of GOX immobilized on “corner” PU/PPY foam samples. As it is seen from the figure, as the pressure is increased from 75 to 105 bar, the activity of the immobilized enzyme increases. However further increase in pressure from 105 to 150 bar causes a decrease in activity. Ye *et al.* (2000) studied the absorption of low molecular weight solutes into polyurethane scCO₂. In their study, at constant temperature (40 and 60 °C) the amount of solutes absorbed in PU initially increased as the pressure increased from 8 to 10 MPa and then started to decrease when the pressure was increased to 12 MPa, showing a similar trend to our results.

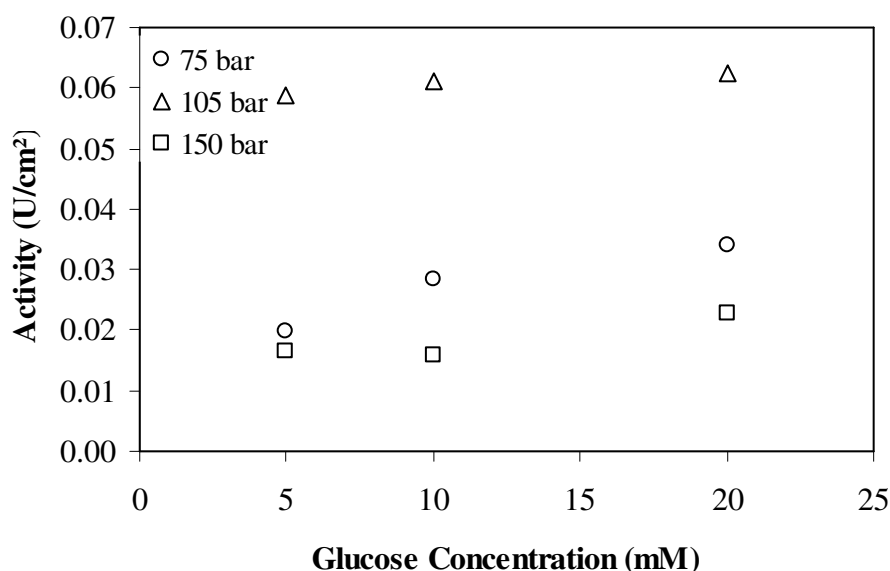


Figure 6.1. Activity of GOX immobilized PU/PPY composite foams from “corner” samples

Figure 6.2 depicts the activity of GOX immobilized PU/PPY composite foams from “inner” samples. As Figure 6.2 shows, the activity of PU/PPY samples, which were processed at 75 and 105 bar are nearly same, but the activity of the sample processed at 150 bar was lower as in the case of “corner” samples. The concave shape of the activity versus concentration data seemed to be a common characteristic for “inner” samples.

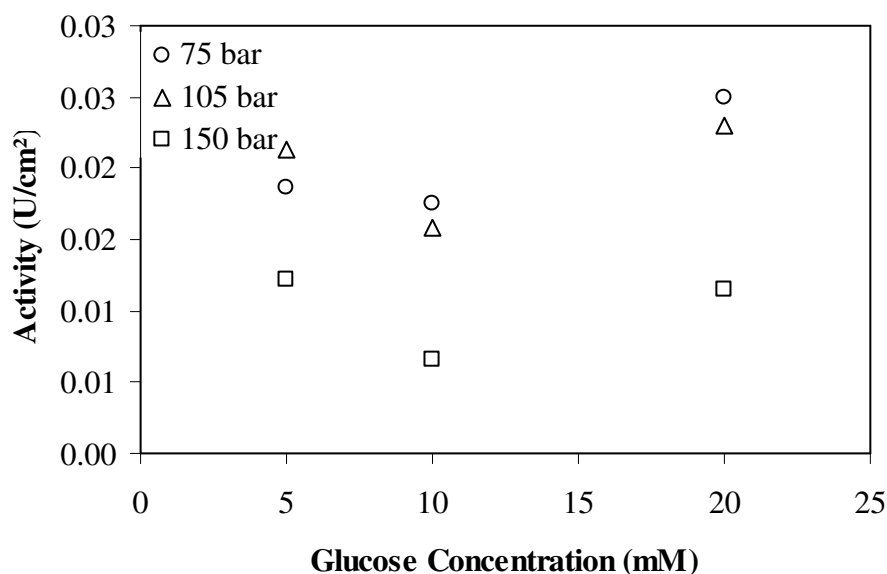


Figure 6.2. Activity of GOX immobilized PU/PPY composite foams from “inner” samples

In preliminary experiments, at 105 bar and 40 °C, PU/PPY samples from three different locations (corner, side, and inner) were used for immobilization. The activities of these three samples are shown in Figure 6.3. It is seen that “inner” and “side” samples, which had smaller surface area exposed directly to I₂ and pyrrole compared to the “corner” sample, both have first decreasing, and then increasing activity values as the glucose concentration increased from 5 to 10 then 10 to 20 mM glucose concentration. However, the “side” sample had higher activity than the “inner” sample. The highest activity values were obtained with “corner” sample within these three samples. The results indicated that there was a relation between the activity and the surface area directly exposed to pyrrole vapor, thus the polypyrrole concentration. It was obvious that the polypyrrole formation on the PU foam surface was more than it was which took place on the inner parts of the foam.

Runs PU/PPY1^{PR} and PU/PPY2^{PR} were performed at the same experimental conditions except for their depressurization rate. Depressurization of PU/PPY2^{PR} was 10 times faster (30 bar/min) than the depressurization step of PU/PPY1^{PR} (3 bar/min). The polymer sample used in Run PU/PPY2^{PR} did show any enzymatic activity. This result shows that fast depressurization was detrimental to the enzyme.

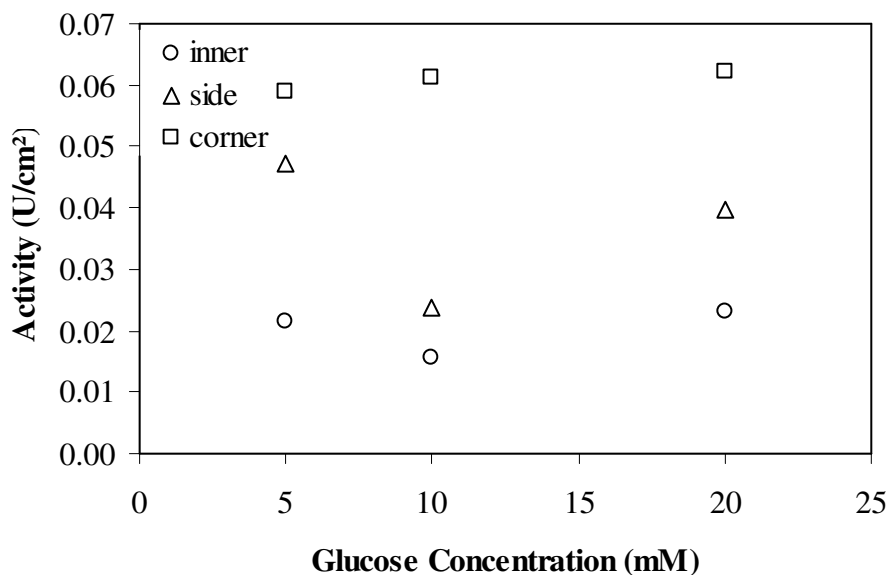


Figure 6.3. Activity comparison of GOX immobilized PU/PPY composite foams from “inner”, “side” and “corner” samples

Figures 6.1, 6.2 and 6.3 show the results at supercritical conditions. The immobilization process is also conducted at atmospheric pressure; all other experimental conditions remaining the same as for the experiments conducted at supercritical conditions. When the immobilization process is conducted at atmospheric pressure, there are GOX particles immobilized on the PU/PPY composite and they give reaction with glucose. The samples were incubated for 2 min, 4 min and 6 min periods, in different glucose solutions consecutively, i.e. first 2 min incubation was carried out, then the polymer support is taken out and put in a new glucose solution for 4 min incubation. The amount of glucose reacted is expected to increase as the reaction time increases, i.e. the immobilized enzyme should convert higher amount of glucose in 6 min incubation period than it converts in 2 and 4 min incubation periods. However, the reacted amount of glucose decreases with time when GOX immobilized sample that was obtained as a result of atmospheric immobilization, although it must increase as it is the case for the samples immobilized at supercritical conditions, i.e. in the 6 min run, less reaction was noticed as compared to 2 and 4 min runs. The decrease in the reacted glucose amount for successive incubation times may be explained by the loss of GOX particles from the polymer matrix. Since the reacted glucose amount decreases with time, the absorbance vs time graph has a negative slope, thus

activity cannot be calculated. On the other hand, the supercritical immobilization process gives stable PU/PPY polymer samples.

At the end of experiments, when the test tubes, containing PU/PPY composite immersed in buffer-GOX solution, was taken out of the pressure cell, it was seen that the solution was no more in its original yellow color. The color of solution became brown-black. Although the PU/PPY composites were dried in a fume hood for 7 days until the excess monomer/oxidant evaporated from the sample, as composites were immersed in GOX solution, the color of solution became brown-black even at atmospheric pressure (without CO₂). Therefore, it is thought that the monomers, dimers, trimers, or I₂, which cannot diffuse out of the composite when exposed to air, come out of the composite as it is immersed in the solution. The color of the solution was darker when immobilization was conducted at supercritical conditions compared to immobilization at atmospheric pressure. This may be due to swelling of PU/PPY composite by scCO₂ which makes the monomers, dimers, trimers, or I₂, to come out of the composite more easily. Additionally, scCO₂ may dissolve the pyrrole monomers, dimers, trimers or I₂ and take them out of the composite. The chemicals coming into the GOX solution can decrease the enzyme activity. Therefore, after two months following the “first immobilization”, the same polymer supports used in “first immobilization” were again used for supercritical CO₂ immobilization of GOX at the same conditions as in the “first immobilization”. This is called “second immobilization”. In the “second immobilization” the polymer sample is the one used in “first immobilization” but the GOX solution used for immobilization was freshly prepared. “Second immobilization” was only performed for “corner” samples. Figure 6.1 shows the results of the “first immobilization” and Figure 6.4 shows the results of “second immobilization”. The results show that after the “second immobilization”, the activity of the polymer sample processed at 150 bar increased between 42-160% for three different glucose concentrations compared to the “first immobilization”. However, after the “second immobilization” the activities of the polymer sample processed at 75 bar show a slight decrease and the activities of the polymer sample processed at 105 bar show a sharp decrease compared to their “first immobilization” activity values. These decreases, seen for 75 and 105 bar processing pressures, do not mean that the activities of the polymer samples decrease as a result of “second immobilization”, it is more logical to say that except at 150 bar, the “second immobilization” does not enhance the activity values of polymer samples

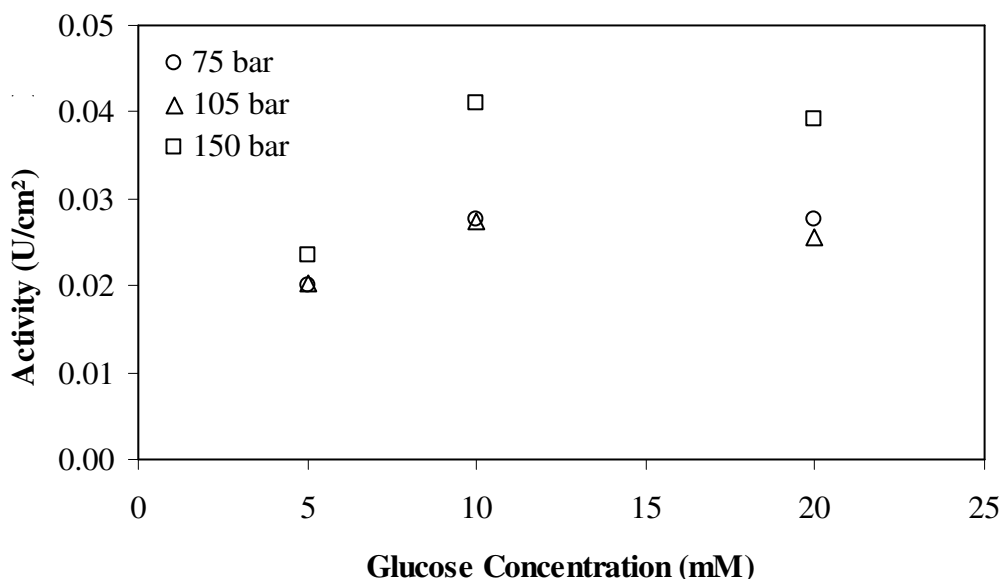


Figure 6.4. Activities of GOX immobilized PU/PPY composite foams from “corner” samples after second immobilization.

obtained from the “first immobilization”. Since the “second immobilization” was performed after two months following the “first immobilization”, during this period there are certain losses in the activities of the samples. After the “first immobilization” of polymer samples, the activity of the samples were immediately analyzed, and then they were kept in buffer solution at +4 °C temperature. The average activity losses after 15 days storage were about 50, 70, and 25% for the samples obtained at 75, 105, and 150 bar, respectively. Although the activity values obtained from polymer samples at the “second immobilization” were lower than their initial (“first immobilization”) activities at 75 and 105 bar, they were higher than the activities measured after 15 days storage following the “first immobilization” (Figure 6.5). For the “first immobilization” the highest activity values were obtained at 105 bar, and among the “second immobilization” the highest activity values were obtained at 150 bar. However, these activity values (“second immobilization”, 150 bar) are still lower than the values measured after the “first immobilization” at 105 bar (Figure 6.5). All these results reveal that, at the first immobilization at 105 bar, most of the available sites were occupied by GOX particles. Whereas, at 150 bar, the still unoccupied sites on the polymer after “first immobilization” were also occupied during the “second immobilization”.

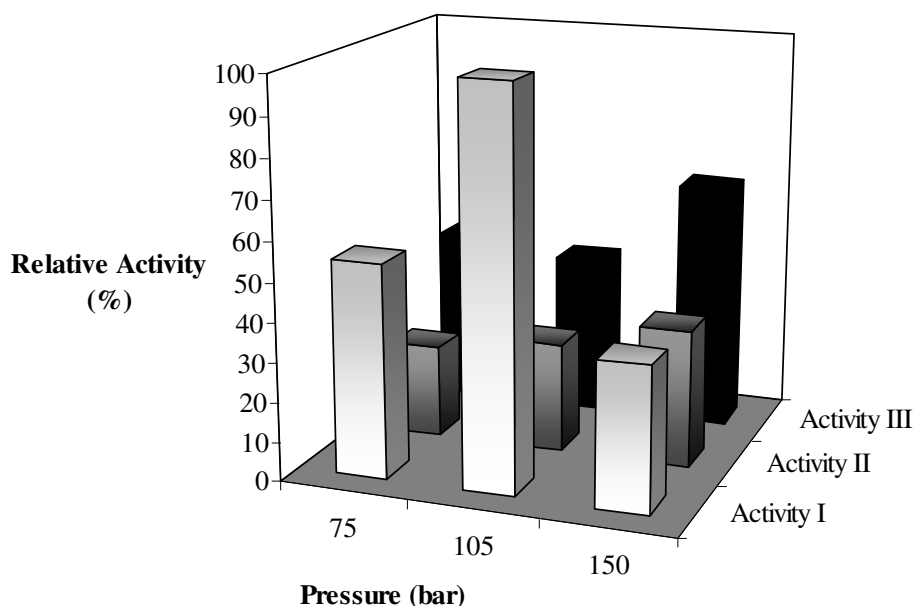


Figure 6.5. Activity comparison of GOX immobilized PU/PPY composite foams from “corner” samples after first and second immobilization (Activity I: Activity of PU/PPY sample after 1st immobilization, Activity II: Enzymatic activity of the same PU/PPY sample after 15 days following the 1st impregnation, Activity III: Enzymatic activity of the same PU/PPY sample after 2nd impregnation)

In preliminary experiments, the real GOX amounts immobilized onto the polymer samples were not measured, but the equivalent enzyme amounts on each sample were calculated. A calibration chart was prepared with free-enzyme (activity vs. free-enzyme amount) in order to evaluate the equivalent amount of enzyme on each polymer sample. The calibration chart prepared with free-enzyme is given in Appendix C. The equivalent active enzyme amounts on “corner” polymer sample are given in Table 6.1. The protein amount given in Table 6.1 is not the absolute amount of protein on the polymer matrix. It corresponds to the free enzyme amount, which will show the same activity with that sample. Therefore it is termed as the “equivalent active enzyme amount”. The calibration chart used to calculate the “equivalent active enzyme amount” is given in Appendix C.

Table 6.1. Equivalent enzyme amounts of immobilized GOX on PU/PPY composites from “corner” samples (Immobilization temperature: 40 °C, Immobilization time: 24 h)

Run No.	Pressure (bar)	Equivalent Active Enzyme Amount (µg)
PU/PPY6 ^{PR}	75	0.30
PU/PPY5 ^{PR}	105	1.05
PU/PPY9 ^{PR}	150	0.14
PU/PPY6 ^{PR} -2 nd	75	0.27
PU/PPY5 ^{PR} -2 nd	105	0.26
PU/PPY9 ^{PR} -2 nd	150	0.44

Up to this point, the results of preliminary experiments performed with ready made PU/PPY samples are given. The results SFI experiments performed with home-made PU/PPY composite foams are given and discussed in the rest of this section.

6.2. Effect of Immobilization Solution Concentration on the Activity of PU/PPY Foam Samples

Three different immobilization solution concentrations were used for SFI immobilization on PU/PPY foams. The immobilization solution concentrations were 6.25, 0.625 and 0.0625 mg/ml. The SFI experiments to determine the effect of immobilization solution concentrations were proceeded at 100 bar and 40 °C for 24 h. In preliminary experiments, the immobilized GOX amount could not be determined by the Bradford method since the immobilization solution became brown-black after the immobilization period. Therefore, it was decided to wash the polymer samples prior to the immobilization process. As a first attempt, they were washed with water in a shaking bath at 40 °C for 30 h while investigating the effect of immobilization solution concentration on the SFI process.

The protein amounts in the immobilization solution before and after the immobilization process were determined with the Bradford method. After the samples were taken from the immobilization solution they were washed with buffer for 10 h. The protein amounts in the washing solutions were also determined. These values were incorporated in the calculation of the immobilized amount of protein although they were very small compared to the immobilized amount. The total amount of protein in the

washing solutions was deducted from the amount of protein difference in the immobilization solution before and after the experiment to find out the immobilized amount of protein. The average of immobilized GOX amount on PU/PPY sample from Runs PU/PPY1 and PU/PPY1^R show that at 105 bar, 40 °C, when the concentration of immobilization solution was 6.25 mg/ml, the immobilized amount of GOX into the PU/PPY matrix is 380 µg. When the initial concentrations of GOX-buffer solutions were 0.625 mg/ml and 0.0625 mg/ml, the enzyme amount immobilized on the polymer matrix was 68 µg and 15 µg. The immobilized enzyme amount decreased as the initial concentration of GOX-buffer immobilization solution decreased as expected. In this respect, the expected situation for activity values of the PU/PPY samples processed at different immobilization solution concentrations was that the activity would decrease as the concentration decreased. However, surprisingly that was not the case. As it is depicted in Figure 6.6, the activity values of the PU/PPY samples from were almost same when the immobilization concentration decreased from 6.25 mg/ml to 0.625 mg/ml. Moreover, decreasing the concentration to 0.0625 mg/ml causes an increase (12.5 percent) in activity compared to the activity of the sample processed at the concentration to 6.25 mg/ml, although the immobilized amount of enzyme decreased significantly. The activity value comparisons are made according to the activity values measured at 20 mM glucose concentration. The specific activities of immobilized enzyme were 4.7 (0.625 mg/ml case) and 25 (0.0625 mg/ml case) times more than the specific activity when immobilization was performed from an initial concentration of 6.25 mg/ml GOX solution.

The inverse relation between the immobilized enzyme amount and activity values are thought to be caused by two possible reasons. ESEM images showed that GOX particles immobilized not only on the surface of the polymer support but also inside the polymer foam. 380 µg is the total amount both for the enzyme immobilized on the surface and in the inner part of the polymer. The particles immobilized on the inner layers may not be accessible for reaction. In enzymatic activity determination, only the enzymes immobilized on surface of the polymer may catalyze the reaction, not the enzymes immobilized on the inner part of the polymer matrix. This can be one reason of low activity at the high enzyme amount. Another reason, thought to cause the inverse relation between the immobilized enzyme amount and activity was agglomeration. Too much enzyme loading can cause agglomeration. In enzymatic activity determination, only the upper surface of the

agglomerated enzymes may catalyze the reaction, agglomeration may hinder the active sites of the surface enzymes. Thus the entire immobilized enzyme did not contribute to the catalysis during reaction.

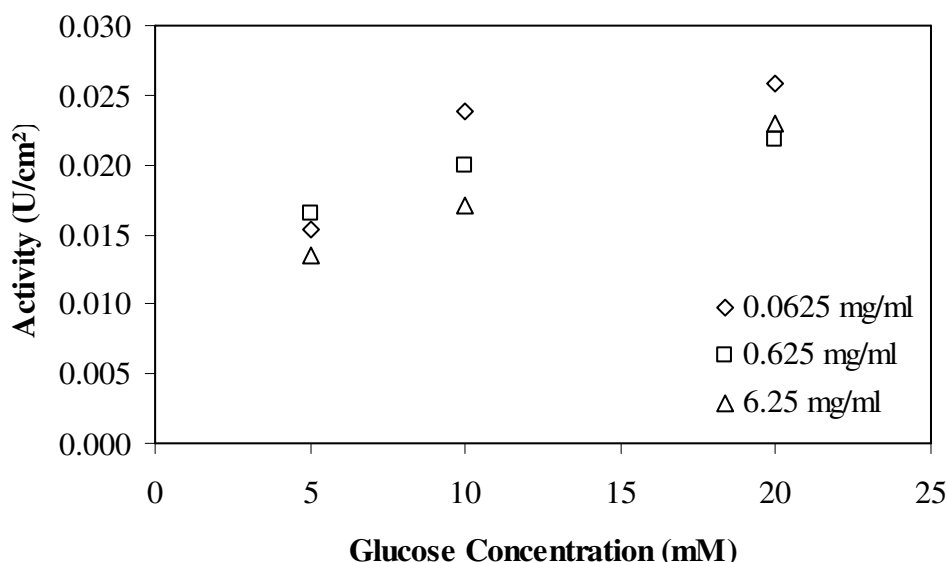


Figure 6.6. Activities of GOX immobilized PU/PPY composite foam samples at different immobilization solution concentrations

6.3. Effects of “Washing Before Immobilization” and “Washing After Immobilization” on the Activity of PU/PPY Foam Samples

In this subsection the effects of “washing before immobilization” and “washing after immobilization” on the activity of PU/PPY foam samples are presented and discussed. The PU/PPY composite foam samples were washed both before and after the SFI process. Following the composite formation of PU/PPY, the samples were dried in a fume hood for 7 days to evaporate the excess monomer/oxidant from the sample. However, as composites were immersed in GOX solution for immobilization, the color of solution became brown-black. It was thought that the monomers, dimers, trimers, or I_2 , which cannot diffuse out of the composite when exposed to air, come out of the composite as it is immersed in the solution. Such chemicals can reduce the enzyme activity of the GOX solution, and furthermore the protein amount in the immobilization solution could not be correctly determined. Therefore “washing before immobilization” step was also encountered in the

immobilization process. Although, “washing before immobilization” was first performed in order to accurately measure the immobilized GOX amount from the immobilization solution, then it was observed that different washing methods lead to differences in activity values of the samples. Three different procedures were followed for the “washing before immobilization” step. In the first procedure, PU/PPY samples (Runs PU/PPY1 and PU/PPY1^R) were washed in 3 ml of water at 40 °C for 30 h prior to the immobilization procedure. In the second procedure, PU/PPY samples (Runs PU/PPY5 and PU/PPY5^R) were washed in 3 ml of buffer at 40 °C for 30 h. In the third procedure, the PU/PPY samples (Runs PU/PPY6 and PU/PPY6^R) were first washed in 3 ml of buffer at 40 °C for 30 h, then they were put in new unused buffer solution (3 ml) and exposed to scCO₂ at 100 bar and 40 °C for 24 h.

Figure 6.7 shows the activity values of PU/PPY samples which were washed at different conditions including the non-washed case. In Figure 6.7, in addition to experimental data, the results of the model are also given. For the model the single substrate form of Michaelis-Menten equation was used. As it is seen, the lowest activity was measured for the non-washed sample and the highest activity was calculated for the sample washed first with buffer then with buffer in scCO₂. The experimental activity data of the samples washed with buffer and water are very similar but the model value at high glucose concentration of the buffer washed sample is higher than the water washed sample. The increase in activity with “washing before immobilization” may be concerned with the removal of the monomers, dimers, trimers, or I₂ which could be harmful for the enzyme activity. Additionally, washing especially with scCO₂ could modify the surface of the polymer matrix making it for favorable for immobilization.

Although the highest activity was obtained for the sample washed first with buffer then with buffer in scCO₂, washing only in buffer was chosen for the investigation of the effects of “washing after immobilization” and immobilization time on the activity in order to optimize the time consumed for washing each sample before immobilization.

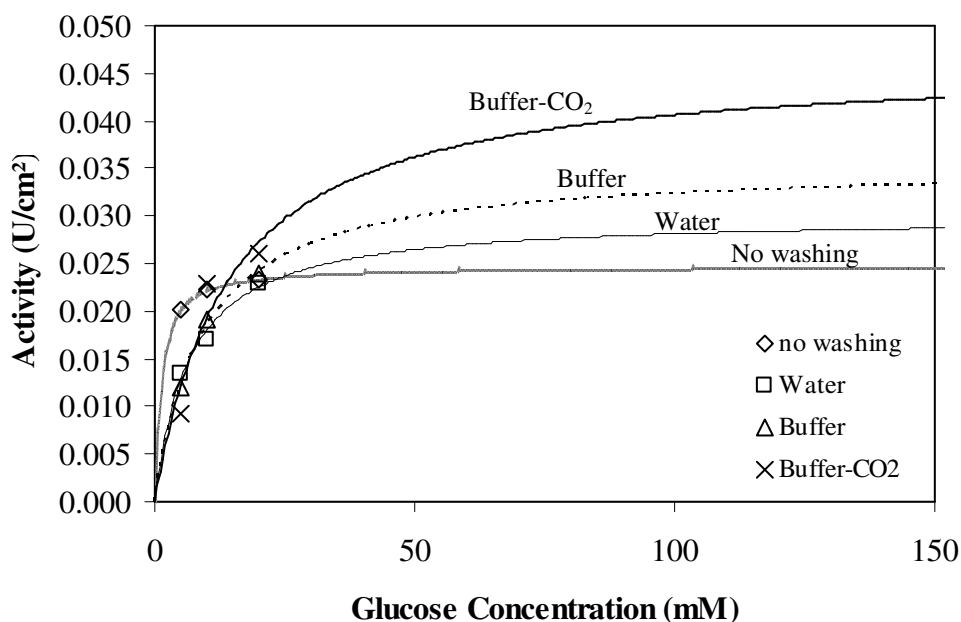


Figure 6.7. Activities of GOX immobilized PU/PPY composite foam samples washed with different washing procedures before immobilization (immobilization at 40 °C, 100 bar, for 24 h, washing time after immobilization: 10 h).

The GOX immobilized PU/PPY samples were washed after the immobilization process to get rid of excess GOX particles which were not really immobilized on the polymer matrix. As it is explained in Section 3.4, experiments were done to determine the washing period after which the washing solutions did not possess any activity, meaning that no GOX particles fall into the washing solution. As it is shown in Figure 3.3, by washing the sample 30 min at 30 °C, 90 per cent of the unbonded GOX was released. The remaining 10 per cent was washed away totally at the end of 4 h (Figure 3.3). In preliminary experiments, the enzymatic analysis was performed after washing the sample for 30 min. But in the rest of the experiments done with PU/PPY, longer washing periods were preferred. Ten hours was the maximum “washing after immobilization” period carried out during runs, although it was proved that after 4 h, no GOX fell into the washing solution (Figure 3.3). Actually, enzymatic activity was only detected when the concentration of immobilization solution was 6.25 mg/ml. At lower enzyme concentrations, no activity was determined in the washing solutions, but still they were washed for long periods, i.e. 4 and 10 h.

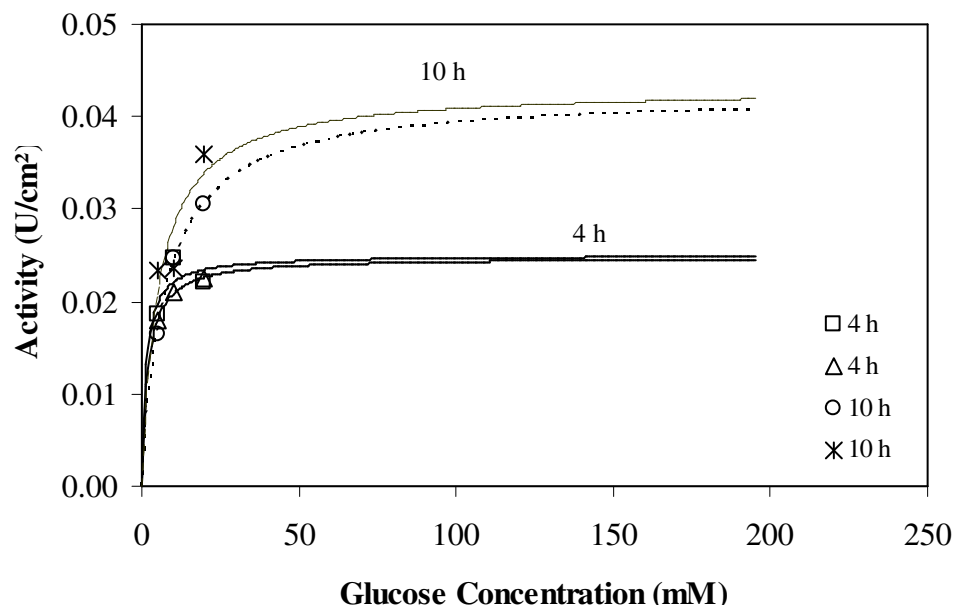


Figure 6.8. Activities of GOX immobilized PU/PPY composite foam samples washed for different washing periods after immobilization (immobilization performed at 40 °C, 100 bar, for 24 h).

Figure 6.8 shows the activity of PU/PPY samples that were washed for 4 and 10 h after immobilization at 30 °C. For these experiments (Runs PU/PPY7, PU/PPY7^R, PU/PPY8, and PU/PPY8^R) GOX-buffer immobilization solution was 0.0625 mg/ml, and the SFI was performed at 100 bar, 40 °C. Since four runs (actually two runs and two replicates) were performed to investigate the effect of “washing after the immobilization period”. Since the number of runs was relatively few, the results of each run were given separately. When the number of runs is high, in order to avoid confusion on the figures, the results are given on the basis of average values (i. e. the average result is calculated for a run and its replicate). As it is seen from Figure 6.8, it was interesting to observe that as the “washing after immobilization” period increased, the activity of the samples also increased. Since it was proven that there were no GOX particles falling into the washing solution, it was expected that the activity values of the samples washed for 4 h and 10 h after immobilization would be same. The increase in activity with an increase in washing period reveals that washing took immobilized GOX to a favorable position for reaction. The “washing after immobilization” was performed at 30 °C. When investigating the effect of temperature on free GOX-buffer solution activity, it was observed that keeping GOX-

buffer solution in a test tube at 30 °C water bath for 24 h caused a slight increase in activity (Figures 4.3 and 4.4) . Washing the GOX immobilized PU/PPY sample at 30 °C after immobilization made the same effect on immobilized GOX activity with keeping the free GOX-buffer solution at 30 °C. As it is mentioned in Section 4, water does not act only as a solvent for the reactant and the products, but it also takes part in the reaction. The slight enhancement in activity after washing at 30 °C may be attributed to the increase of the hydration shell around the enzyme, which may facilitate the enzymatic reaction taking place after washing.

6.4. Effect of Immobilization Time on PU/PPY Foam Sample Activities

The SFI process was performed at three different immobilization periods, namely for 4, 24 and 72 h immobilization periods. Figure 6.9 shows the activity values of PU/PPY samples immobilized for 4, 24, and 72 h. The immobilization processes were performed at 100 bar, 40 °C using 0.0625 mg/ml GOX solution. It is observed that when the immobilization time was increased from 4 h to 24 h, the increase in activity was 45 per cent. Further increase of immobilization time from 24 h to 72 h causes 9.4 per cent decrease in activity, based on the measured activity values at 20 mM glucose concentration. If model values at 100 mM are considered, it is seen that increase of immobilization time from 4 to 24 h lead to activity enhancement by 71.8 per cent, and an increase from 24 h to 72 h caused 16.4 per cent decrease in activity. The best immobilization period among the tested ones was 24 h. As the immobilization time increased from 4 to 24 h, there was a considerable increase in activity caused by increase in the immobilized enzyme amount, but further increase from 24 to 72 h did not enhance the activity possibly due to the high enzyme loading that caused agglomeration. İnoğlu (2007) also reported that 24 h of immobilization time lead to highest immobilized enzyme activity among 4, 24, and 72 h immobilization periods for immobilization of GOX on PU film.

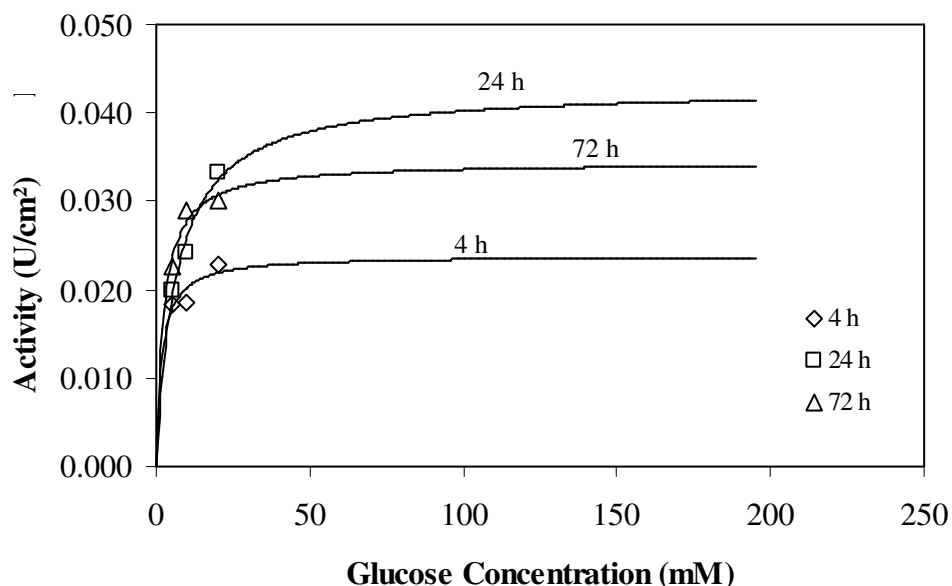


Figure 6.9. Activities of GOX immobilized PU/PPY composite foam samples for which immobilization was performed at different immobilization periods (immobilization performed at 40 °C, 100 bar, for 24 h).

6.5. Effect of Pressure on SFI Process of PU/PPY Foam Samples

Up to this point, the PU/PPY samples were washed in 40 °C buffer solution for 30 h. However, some problems were still encountered during the protein determination method. Therefore, at this point, two other washing procedures were decided to be used. In the first method, the samples were put in a 3 ml buffer solution, which were kept for 24 hours at 50 °C. In the second way, the samples were exposed to scCO₂ at 150 bar and 50 °C, again in 3 ml buffer solution for 24 h. The polymer samples washed with both methods were used for SFI, and the resulting activities of the samples are given in Figure 6.10. As it is seen from Figure 6.10, the activity of the scCO₂ washed sample was higher than the activity of the sample washed only with buffer at atmospheric conditions, which coincides with the results given in Section 6.3. Regarding to the higher activities and more consistent results obtained in protein determination, it was decided to wash the PU/PPY samples in sc CO₂ at 150 bar and 50 °C for 24 h before GOX immobilization.

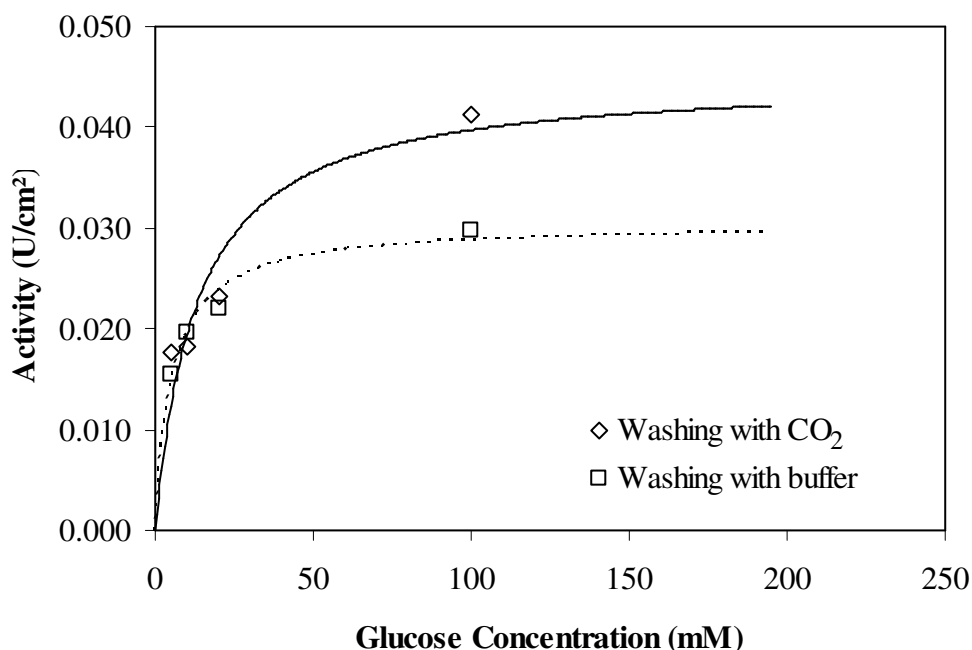


Figure 6.10. The effect of different washing procedures (before immobilization) of PU/PPY samples on the activity of samples after immobilization (immobilization performed at 40 °C, 100 bar, for 24 h).

Generally, in the enzymatic studies mentioned in the literature, the activity values are reported only at one substrate concentration, which is well above the K_m value. Only in experiments in which the aim is to determine the kinetic parameters, the enzymatic activities are measured at several substrate concentrations. In this study, the activities of the GOX immobilized polymer samples, and also of free GOX, were determined and presented at several glucose concentrations for each experimental run. Up to this point, the evaluation of the performance of GOX immobilized PU/PPY samples was made by determining their enzymatic activity at 5, 10 and 20 mM glucose concentrations, in a range which includes the lower and upper limits of human blood glucose concentration. In the rest of the study, the activities of GOX-immobilized PU/PPY samples were analyzed at glucose concentrations of 5, 10, 20 and 100 mM. Normally, the activities measured at 100 mM glucose concentration may only be investigated and reported, because at lower glucose concentration, the activities of GOX-immobilized samples did not differentiate as they did at 100 mM. However, the main aim of immobilizing GOX on to a polymer matrix was to prepare a biosensor that would measure the blood glucose level, and the human

blood glucose level is between 5-10 mM. Therefore, the behavior of the activities measured at lower glucose concentrations should also be investigated.

The effect of pressure and temperature on the activities of the GOX-immobilized PU/PPY samples were mainly evaluated according to the experimental data, and model predicted value at 100 mM glucose concentration. The experimental data at lower glucose concentrations were also examined, even though they are less differentiating. The activity values given in this and following subsections are the averages of two runs for each data point. The experimental data was fit to the single substrate form of Michaelis-Menten equation without transformation of data, the kinetic parameters (K_m and v_m) were found. The model results are also given on Figures 6.11-15, along with the experimental data.

The pressure effect (80, 100 and 150 bar) on immobilization was investigated at two different temperatures, 30 and 40 °C for 24 h of immobilization time. Figures 6.11 and 6.12 show the activities of GOX-immobilized samples, prepared at 30 and 40 °C, respectively. As it is seen on (the main frame) Figure 3, at 30 °C, when immobilization was conducted at 80 and 100 bar, the activities of the samples at 100 mM glucose concentration were nearly the same and they were approximately 50 and 80 % higher than the activity values obtained at 150 bar-30 °C and 1 bar-30 °C, respectively. When the model values were inspected, it was seen that the predicted activity values at high glucose concentrations (> 50 mM) were higher for the samples produced at 100 bar than 80 bar. Zooming on the low glucose concentrations of the Figure 6.11, it was observed that the activity of samples prepared at 1, 100 and 150 bar exhibit the same behavior, but the slope of activity increase between 5 and 10 mM glucose concentrations was higher for 100 and 150 bar than for 1 bar. At 80 bar, the behavior was different, and was not suitable for measuring blood glucose level since it decreased as glucose concentration increased from 5 to 10 mM.

At 40 °C immobilization temperature, 80 and 100 bar immobilization pressure, the activities of the resultant samples at 100 mM glucose concentration were nearly the same. They were approximately 30 and 120 % higher than the activity values of the samples obtained at 150 bar and 1 bar, respectively (Figure 6.12). At low glucose concentrations (the inner frame of Figure 6.12 shows the zoomed region), the activities of the samples prepared at 1 bar decreased as the glucose concentration increased from 5 to 10 mM, which

is not a preferred case in glucose sensing. Among 80, 100 and 150 bar, the highest slope in activity increase between 5 and 10 mM glucose concentration was measured for the sample prepared at 100 bar, 40 °C.

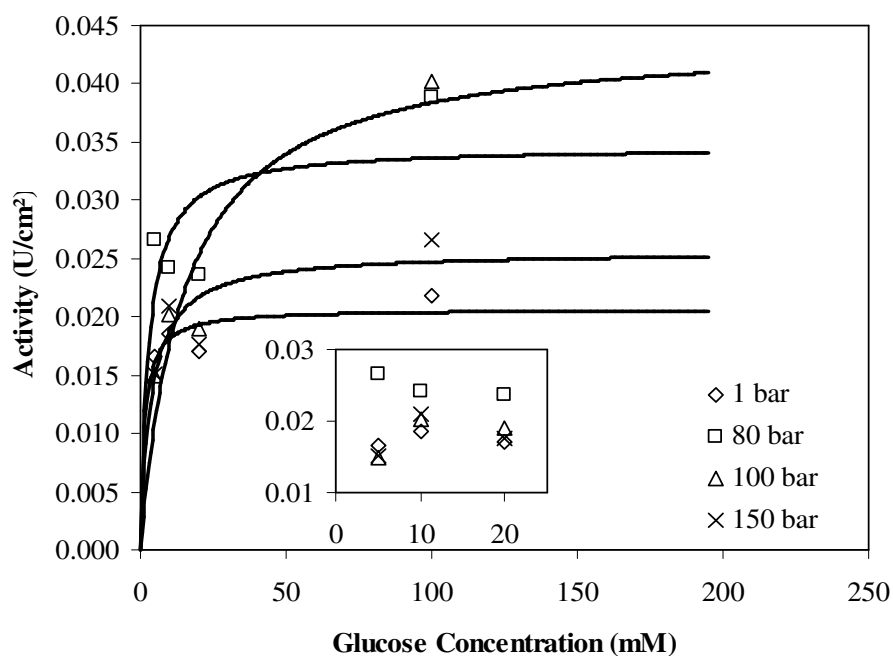


Figure 6.11. The pressure effect on the activity of GOX-immobilized PU/PPY composite foams at 30 °C

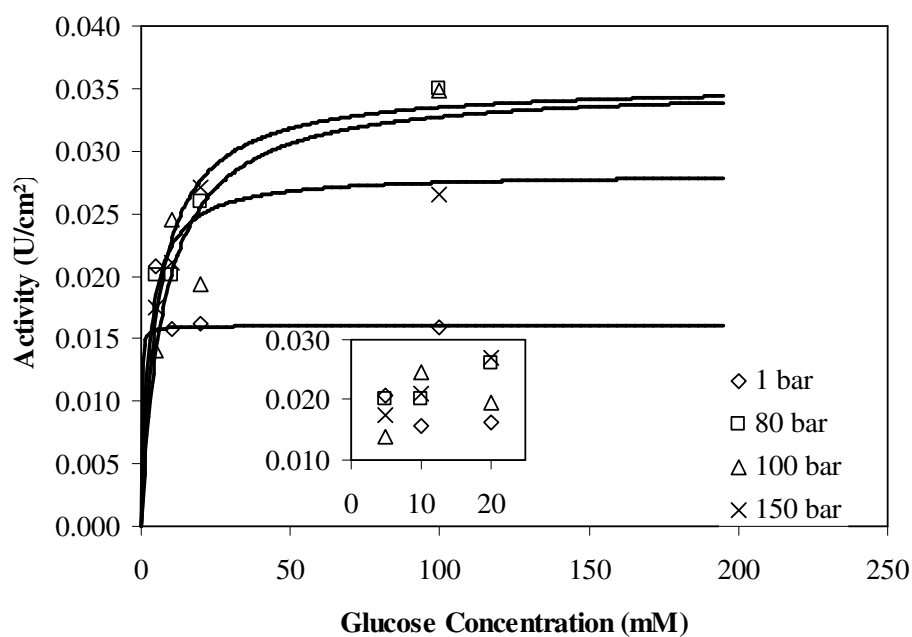


Figure 6.12. The pressure effect on the activity of GOX-immobilized PU/PPY composite foams at 40 °C

From these results, it is obvious that at 30 and 40 °C, immobilizations performed in scCO₂ environment gave much better activity values as compared to immobilizations performed at atmospheric conditions. In order to better visualize the effect of pressure on activity of the samples, activity values were plotted as a function of pressure (Figure 6.13), the reported activities in Figure 6.13 are the model values at 100 mM glucose concentration. As it is seen from Figure 6.13, at both immobilization temperatures (30 and 40 °C), the activity values of PU/PPY foam samples processed at supercritical conditions were always much higher than the ones processed at 1 bar. Additionally, at supercritical conditions, up to 100 bar, the pressure enhanced the observed activity values. However, further increase in pressure did not enhance the activity any further.

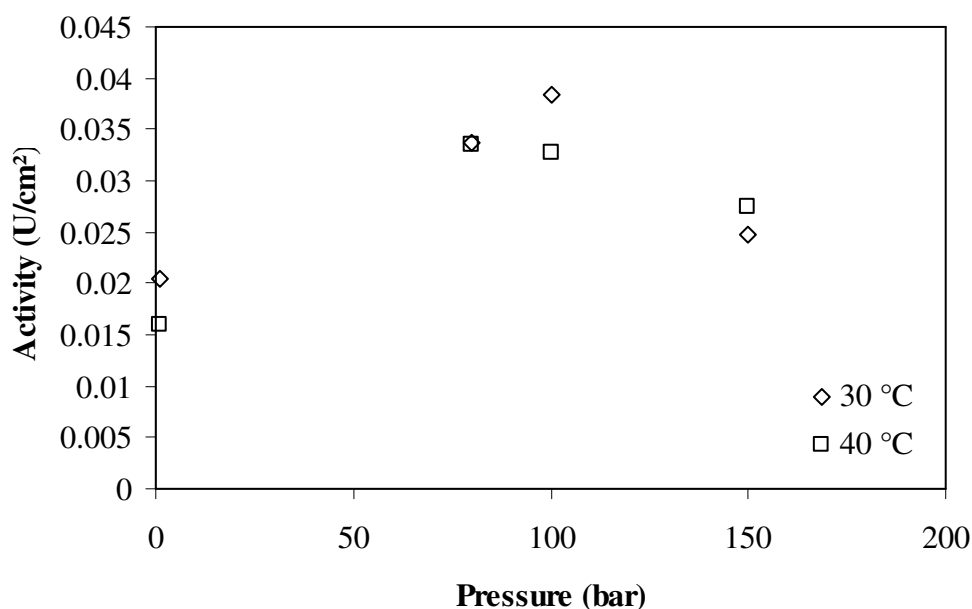


Figure 6.13. The pressure effect on the activity of GOX-immobilized PU/PPY composite foams at 100 mM glucose concentration

The immobilized GOX amount on PU/PPY samples was also determined by the Bradford Method, the details of the procedure are given in Section 3 and Appendix C. The immobilized GOX amount on PU/PPY sample at different experimental conditions are given in Table 6.2 and shown on Figure 6.14 as a function of pressure. Figure 6.14 reveals that as the pressure increased, the immobilized GOX amount on the polymer support was decreased. At constant temperature, the density of CO₂ increases as the pressure increases. Increase in CO₂ density may affect the immobilized GOX amount. Figure 6.15 shows the

CO₂ density dependence of immobilized GOX amount. As it is observed from Figure 6.15, the immobilized GOX amount decreased as the CO₂ density increased. ScCO₂ could interact with both GOX and the polymer. The increase in CO₂ amount may increase the interaction between CO₂-enzyme and CO₂-polymer, and thus may introduce a barrier for higher amounts enzyme immobilization by lowering the interaction of polymer matrix and GOX. From Figure 6.15, it is seen that CO₂ density has significant effect on the immobilization process, but not the only effect, temperature has also influence on the immobilization process.

Table 6.2. Immobilized GOX amount and specific activity of PU/PPY samples processed at different experimental conditions

Pressure (bar)	Temperature (°C)	Immobilized GOX Amount (μg)	Specific Activity (U/mg)
1	30	30.1	1.8
80	30	21.4	4.0
100	30	19.7	4.9
150	30	15.9	4.0
1	40	42.1	1.0
80	40	39.3	2.0
100	40	29.8	2.7
150	40	23.3	2.7
100	50	30.2	1.8

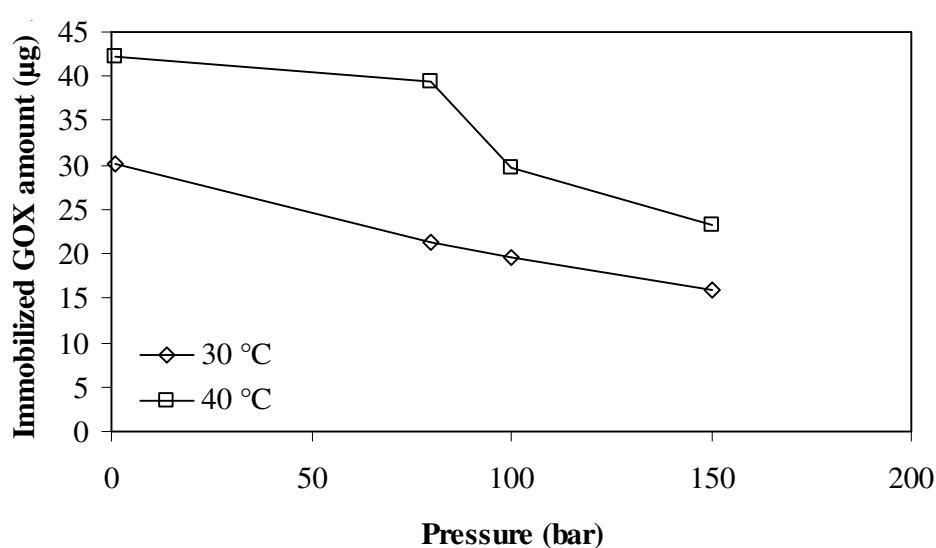


Figure 6.14. The immobilized GOX amount on PU/PPY composite foams at different pressure values

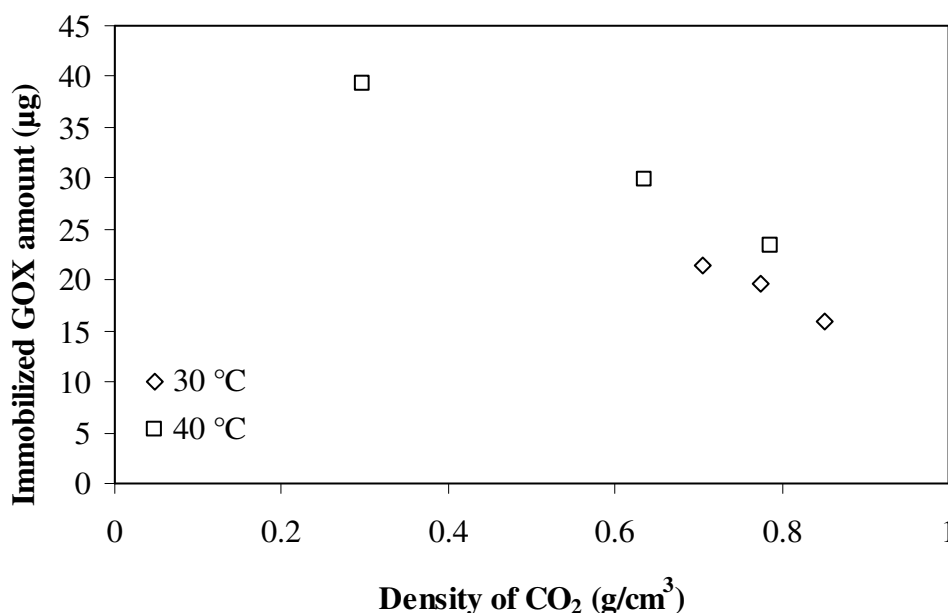


Figure 6.15. The immobilized GOX amount on PU/PPY composite foams at different density values

The activities observed (U/cm^2) and the immobilized GOX amounts were presented in previous paragraphs. The specific activity (U/mg) was also calculated in order to better evaluate the SFI process. Although the immobilized GOX amounts were lower in samples prepared using the SFI process than in immobilization proceeded at atmospheric conditions, the activities of the PU/PPY samples from SFI were higher. This indicates that specific activities of the GOX immobilized PU/PPY samples obtained from SFI process were much higher than the samples obtained from atmospheric immobilization. The specific activity values as a function of pressure are given in Figure 6.16.

At constant temperature, the specific activities of PU/PPY samples on which GOX immobilized at supercritical conditions were always higher (at least 2 folds) than the specific activities of the samples on which GOX immobilized at atmospheric conditions. In the supercritical region, as the pressure increased from 80 to 100 bar, the specific activity increases. However, further increase to 150 bar, did not cause any increase in the specific activity. It cannot be said that, at pressures higher than 100 bar, pressure and CO_2 had an adverse effect on enzyme activity so that at 150 bar the activity and specific activity of immobilized GOX was lower. Actually, the activity of GOX-buffer solution exposed to

150 bar, 40 °C was higher than the GOX-buffer solution exposed to 100 bar (Figures 4.7 and 4.8). However, in the immobilization process, the only interaction was not between scCO_2 and GOX-buffer solution; there were also interactions between the polymer matrix and scCO_2 , and between polymer and GOX-buffer solution. Although, the activity of GOX-buffer solution that was exposed to scCO_2 at 100 bar was slightly lower than the activities of the solutions exposed to scCO_2 at 80 and 150, highest activity and specific activity for immobilized GOX were highest at 100 bar, which also indicated the existence of interactions other than the one between scCO_2 and GOX-buffer solution.

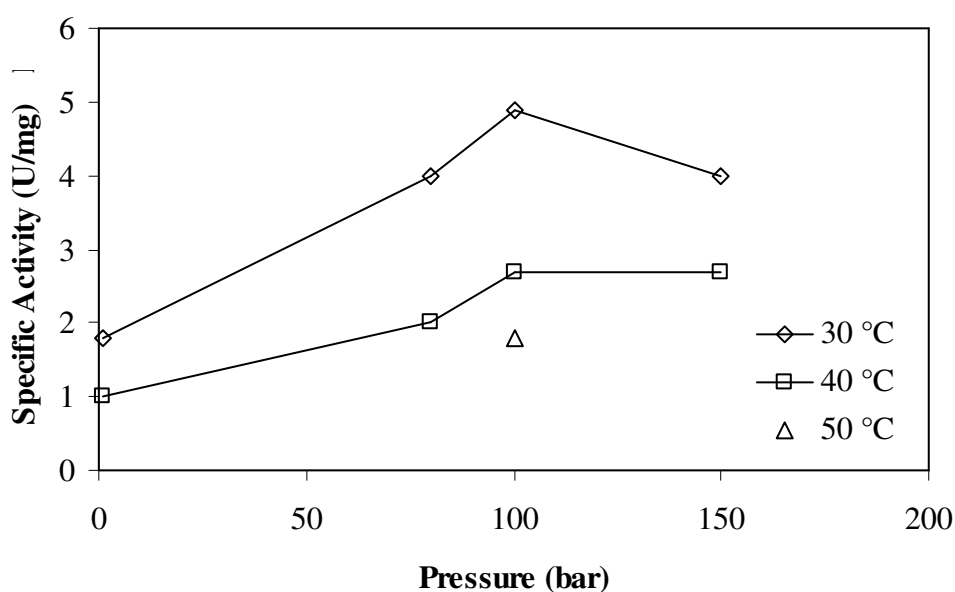


Figure 6.16. The specific activity of GOX immobilized on PU/PPY composite foams as a function of pressure

It is known that scCO_2 did not cause detrimental changes to the enzyme. The reason for better activity values obtained as a result of SFI than the immobilization done at atmospheric conditions can be attributed to the changes caused by scCO_2 on the microenvironment of the enzyme and the polymer surface. The microchanges done by scCO_2 on the enzyme and the polymer might have made the immobilized enzyme active site more favorable for reaction.

6.6. Effect of Temperature on SFI Process of PU/PPY Foam Samples

The effect of temperature on SFI process was investigated at 30, 40 and 50 °C immobilization temperatures. At 1, 80 and 150 bar, the immobilization procedure was conducted at 30 and 40 °C for 24 h.

Among all pressures investigated, the PU/PPY foam sample on which GOX was immobilized at 100 bar was found to have the highest activity (at high glucose concentration, i.e. 100 mM), the highest activity increase between 5–10 mM glucose concentration, and also highest specific activity. Therefore, the effect of temperature was investigated at 30, 40 and 50 °C for 100 bar immobilization pressure and 24 h of immobilization time.

As the immobilization temperature increased from 30 to 40 °C, and from 40 to 50 °C, the activities of the GOX-immobilized (at 100 mM glucose concentration) composite samples decreased 13.5 per cent and 31 per cent, respectively (Figure 6.17). Increasing temperature from 40 to 50 °C caused a more pronounced decrease in activity compared to the activity decrease between 30 and 40 °C. At 100 bar, decrease in activity with increasing temperature might be caused by a slight decrease in free enzyme activity. At low glucose concentrations, all the samples processed at 100 bar and at different temperatures showed the same trend, but the activity increase between 5 and 10 mM glucose concentration was the steepest at 40 °C (the inner frame of Figure 6.17).

The activity of the GOX immobilized PU/PPY samples decreased as the immobilization temperature increased at 100 bar, however at other immobilization pressures tested, the situation is not exactly the same. At 1 bar, the activity of GOX immobilized PU/PPY sample decreased as the immobilization temperature increased, as in the case of 100 bar, but it was not valid for 80 bar and 150 bar (Figure 6.18).

The effect of temperature on specific activity was more consistent (Figure 6.19) than its effect on activity. At constant pressure, as temperature increased, the specific activity decreased for all pressures (1, 80, 100 and 150 bar).

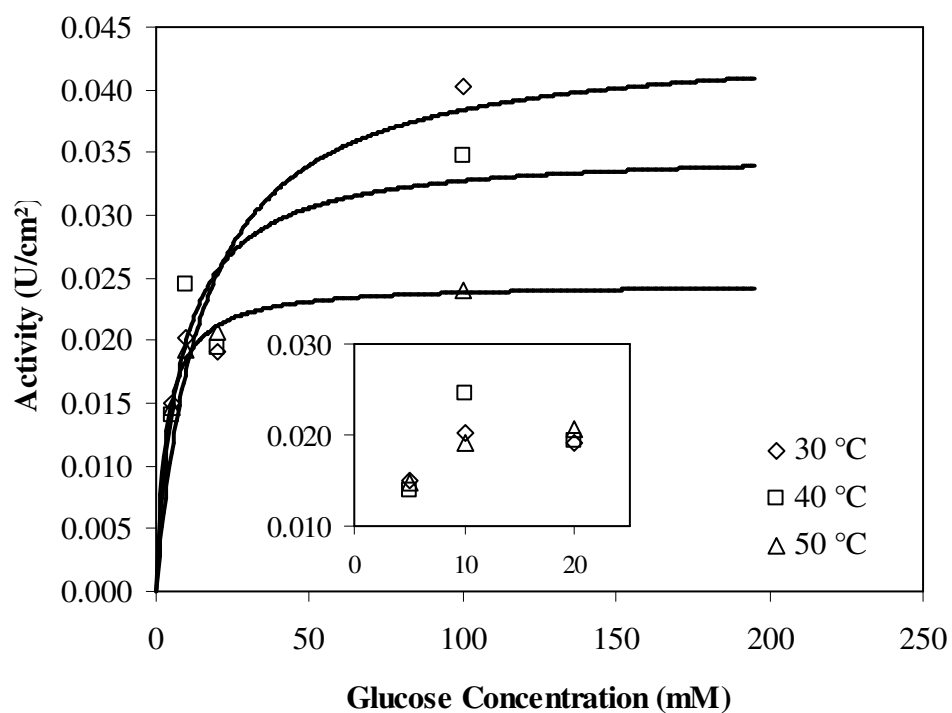


Figure 6.17. The temperature effect on the activity of GOX-immobilized PU/PPY composite foams at 100 bar.

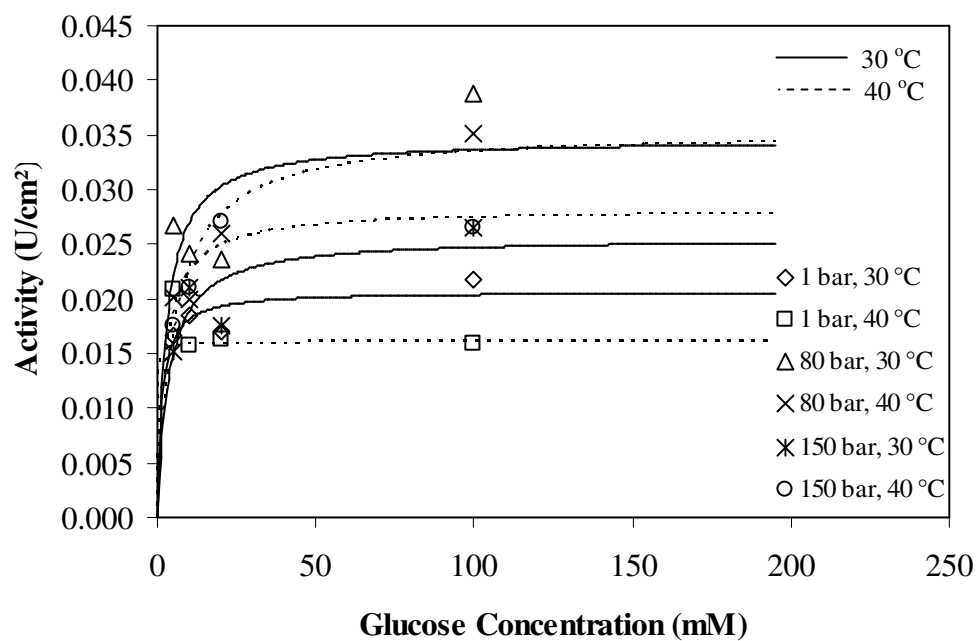


Figure 6.18. The temperature effect on the activity of GOX-immobilized PU/PPY composite foams at 1, 80 and 150 bar

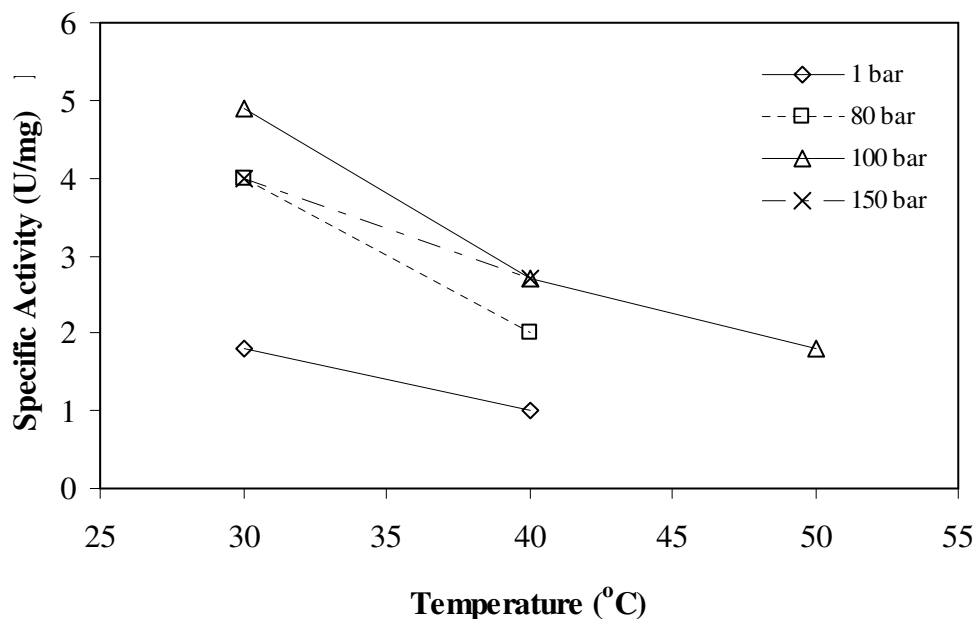


Figure 6.19. The specific activity of GOX immobilized on PU/PPY composite foams as a function of temperature

6.7. Kinetic parameters and long term stability of PU/PPY foam samples

The kinetic parameters of Michaelis-Menten equation were found by nonlinear curve fitting without transformation of the data, and the results are given in Table 6.3. The lowest and the highest v_m values were found as 0.016 and 0.044 $\mu\text{mol}/(\text{min}.\text{cm}^2)$ for the GOX-immobilized PU/PPY samples processed at 1 bar-40 °C and 100 bar-30 °C, respectively. The lowest K_m values were obtained for the samples processed at 1 bar, and the highest values obtained for the samples processed at 100 bar. All K_m values found for the samples processed at pressures 80, 100 and 150 bar were higher than the K_m values of the samples processed at atmospheric pressure without CO_2 . This may indicate that the scCO_2 modified the polymer matrix surface that effects the interaction between the enzyme and the immobilization matrix. Despite the higher K_m values, the activities observed with the samples immobilized via SFI process are much higher than the activity values obtained at 1 bar.

In order to investigate the stability of the GOX-immobilized PU/PPY samples 10 consecutive enzymatic analysis were done at 100 mM glucose concentration. The results of

these consecutive analyses are shown in Figure 6.20. It is seen that, there was no gradual decrease in the activity of the sample as the analysis number was increased. The mean value of the data was 0.030 U/cm^2 (shown as straight line in Figure 6.20) and lower and upper limits of 95 per cent confidence interval were 0.32 and 0.27 U/cm^2 (shown as dashed line on Figure 6.20), respectively. Long term stability of PU/PPY was also investigated for a sample immobilized at 100 bar and 40°C . At the end of 21 days the activity of the sample decreased by 20 per cent when it was kept in $+4^\circ\text{C}$ (Figure 6.21).

Table 6.3. Kinetic parameters of immobilized GOX on PU/PPY composites

Pressure (bar)	Temperature ($^\circ\text{C}$)	v_m $\mu\text{mol}/(\text{min}.\text{cm}^2)$	K_m (mM)
1	30	0.021	1.41
80	30	0.035	2.84
100	30	0.044	14.84
150	30	0.026	3.60
1	40	0.016	0.50
80	40	0.035	5.55
100	40	0.035	7.54
150	40	0.028	2.65
100	50	0.025	3.23

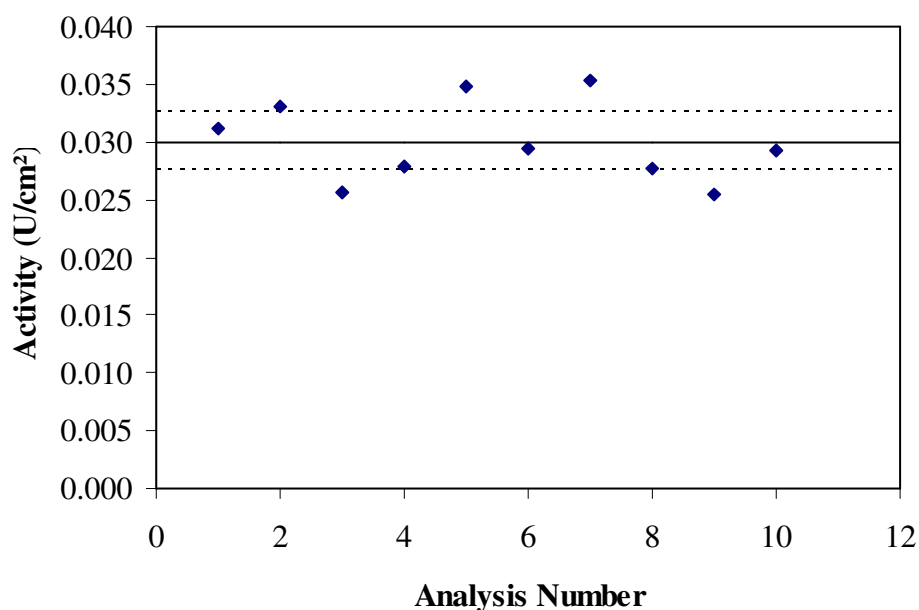


Figure 6.20. The activity results of successive enzymatic analyses

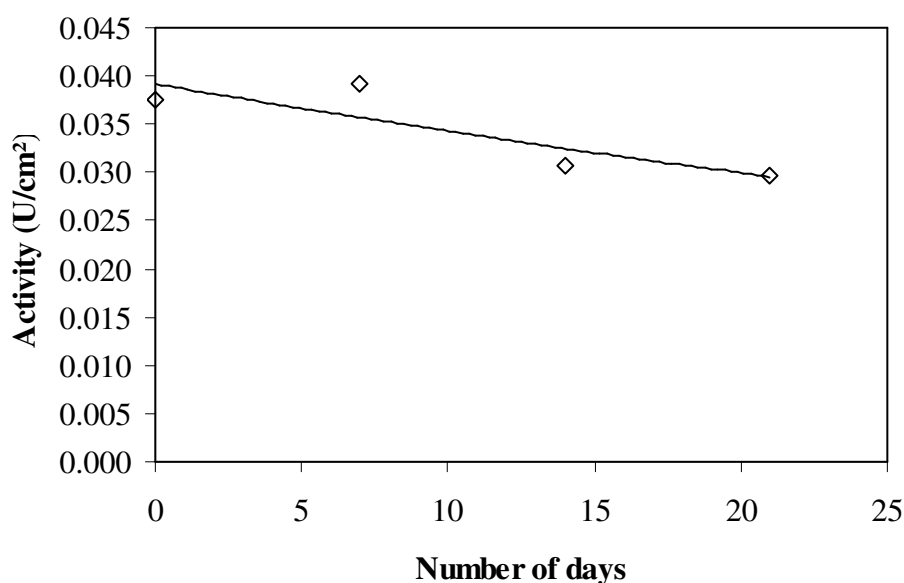


Figure 6.21. Long term stability of GOX immobilized PU/PPY composite stored at +4 °C.

6.8. SFI of GOX on PU/PPY Composite Film

The maximum and minimum GOX-buffer solution concentrations used for SFI of GOX on PU/PPY composite foam was 6.25 and 0.0625 mg/ml. First of all, PU/PPY films were washed with buffer at 40 °C for 35 h prior to immobilization. The washing solution become brown-black, but different from the PU/PPY foams, there were sticky and greasy black particles on the walls of the test tubes used for washing in PU/PPY film case. The PU/PPY particles were washed with 4 ml of unused fresh buffer solution after they were taken out of the washing solutions to get rid of the these black particles. In Runs PU/PPYF1 and PU/PPYF2, the washed PU/PPY films were used for immobilization in 6.25 and 0.0625 mg/ml enzyme solutions at 100 bar and 40 °C for 24 h, respectively. The aim was to optimize “washing after immobilization” period. When the immobilization time was over, the PU/PPY films were taken out of the pressure cell and washed with 4 ml of buffer solution. It was observed that there were again sticky black particles in the immobilization solution.

The sample taken out of the pressure cell was put in a test tube containing 2 ml buffer and kept in that buffer solution in a constant temperature water bath equipped with a shaking apparatus at 30 °C for 10 min. At the end of 10 minutes, the sample was taken out

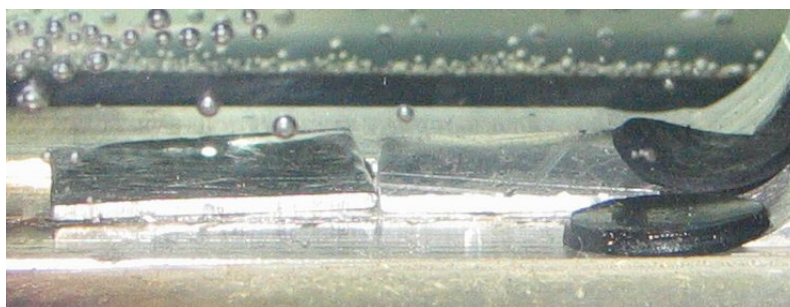
of that buffer solution, and put in 2 ml new (unused) buffer solution for an additional 20 minutes at 30 °C constant temperature water bath with a shaking apparatus. At the end of 20 min washing, the sample was taken out of the buffer solution and put in a new (unused) 2 ml buffer solution for additional 30 minutes. It was hoped that the washing procedure continued in increased durations (1st washing: 10 min, 2nd washing: 20 min, 3rd washing: 30 min, 4th washing: 1 h, 5th washing: 2 h, etc.) until no activity was observed in the washing solution. The washing procedure proceeded until 2 h total washing time (1st washing: 10 min, 2nd washing: 20 min, 3rd washing: 30 min, 4th washing: 1 h). No activity was observed for these washing solutions, therefore at the end of fourth step, washing was stopped and enzymatic analysis of the PU/PPY film sample was performed. As it was mentioned in Section 3, for PU/PPY foam samples, if the immobilization solution concentration was 6.25 mg/ml, then all of the unbond GOX particles were dropped to the washing in the first 4 h of washing period, but there were no unbound particles that fell into the washing solution when the concentration of the immobilization solution was low (0.0625 mg/ml). However, for PU/PPY films there were no fallen GOX particles in the washing solution even if the concentration of the immobilization solution was 6.25 mg/ml, thus no activity was observed in the washing solutions.

İnoğlu (2007) found that GOX immobilized PU films showed higher activity values than the GOX was immobilized PU foams, where the immobilization processes were conducted exactly at the same conditions for both polymer matrices. Moreover, when the activities of GOX immobilized PU foams and PU/PPY foams were compared it was observed that the activities of the PU/PPY foams were higher than the activities of PU foams. Therefore based on these facts, it was expected that PU/PPY films would have the highest activity among these four polymer matrices (PU film and foam, PU/PPY film and foam). However the results of enzymatic activity analysis of PU/PPY film samples from Runs PU/PPYF1 and PU/PPYF2 were totally surprising. The enzymatic activity analysis started with the lowest substrate concentration (5 mM) and at 5 mM of glucose concentration; there were no reactions at the end of 2, 4 and 6 min of incubation time for both samples. After this disappointment, analysis at 10 mM glucose concentration was omitted and the samples were incubated at 20 mM glucose concentration for 30 min. At the end 30 min the activity for the sample from Run PU/PPYF1 was very low (0.001 U/cm²), and no activity was observed for the sample from Run PU/PPYF2. The difference between

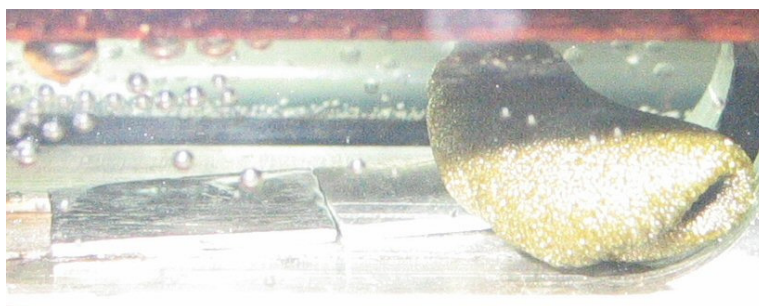
Runs PU/PPYF1 and PU/PPYF2 was their initial concentration of immobilization solutions. Very low activity was measured from the sample on which GOX immobilized from 6.25 mg/ml GOX solution.

The next step was to repeat the immobilization process on PU/PPY films. Four more experiments (two runs and two replicates) were conducted. Two non-washed and two washed samples were used. The samples used in Runs PU/PPYF3 and PU/PPYF3^R were first washed in buffer at 40 °C for 50 h and then they were put in 3 ml of unused buffer solution and kept in scCO₂ environment for 24 h. The concentration of immobilization solution was 6.25 mg/ml for Runs PU/PPYF3, PU/PPYF3^R, PU/PPYF4 and PU/PPYF4^R. The immobilization temperature and pressure were 40 °C and 100 bar. The samples were washed for only 30 min after immobilization, since no activity was observed in the washing solutions. At 10 mM glucose concentration very low activity was observed for samples of PU/PPYF3 and PU/PPYF3^R, which were 0.015 and 0.010 U/cm². The samples of PU/PPYF4 and PU/PPYF4^R reacted with glucose, but the amount of glucose converted at 2 min was higher than the amount converted at 4 and 6 min, which should not be the case. Therefore, activity values for those samples could not be calculated. Since very low or no activity was observed for PU/PPY film samples, no more runs were performed.

It must be also noted that, the sample from Run PU/PPYF4^R was swelled after the immobilization process. In order to verify that, two PU/PPY film samples were put in a view-cell and monitored in scCO₂ environment. The PU/PPY films were kept in the view-cell at 40 °C and 100 bar for 5 h. During that period, no obvious swelling was observed, but the sharp edges of the samples were softened. However, during depressurization one of the samples got larger approximately by 400 per cent of its original surface area (Figure 6.22), which was thought to be foaming.



(a)



(b)

Figure 6.22. The images of PU/PPY film (a) in scCO_2 environment, (b) after depressurization

In this work, the effect of scCO_2 on GOX-buffer solution was investigated and it was found that scCO_2 has no negative effect on the enzyme up to 150 bar. SCF immobilization was applied to PPY films, PU/PPY composite foams and films. The highest activity was obtained for PU/PPY composite foams. The SFI process approximately doubled the activity values of the polymer matrices compared to atmospheric immobilization.

7. CONCLUSIONS AND RECOMMENDATIONS

This work lays the foundations on the use of SCF techniques (e.g. SFI) in biosensor development and production. This study is aimed to provide useful information and data for immobilization of GOX on a conducting polymer in supercritical fluid environment. It especially encourages further research both on using PU/PPY composites and Supercritical Fluid Immobilization (SFI) in biosensor production. The study is mainly divided into two parts: first, the effect of scCO_2 on the GOX was studied, and it was observed that scCO_2 had no adverse effect GOX. Then, supercritical fluid immobilization was applied on conducting PPY and PU/PPY composite polymer matrices in order to immobilize GOX on these matrices. For each polymer matrix, the studies were also divided into two parts; first preliminary experiments that gave information on further steps to do for sample preparation, analytical conditions were performed before deciding on the experimental procedure to be adapted. Preliminary experiments also provided the initial observations on GOX-Polymer matrix- scCO_2 system.

7.1. Conclusions

Initially, the solubility of GOX in scCO_2 was investigated by gravimetric methods. It was observed that the amount of free GOX was decreased 200 μg after exposure to scCO_2 at 100 bar 40 °C for 24 h, but it was not obvious that whether the whole GOX molecule was dissolved or a part of it such as its carbohydrate content was dissolved in scCO_2 which causes weight loss. GOX was impregnated into PMMA matrix from a solid phase in scCO_2 environment two times, the mass gain of the polymer matrices at the end of one month showed that one sample gained 200 μg , whereas the other did not, but the color of each sample turned to be light yellow, the color of GOX. Therefore, an exact result for the solubility of GOX in scCO_2 could not be given, but it was shown that GOX can be transferred to the polymer matrix when it was captive in a filter paper enclosure in supercritical environment, even though its solubility in scCO_2 is immeasurably low.

The effects of temperature and pressure on GOX-buffer solution were investigated in order to better understand the behavior of the system studied. GOX-buffer solution was

kept in a water bath at different temperatures between 30-60 °C for 24 h and then the activity of the solution was measured in order to evaluate the temperature effect. Moreover, the GOX-buffer solution was also exposed to scCO₂ at 40 °C and 80, 100 and 150 bar for 24 h in order to investigate the pressure and CO₂ effect on GOX-buffer solution. Taking into account both average values and standard deviations, it was found that keeping GOX-buffer solution at 30 °C enhanced its activity by 8.2 per cent, while 40 and 50 °C temperatures did not have a lowering effect on GOX-buffer solution activity. Higher temperatures (55 and 60 °C) caused decreases in GOX-buffer solution activity (29 and 95 per cent respectively) and slight gelation which were mainly attributed to the dissociation of cofactor FAD. Decrease in activity above 50 °C suggested that further work on enzyme immobilization should be performed under 50 °C. As a result of exposure of GOX-buffer solution to scCO₂, it was concluded that there was no negative effect of pressure and scCO₂ on activity up to 150 bar and 40 °C. Indeed at 80 and 150 bar, the activity approximately increased 6 per cent compared to the original GOX-buffer solution activity. Although there was no decrease in activity values after exposure to scCO₂, there was a decrease in GOX amount measured by the protein determination method. At all pressures and temperatures tested, there was a decrease in available protein values, but the highest decrease was at 100 bar. The decreases in GOX amount were 18, 14.3 and 49.5 per cent respectively compared to the original value at 100 bar, 30, 40 and 50 °C, respectively.

GOX immobilizations on PPY films were done via SFI in four different immobilization mediums namely “Dry”, “Semi-Dry”, “Wet”, and “Wet-b”. ESEM investigations showed that GOX particles were seen in very different forms when immobilizations were performed in different mediums. When immobilization was done in “Dry” medium, GOX were seen as particles of well-defined shape, but in “Wet” and “Wet-b” mediums, GOX looked like a very thin layer of film or as a cloud. The maximum activity for GOX immobilized PPY film was observed in “Wet-b” medium. In “Wet” medium at 150 bar and 40 °C, the activities of the GOX immobilized PPY films increased as the immobilization time increased from 5 to 72 h. Using SDS as a electrolyte in electropolymerization of PPY was a better choice than PTSA if immobilization would be performed via SFI. The activity of PPY film from SFI process was 136 per cent higher than the sample obtained from atmospheric immobilization. Fast decompression was also found to be detrimental to immobilized GOX.

The preliminary experiments for GOX immobilization on PU/PPY composites via SFI was done in “Wet-b” medium at 40 °C, for 24 h, at 75, 105 and 150 bar. In preliminary SFI experiments done on PU/PPY composite foam, it was seen that the degree of PPY polymerization on PU foam was an important parameter in immobilization. Immobilization from corner and inner parts of a larger sample gave different activity values. Samples from corner gave higher activity values. These differences were attributed to variations in PPY amount on the PU foam samples.

Immobilization on PU/PPY samples were done on at three different GOX-buffer solution concentrations; at 6.25, 0.625 and 0.0625 mg/ml. It should be also noted that decreasing the initial concentration of GOX solution two orders of magnitude enhanced the specific activity of the immobilized on PU/PPY composite GOX by 25 fold. Washing the PU/PPY samples before and after immobilization was necessary to correctly perform the enzymatic and protein determination analysis. Moreover, the “washing before immobilization” and “washing after immobilization” steps affected the enzymatic activity values. As the washing step got longer in both steps, the activity of the sample increased. Washing the sample in scCO₂ before immobilization also increased the activity compared to non-CO₂ washing procedure. The SFI process was performed at three different immobilization periods, namely for 4, 24 and 72 h immobilization periods. It was observed that increase of immobilization time from 4 to 24 h lead to the activity enhancement by 71.8 per cent, and an increase from 24 h to 72 h caused 16.4 per cent decrease in activity. As the immobilization time increased from 4 to 24 h, there was a considerable increase in activity caused by increase in the immobilized enzyme amount, but further increase from 24 to 72 h did not enhance the activity, possibly due to the high enzyme loading that caused agglomeration. Therefore, the best immobilization period among the tested ones was concluded to be 24 h.

The pressure effect (80, 100 and 150 bar) on immobilization was investigated at two different temperatures, 30 and 40 °C for 24 h of immobilization. At both temperatures, the best immobilization pressure was found to be 100 bar by considering the activity values at both high and low glucose concentration regions. The activity value (U/cm²) reached a maximum at 100 bar and then decreased with further increase in pressure. Additionally, the

activity of the PU/PPY samples processed at supercritical conditions were always much higher than the ones processed at atmospheric conditions.

The immobilized amount of GOX on PU/PPY composite increased as the temperature increased at all pressures and decreased as the pressure increased at all temperatures. In supercritical region, the specific activity value (U/mg) increased as the pressure increased from 80 to 100 bar (22.5 and 35 per cent increase at 30 and 40 °C, respectively), however further increase to 150 bar did not cause any increase in specific activity. At constant pressure, as the temperature increases, the specific activity decreased.

The highest activity and highest specific activity for the samples processed at supercritical conditions were obtained at 100 bar, 30 °C as 0.040 U/cm² and 4.9 U/mg, respectively. The highest activity and specific activity for the samples immobilized with GOX at 1 bar without CO₂ were 0.022 U/cm² and 1.8 U/mg, respectively. When these results were compared, it can be said that by performing immobilization via SFI, the activity values were doubled compared to the immobilization at atmospheric conditions. The superior enhancement of SFI may be attributed to the changes done by scCO₂ on the microenvironment of the enzyme and the polymer surface. When 10 successive assays were performed for GOX-immobilized PU/PPY sample, there was no gradual decrease in the activity of the sample as the assay number was increased and 80 per cent of its activity remained after 21 days of storage at +4 °C.

No satisfactory activity values of was obtained for immobilization on PU/PPY composite films, but it was found that during depressurization from 100 bar 40 °C, the PU/PPY film sample got larger approximately by 400 per cent of its original surface area. GOX was also immobilized on PPY film via SFI, but there were some reproducibility problems due to the inhomogeneous surfaces of the PPY films. Additionally, the PPY films were sometimes found to be cracked after the experiments. Moreover, activity values for PU/PPY composite foams measured and calculated in U/cm² are higher than the activity values of PPY and PU/PPY films. Therefore, the most suitable matrix for GOX immobilization via SFI was found to be PU/PPY composite foam in terms of the higher activity values. The immobilization via SFI was suggested to be a combination of adsorption and impregnation since PPY surfaces are able to adsorb proteins.

7.2. Recommendations

The effect of scCO_2 on GOX activity was determined by measuring its residual activity and protein amount after being subjected to scCO_2 . From the results of these experiments, it was determined that scCO_2 did not cause negative changes on GOX which could affect GOX activity, on the contrary it slightly enhanced the activity and there was a decrease in GOX amount after exposure to scCO_2 . For further study, the possible change in GOX structure can be investigated by spectroscopic methods and additionally in situ conformational changes can be observed by spectroscopic methods such as fluorescence spectroscopy. If the possible irreversible and permanent changes in structure are determined, the explanations on activity enhancement of both free and immobilized GOX after exposure to scCO_2 can be more clearly made.

The solubility of GOX in scCO_2 was immeasurably low. At this low solubility, the immobilization process was performed successfully. However, a surfactant which can form micelles and increase the solubility of GOX in scCO_2 can be added to immobilization medium and the effect of solubility GOX in scCO_2 on the immobilization process can be further investigated.

The thickness of PPY films, PU/PPY foams and PU/PPY films used in immobilization were approximately, 50 μm , 3.6 mm and 1 mm, respectively. The thickness of PU/PPY foams were too large for use in biosensor production. It was quite impossible to obtain thinner and smooth PU foam samples to be used in PU/PPY composite formation, additionally if thinner slices were cut from PU sample, it was impossible to obtain the same thickness every time. If PU foams at different thicknesses can be casted for PU/PPY formation, the effect of thickness can be also investigated. Thinner samples will prevent the diffusion barrier for enzymatic reaction, and also higher portion of the enzyme immobilized on the polymer including the inner parts will contribute to the enzymatic reaction. Many other conducting polymer composites can be also used for SFI process in order to verify the applicability of the process to a broader range of polymer matrices. Finally, if in situ pH changes of the immobilization medium under scCO_2 can be measured, further investigations on the effect of CO_2 on immobilization process could be performed.

APPENDIX A: PROPERTIES OF GLUCOSE OXIDASE

Glucose oxidase (GOX) (β -D-glucose : oxygen 1-oxido-reductase, E.C. 1.1.3.4) catalyzes the oxidation of β -D-glucose by molecular oxygen to δ -gluconolactone, which subsequently hydrolyzes spontaneously to gluconic acid and hydrogen peroxide (Hecht *et al.*, 1993). In this study, GOX from *Aspergillus niger* was used.

Glucose oxidase from *Aspergillus niger* is a homodimer of molecular weight 150 to 180 kDa containing two tightly bound FAD molecules (Pazur *et al.*, 1965). The structure of GOX is shown in Figure A.1. The FAD is not covalently bound to the holo protein. Dissociation of the subunits is only possible under denaturing conditions and is accompanied by the loss of cofactor FAD. The native protein is acidic having an isoelectric point (pI) of 4.2.

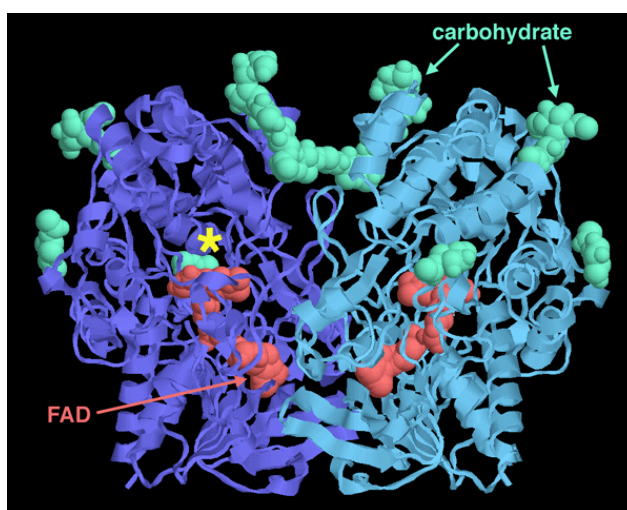


Figure A.1. The structure of GOX

APPENDIX B: ABSORBANCE VALUES OBTAINED IN ENZYMATIC ACTIVITY ANALYSES

During the enzymatic analysis of immobilization experiments, absorbance values were read at 530 nm by the double-beam spectrophotometer (Analytic Jena, Specord 200). The values are read after reaction times of 2 min, 4 min and 6 min.

Table B.1. Absorbance values for Run PPY6

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
10	0.0199	0.0388	0.0617
50	0.0316	0.0532	0.0931
100	0.0423	0.0631	0.1102

Table B.2. Absorbance values for Run PPY7

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
10	0.0389	0.0786	0.0992
50	0.0945	0.1304	0.1773
100	0.0651	0.1379	0.1848

Table B.3. Absorbance values for Run PPY8

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0076	0.0184	0.0328
10	0.0171	0.0266	0.0536
100	0.0651	0.0360	0.0858

Table B.4. Absorbance values for Run PPY9

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
10	0.0598	0.1371	0.2217
50	0.1449	0.2300	0.4044
100	0.1884	0.2455	0.4149

Table B.5. Absorbance values for Run PPY10

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0093	0.0251	0.0408
10	0.0148	0.0527	0.0671
50	0.0445	0.0577	0.1179
100	0.0458	0.0972	0.1025

Table B.6. Absorbance values for Run PPY10^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0120	0.0237	0.0344
10	0.0197	0.0368	0.0525
50	0.0394	0.0458	0.0838
100	0.0318	0.0666	0.1096

Table B.7. Absorbance values for Run PPY11

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
10	0.0320	0.0565	0.0773
50	0.0484	0.0836	0.1249
100	0.0560	0.0922	0.1371

Table B.8. Absorbance values for Run PPY12

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0156	0.0226	0.0343
10	0.0156	0.0405	0.0468
50	0.0400	0.0660	0.0735
100	0.0336	0.0630	0.0997

Table B.9. Absorbance values for Run PPY12^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0151	0.0237	0.0277
10	0.0184	0.0290	0.0448
50	0.0367	0.0672	0.0626
100	0.0329	0.0612	0.0930

Table B.10. Absorbance values for Run PU/PPY1^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0139	0.0592	0.0503
10	0.0391	0.0250	0.0412
20	0.0388	0.0633	0.0709

Table B.11. Absorbance values for Run PU/PPY5^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0316	0.2949	0.3645
10	0.1561	0.3074	0.5025
20	0.1282	0.3216	0.4823

Table B.12. Absorbance values for Run PU/PPY6^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0522	0.0933	0.1422
10	0.0447	0.2992	0.1897
20	0.0697	0.1575	0.2496

Table B.13. Absorbance values for Run PU/PPY7^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0276	0.0706	0.1027
10	0.0428	0.0980	0.1112
20	0.0457	0.1102	0.1595

Table B.14. Absorbance values for Run PU/PPY8^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0197	0.0634	0.1075
10	0.0370	0.0572	0.0917
20	0.0350	0.0825	0.1328

Table B.15. Absorbance values for Run PU/PPY9^{PR}

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0132	0.0547	0.0807
10	0.0243	0.0770	0.0874
20	0.0313	0.0782	0.1401

Table B.16. Absorbance values for Run PU/PPY5^{PR}-2nd

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.2773	0.3007	0.3049
10	0.0835	0.1426	0.1899
20	0.0793	0.1607	0.1800

Table B.17. Absorbance values for Run PU/PPY6^{PR}-2nd

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.1122	0.2563	0.2772
10	0.3693	0.2050	0.5424
20	0.1244	0.2676	0.3000

Table B.18. Absorbance values for Run PU/PPY9^{PR}-2nd

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.1357	0.2334	0.2964
10	0.0915	0.1911	0.3354
20	0.0897	0.1857	0.3124

Table B.19. Absorbance values for Run PU/PPY1

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0039	0.0218	0.0380
10	0.0188	0.0515	0.0915
20	0.0388	0.0746	0.1606

Table B.20. Absorbance values for Run PU/PPY1^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0475	0.0904	0.1524
10	0.0651	0.1382	0.1881
20	0.0921	0.1510	0.2590

Table B.21. Absorbance values for Run PU/PPY2

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0289	0.0727	0.1095
10	0.0423	0.1193	0.1484
20	0.0614	0.1378	0.1810

Table B.22. Absorbance values for Run PU/PPY3

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0438	0.1022	0.1159
10	0.0343	0.1170	0.1680
20	0.0672	0.1491	0.2146

Table B.23. Absorbance values for Run PU/PPY4

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0594	0.1151	0.1820
10	0.0834	0.2190	0.2440
20	0.1055	0.2362	0.2754

Table B.24. Absorbance values for Run PU/PPY4^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0511	0.1050	0.1312
10	0.0601	0.1231	0.1696
20	0.0832	0.1362	0.1588

Table B.25. Absorbance values for Run PU/PPY5

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0685	0.0863	0.1178
10	0.0488	0.1002	0.1488
20	0.0593	0.1554	0.1906

Table B.26. Absorbance values for Run PU/PPY5^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0850	0.1283	0.1356
10	0.0698	0.1272	0.1778
20	0.0852	0.1851	0.2321

Table B.27. Absorbance values for Run PU/PPY6

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0768	0.0637	0.1021
10	0.0447	0.1045	0.1563
20	0.0627	0.1459	0.1917

Table B.28. Absorbance values for Run PU/PPY6^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0852	0.0863	0.1140
10	0.0492	0.1201	0.1919
20	0.0559	0.1754	0.2253

Table B.29. Absorbance values for Run PU/PPY7

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0326	0.0808	0.1389
10	0.0577	0.1231	0.1845
20	0.0813	0.1463	0.1905

Table B.30. Absorbance values for Run PU/PPY7^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0436	0.1042	0.1317
10	0.0511	0.1125	0.1649
20	0.0680	0.1655	0.1944

Table B.31. Absorbance values for Run PU/PPY8

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0310	0.0741	0.1256
10	0.0442	0.1241	0.1837
20	0.0686	0.1569	0.2501

Table B.32. Absorbance values for Run PU/PPY8^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0574	0.1301	0.1786
10	0.0803	0.1621	0.2037
20	0.0920	0.2309	0.3000

Table B.33. Absorbance values for Run PU/PPY9

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0528	0.0895	0.1110
10	0.0643	0.1359	0.1480
20	0.0643	0.1474	0.1898

Table B.34. Absorbance values for Run PU/PPY9^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0468	0.0907	0.1256
10	0.0463	0.1035	0.1339
20	0.0622	0.1672	0.1646

Table B.35. Absorbance values for Run PU/PPY10

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0538	0.1239	0.1635
10	0.0698	0.1656	0.2101
20	0.0872	0.1580	0.2413

Table B.36. Absorbance values for Run PU/PPY10^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0583	0.1240	0.1863
10	0.0669	0.1556	0.1995
20	0.0766	0.1842	0.2404

Table B.37. Absorbance values for Run PU/PPY11

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0401	0.0917	0.1060
10	0.0449	0.1302	0.1970
20	0.0661	0.1232	0.1653
100	0.0657	0.1729	0.2811

Table B.38. Absorbance values for Run PU/PPY11^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0416	0.0794	0.1364
10	0.0560	0.1086	0.1317
20	0.0660	0.1663	0.2312
100	0.1141	0.2330	0.2829

Table B.39. Absorbance values for Run PU/PPY12

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0472	0.0960	0.1618
10	0.0686	0.1253	0.1557
20	0.0957	0.1454	0.2157
100	0.0832	0.2677	0.3660

Table B.40. Absorbance values for Run PU/PPY12^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0628	0.1088	0.1222
10	0.0592	0.1359	0.1548
20	0.0739	0.1693	0.2070
100	0.0920	0.2486	0.3223

Table B.41. Absorbance values for Run PU/PPY13

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0481	0.1053	0.1541
10	0.0571	0.1161	0.1537
20	0.0704	0.1312	0.1399
100	0.0971	0.1585	0.3068

Table B.42. Absorbance values for Run PU/PPY13^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0488	0.1080	0.1484
10	0.0493	0.1030	0.1571
20	0.0537	0.1816	0.2727
100	0.0942	0.1861	0.2951

Table B.43. Absorbance values for Run PU/PPY14

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0408	0.0974	0.1142
10	0.0790	0.1171	0.2336
20	0.0686	0.1379	0.1745
100	0.0813	0.1436	0.3043

Table B.44. Absorbance values for Run PU/PPY14^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.1006	0.1426	0.1645
10	0.0741	0.1302	0.1846
20	0.0610	0.1072	0.1491
100	0.0752	0.1953	0.2615

Table B.45. Absorbance values for Run PU/PPY15

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0986	0.1202	0.1794
10	0.0723	0.1236	0.1796
20	0.0937	0.2020	0.1787
100	0.1101	0.2756	0.2756

Table B.46. Absorbance values for Run PU/PPY15^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0310	0.0737	0.1214
10	0.0791	0.1350	0.2048
20	0.0454	0.1572	0.2685
100	0.0859	0.1694	0.2301

Table B.47. Absorbance values for Run PU/PPY16

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0411	0.1150	0.1850
10	0.1105	0.1568	0.1729
20	0.1128	0.1908	0.1980
100	0.1363	0.2357	0.1863

Table B.48. Absorbance values for Run PU/PPY16^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0567	0.1031	0.1470
10	0.0829	0.1406	0.1813
20	0.0623	0.1561	0.1471
100	0.1157	0.2362	0.2416

Table B.49. Absorbance values for Run PU/PPY17

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0517	0.1080	0.1852
10	0.0473	0.1325	0.1655
20	0.0850	0.1377	0.1910
100	0.0841	0.1827	0.3080

Table B.50. Absorbance values for Run PU/PPY17^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0360	0.1162	0.1863
10	0.0819	0.1844	0.2133
20	0.0960	0.1815	0.2330
100	0.0724	0.2237	0.3268

Table B.51. Absorbance values for Run PU/PPY18

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0592	0.1050	0.1353
10	0.0438	0.1005	0.1550
20	0.0568	0.1262	0.1620
100	0.0874	0.2012	0.3325

Table B.52. Absorbance values for Run PU/PPY18^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0698	0.0983	0.1353
10	0.0433	0.1131	0.1652
20	0.0679	0.1595	0.1640
100	0.0714	0.2062	0.3402

Table B.53. Absorbance values for Run PU/PPY19

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0505	0.1456	0.1327
10	0.0679	0.1113	0.1713
20	0.0666	0.1819	0.1556
100	0.0719	0.2153	0.2438

Table B.54. Absorbance values for Run PU/PPY19^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0429	0.0938	0.1061
10	0.0445	0.1598	0.1720
20	0.0694	0.1330	0.1616
100	0.0955	0.2013	0.2386

Table B.55. Absorbance values for Run PU/PPY20

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0594	0.1246	0.1333
10	0.0558	0.1645	0.1392
20	0.0803	0.1714	0.1120
100	0.0778	0.2215	0.1802

Table B.56. Absorbance values for Run PU/PPY20^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0500	0.1062	0.1603
10	0.0440	0.1329	0.1753
20	0.0675	0.1461	0.2311
100	0.0845	0.1980	0.2500

Table B.57. Absorbance values for Run PU/PPY21

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0172	0.0509	0.0866
10	0.0391	0.0830	0.1388
20	0.0660	0.1225	0.1793
100	0.0636	0.1503	0.1963

Table B.58. Absorbance values for Run PU/PPY21^R

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0129	0.0511	0.0745
10	0.0455	0.0712	0.1416
20	0.0608	0.1429	0.1637
100	0.0690	0.1598	0.2028

Table B.59. Absorbance values for Run FES1 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0202	0.0340	0.0498
10	0.0296	0.0568	0.0850
20	0.0480	0.0890	0.1310

Table B.60. Absorbance values for Run FES1 (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0167	0.0338	0.0516
10	0.0342	0.0554	0.0872
20	0.0480	0.0870	0.1314

Table B.61. Absorbance values for Run FES1 (3rd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0190	0.0348	0.0525
10	0.0309	0.0593	0.0896
20	0.0510	0.0924	0.1400

Table B.62. Absorbance values for Run FES2 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0199	0.0358	0.0491
10	0.0322	0.0616	0.0909
20	0.0568	0.0980	0.1379
100	0.0872	0.1572	0.2167

Table B.63. Absorbance values for Run FES2 (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0199	0.0337	0.0517
10	0.0354	0.0609	0.0892
20	0.0517	0.0941	0.1338
100	0.0784	0.1500	0.2156

Table B.64. Absorbance values for Run FES2^R (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0129	0.0409	0.0439
10	0.0288	0.0506	0.0748
20	0.0619	0.0792	0.1292
100	0.0775	0.1472	0.2018

Table B.65. Absorbance values for Run FES2^R (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0147	0.0327	0.0418
10	0.0257	0.0484	0.0817
20	0.0804	0.1394	0.2010

Table B.66. Absorbance values for Run FES3

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0208	0.0381	0.0454
10	0.0308	0.0533	0.0773
20	0.0466	0.0795	0.1207

Table B.67. Absorbance values for Run FES3^R (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0144	0.0281	0.0415
10	0.0237	0.0485	0.0730
20	0.0619	0.0748	0.1133

Table B.68. Absorbance values for Run FES3^R (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0132	0.0272	0.0445
10	0.0268	0.0515	0.0826
20	0.0457	0.0779	0.1151

Table B.69. Absorbance values for Run FES4 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0058	0.0125	0.0219
10	0.0113	0.0240	0.0381
20	0.0228	0.0421	0.0655

Table B.70. Absorbance values for Run FES4 (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0025	0.0071	0.0181
10	0.0115	0.0235	0.0358
20	0.0195	0.0401	0.0617

Table B.71. Absorbance values for Run FES4 (3rd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0060	0.0156	0.0212
10	0.0138	0.0232	0.0338
20	0.0216	0.0431	0.0615

Table B.72. Absorbance values for Run FES6 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0182	0.314	0.0518
10	0.0358	0.0591	0.0859
20	0.0570	0.0906	0.1359
100	0.0989	0.1924	0.2377

Table B.73. Absorbance values for Run FES6^R (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0203	0.353	0.0509
10	0.0317	0.0595	0.0816
20	0.0494	0.0896	0.1292
100	0.0986	0.1776	0.2383

Table B.74. Absorbance values for Run FES6^R (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0205	0.331	0.0518
10	0.0328	0.0550	0.0814
20	0.0446	0.0798	0.1193
100	0.0833	0.1477	0.2194

Table B.75. Absorbance values for Run FES6^R (3rd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0205	0.0343	0.0495
10	0.0307	0.0576	0.0834
20	0.0566	0.0822	0.1219
100	0.0801	0.1586	0.2110

Table B.76. Absorbance values for Run FES7 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0184	0.0312	0.0458
10	0.0306	0.0531	0.0758
20	0.0454	0.0785	0.0972
100	0.0790	0.1411	0.2060

Table B.77. Absorbance values for Run FES7 (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0227	0.0341	0.0465
10	0.0307	0.0533	0.0834
20	0.0525	0.0851	0.1278
100	0.0780	0.1491	0.2120

Table B.78. Absorbance values for Run FES7 (3rd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0196	0.0353	0.0516
10	0.0358	0.0553	0.0790
20	0.0463	0.0812	0.1244
100	0.0654	0.1184	0.1715

Table B.79. Absorbance values for Run FES8 (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0237	0.0404	0.0560
10	0.0352	0.0560	0.0895
20	0.0444	0.0851	0.1296
100	0.0888	0.1537	0.2234

Table B.80. Absorbance values for Run FES8 (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0362	0.0524	0.0686
10	0.0441	0.0678	0.0956
20	0.0532	0.0904	0.1287
100	0.0864	0.1562	0.2187

Table B.81. Absorbance values for Run FES8 (3rd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0359	0.0520	0.0671
10	0.0441	0.0710	0.0983
20	0.0491	0.0902	0.1281
100	0.0905	0.1571	0.2300

Table B.82. Absorbance values for original GOX-buffer solution (1st analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0105	0.0298	0.0407
10	0.0249	0.0500	0.0792
20	0.0420	0.0827	0.1149
100	0.0981	0.1838	0.2168

Table B.83. Absorbance values for original GOX-buffer solution (2nd analysis)

Glucose Concentration (mM)	Absorbance value obtained after reaction time of 2 min	Absorbance value obtained after reaction time of 4 min	Absorbance value obtained after reaction time of 6 min
5	0.0160	0.0289	0.0430
10	0.0256	0.0521	0.0765
20	0.0442	0.0823	0.1211
100	0.0916	0.1556	0.2259

APPENDIX C: DETERMINATION OF ACTIVITY VALUES AND PROTEIN AMOUNT

C.1. Activity Determination Calculations

As given in Appendix A, the absorbance values were read for different glucose concentrations of 5, 10, 20, 50, and 100 mM and for different reaction times of 2, 4 and 6 min. To determine the change in the absorbance values on the basis of one minute, the absorbance values and the corresponding reaction times were plotted and a linear trend line was fitted. The slope of this line showed the change in absorbance values per minute which was then interpolated to determine the amount of H_2O_2 produced as a result of chemical reaction by using the calibration curve below. The enzymatic activity of GOX immobilized in PU matrices were investigated utilizing the amount of H_2O_2 produced with the fact that one unit of GOX activity is defined as 1 μmol of β -D-glucose oxidized to gluconic acid with simultaneous production of H_2O_2 per minute at pH 5.7 and 30°C .

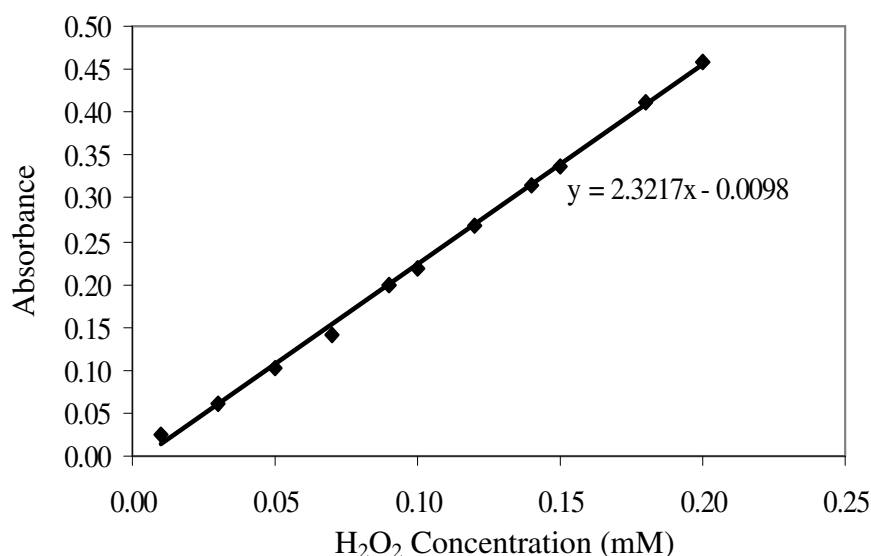


Figure C.1. Calibration chart for enzymatic activity determination

The calibration curve was prepared for 5, 10, 20, 50 and 100 mM glucose concentrations and it was observed that the results were similar to each other. The calibration curve above was obtained by considering the average of the results.

C.2. Protein Determination Calculations

The protein amount in the solution was determined by the Bradford method. Bradford assay is based on the observation that the maximum absorbance of acidic solutions of the anionic dye Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when it binds electrostatically to $-\text{NH}_3^+$ groups in the side chain of amino acids in proteins. It relies on the assumption that all proteins have the same number of lysine or arginine amino acids with suitable $-\text{NH}_3^+$ groups in their side chains.

The immobilized enzyme amount calculations were based on the difference of the amount of enzyme present in original GOX solution in which the sample was placed and exposed to scCO_2 and the amount of enzyme present in the GOX solution after the immobilization process. Also the amounts of non-immobilized enzyme present in washing solutions were taken into account. The amounts of enzyme present in original GOX solution and GOX solution obtained after the immobilization process were measured by mixing 1 ml of Bradford reagent (Sigma) into 1 ml of protein solution. Bradford reagent was used without further dilution. The absorbance value of the resulting solution was measured at 595 nm wavelength. The reading in spectroscopy was done in maximum 20 min after the protein solution and the Bradford reagent were mixed. The calibration chart was prepared with BSA standard solution, is given in Figure C.2, with a linear range between 1-10 μg in 1 ml solution. The GOX solutions were diluted by different dilution factors depending on the concentration of the solution to bring their concentration to the linear range. The protein amount in the solution was calculated in mg/ml as follows:

$$\text{Amount Enzyme} = \frac{\frac{\text{Absorbance}}{\text{Slope}} * (\text{Dilution factor})}{1000} \quad (\text{C.1})$$

Since the volume of the immobilization solution was 2 ml, total amount of enzyme in immobilization solution was evaluated by multiplying the result found from the above equation with two.

The slope appearing in Equation C.1 was the slope of the calibration chart prepared with BSA standard solution and it was 26.809.

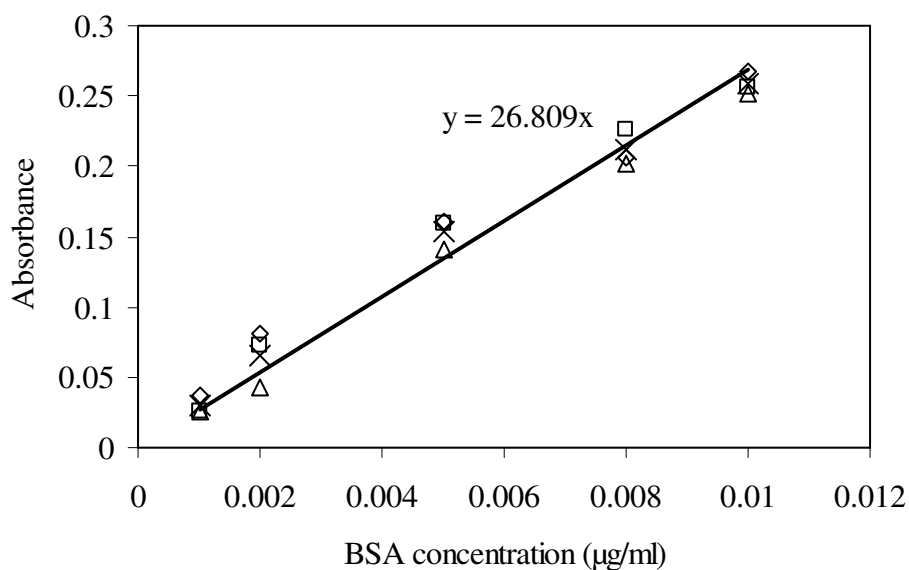


Figure C.2. Calibration chart for protein determination

The enzyme amount in washing solutions were determined by performing the enzymatic activity analysis on these solutions and their activities were obtained. These activity values were then converted into enzyme amount by using the graph below. Since enzymatic analysis was performed by adding 0.1 ml of the washing solution on to 2.9 ml glucose solution, the enzyme amount determined is the one present in 0.1 ml of the washing solution. By multiplying with the total volume of washing solution, total amount of enzyme present in washing solutions was determined.

Finally, the amount of enzyme immobilized by polymer sample was determined by summing the non-immobilized amounts present in washing solutions and the amounts present in GOX solution after immobilization and subtracting these from the total amount of enzyme present in original GOX solution. The equivalent enzyme amount given in Table 6.1 were also calculated from Figure C.3.

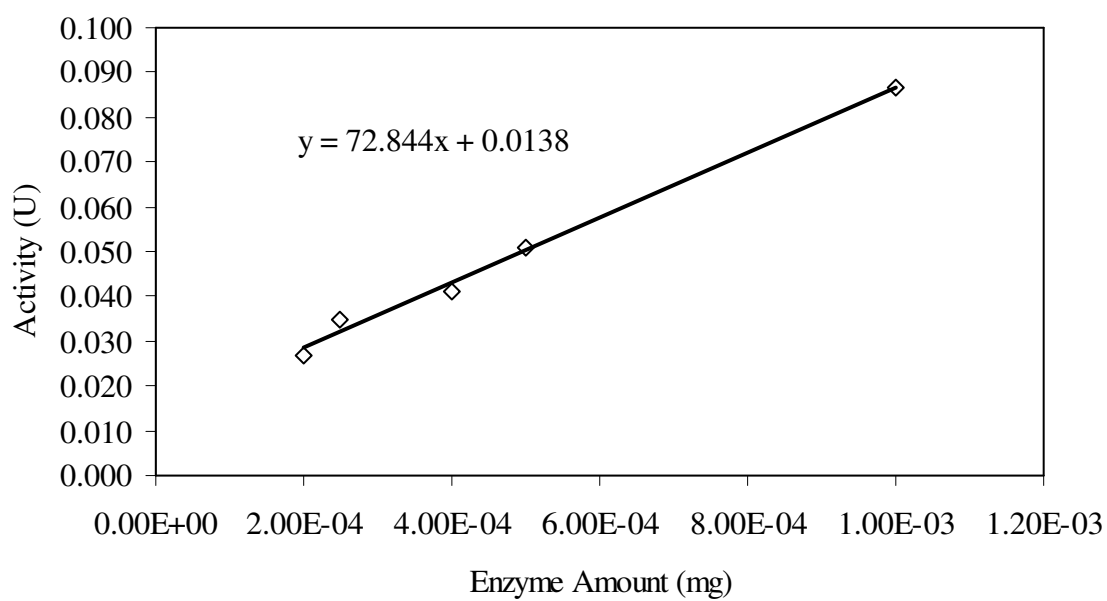


Figure C.3. Calibration chart for determination of equivalent enzyme amount at 20 mM glucose concentration

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