Immobilization of Glucose Oxidase on Polyurethane/Polypyrrole Composite via Supercritical Impregnation

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ABSTRACT

The aim of this work is to immobilize glucose oxidase (GOX) on a conducting composite polymer via supercritical fluid technologies for further use in the preparation of biosensors. The conducting polymer used is polyurethane/polypyrrole (PU/PPY) composite as the immobilization matrix. To understand the system studied, initially the effect of supercritical carbon dioxide ($scCO_2$) (80-150 bar, 40 ^oC) and the effect of temperature (30-60 ^oC) on free enzyme-buffer solution is studied. The exposure time to $scCO_2$ and temperature is set to 24 hours in all experiments. The effect of $scCO_2$ on free GOX-buffer solution is investigated by determining its enzymatic activity before and after its exposure to $scCO_2$. Results show that enzymatic activity of pure GOX after exposure to $scCO_2$ is retained and the activity of GOX-buffer solution does not show a noticeable decrease between 30-50 ^oC. However when the solution is exposed to 55 ^oC and 60 ^oC temperatures, 30 % and 94 % losses in its original activity are noticed, respectively.

Supercritical Fluid Immobilization is used to immobilize the GOX into the PU/PPY composite samples. Based on our past experiences, buffer solution is used as the impregnation medium. GOX immobilized PU/PPY composite foam samples are analyzed via enzymatic activity methods. All the experiments are conducted at 40 ^oC temperature and 24 hours impregnation time. The effect of impregnation pressure (75-150 bar) on the activity of the immobilized GOX are investigated. When the pressure is increased from 75 bar to 105 bar, the immobilized enzyme activity increases significantly, whereas further increase in pressure to 150 bar causes a decrease in the GOX immobilized PU/PPY composite sample activity. The immobilization process is also done at atmospheric pressure, and it is seen that immobilization of GOX onto PU/PPY composite by only adsorption at 1 bar is not feasible. GOX immobilized PU/PPY composite samples are re-used in the immobilization process to investigate possible increase in activity. Additionally, kinetic parameters (K_m and v_{max}) are evaluated and long term stability of the immobilized enzyme in different mediums (in buffer solution at +4 ^oC and at room temperature in a dessicator) are determined.

Keywords: Supercritical carbon dioxide, immobilization, biosensor, glucose oxidase, conducting polymer

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. 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 conventionally are adsorption, covalent bonding, entrapment, and cross-linking. 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. Biological recognition components are usually entrapped into the conducting polymer during its electrochemical synthesis. This is a one step procedure and conducting polymer synthesis and immobilization conditions should not 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) 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 current studies [1-3].

In this work, supercritical fluid immobilization is used to immobilize biological component on a conducting polymer. The high diffusivity, low surface tension, sterilization effect and the ability of $scCO_2$ to swell polymeric matrices 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 [4, 5] or microencapsulation of biological materials [6-8] 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, the 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, PPY has been investigated the most. Therefore, our investigation on supercritical fluid immobilization was started with the GOX and PPY system [9]. 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. 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 as a second immobilization matrix, PU/PPY composite foams were chosen to continue with the project and the results of supercritical fluid immobilization of GOX on PU/PPY composite foam are represented in this paper.

2 EXPERIMENTAL

2.1 Materials

Pyrrole (98% purity) and *p*-Toluenesulfonic acid monohydrate (PTSA, min 98.5%) were obtained from Aldrich. Pyrrole was used without further purification. Sodium dodecyl sulfate (SDS, min 98.5%) was purchased from Sigma-Aldrich. Glucose oxidase from *Aspergillus Niger* (GOX, 200 U/mg) was purchased from Fluka. Potassium phosphate monobasic (Sigma, 98%) and potassium phosphate dibasic (Sigma, min 99%) were used for buffer preparation. β-D-glucose (Sigma, min 97%), peroxidase (POD) from Horseradish (Sigma, Type II, 181 U/mg), *o*-dianisidine (Sigma), sulphuric acid (Fluka, 95-97%), hydrogen peroxide (Riedel de Haen, 30% solution) were used throughout the enzyme activity assay. Sulphuric acid was diluted to 5 M before use. Carbon dioxide (99.7% pure), in cylinders equipped with dip tubes, was supplied by HABAŞ A.Ş. (İstanbul, Turkey).

PU/PPY composites were prepared by Dr. Can Erkey's group (University of Connecticut, Storrs, USA) via chemical polymerization. The preparation method can be found elsewhere [10]. The dimensions of the PU foam samples were 25.4 mm in length, 25.4 mm in width with 3.2 mm thickness. After polymerization of pyrrole on PU foam, 9×6 mm samples were cut from PU/PPY composite for the immobilization process.

2.2 Immobilization procedure

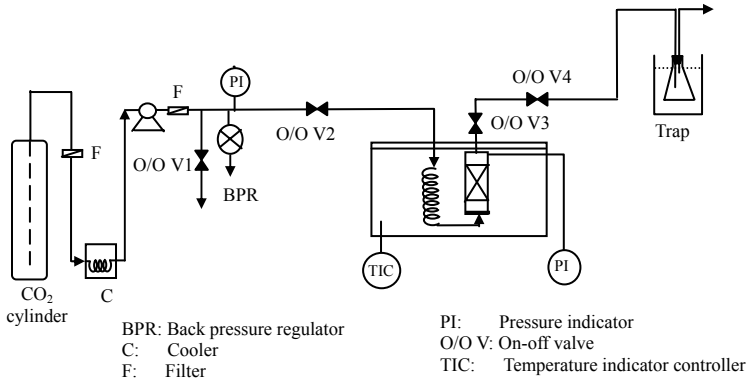


Figure 1. The experimental set-up.

The set-up used for the immobilization experiments can be seen in Figure 1. The set-up consists mainly of a pressure vessel, mounted in a constant temperature bath, having an internal diameter of 45 mm and a depth of 200 mm. In immobilization experiments (called "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 solution and positioned in the pressure vessel. After obtaining the desired temperatures throughout the system, the vessel was filled with CO_2 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 5-72 h. After the desired time 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, they were washed with distilled H_2O and buffer, dried and kept at 4 ^oC in test tubes covered by parafilm for enzymatic analysis. The GOX solution taken out of the pressure vessel were kept at −18 ^oC.

2.3 Free-enzyme Experiments

In order to investigate the effect of pressure on 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. The experimental procedure is the same as the supercritical immobilization procedure given in the previous section. In all experiments, GOX-buffer solution was exposed to $scCO_2$ for 24 h. 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 activity was reported.

2.4 Analysis

GOX immobilized conducting polymer matrices were analyzed by enzymatic activity assay. For enzymatic analysis of GOX immobilized on PU/PPY composite, three different β-D-glucose solutions (5, 10, 20 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 ^oC). After temperature equilibrium was established, polymer matrix was placed in the glucose solution and incubated at 30 ^oC for different reaction times (2, 4 and 6 minutes) while the tubes were shaken. 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) and 2.4 ml o-dianisidine (0.21mM) immediately. 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). In free-enzyme analysis, 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 ^oC) 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. The calibration curve was prepared by different amounts of H_2O_2 (0.01mM-0.2mM). One unit (U) of GOX activity is defined as 1 μmol of β-D-glucose oxidized to gluconic acid with simultaneous production of hydrogen peroxide per minute at pH 5.7 and 30 ^oC.

3 RESULTS AND DISCUSSION

3.1 Effect of Pressure and Temperature on Free-enzyme

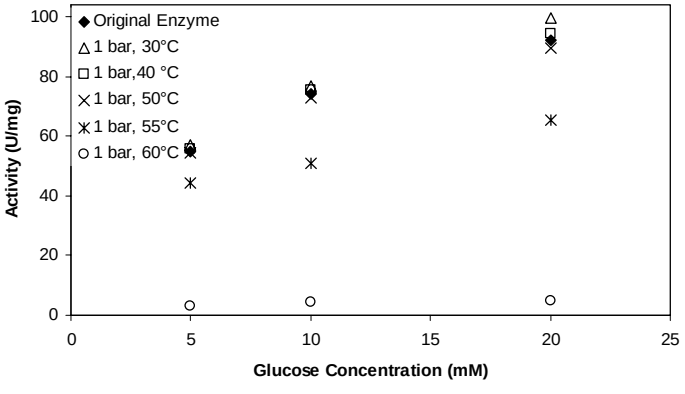


Figure 2. Apparent activity of GOX immobilized PU/PPY composite foams.

The effect 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 solution was left in a water bath at different temperatures between 30-60 ^oC for 24 h and then the activity of the solution was measured. Figure 2 reveals the effect of temperature on GOX-buffer solution activity. The data shown on Figure 2 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. As it is seen on Figure 2, there was no noticeable decrease in the GOX-buffer solution activity when it was subjected to 30, 40 and 50 ^oC; since activities measured at these temperatures for different glucose concentrations are almost indiscernible, more so if the standard deviations noted above are also taken into account. However, the activities of solutions kept at 55 and 60 ^oC decreased by 30 and 60%, respectively as compared to the activities for temperatures 30, 40 and 50 ^oC. Additionally, the original clear yellow GOX-buffer solution became slightly cloudy after exposure to 55 ^oC and cloudy at 60 ^oC.

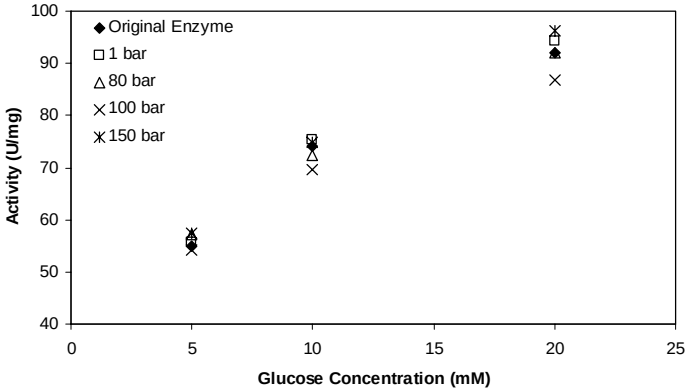


Figure 3. The activities of GOX-buffer solution exposed to different pressures.

Figure 3 shows the effect of $scCO_2$ on the activity of free-enzyme buffer solution. All the solutions were subjected to $scCO_2$ for 24 h at 40 ^oC. Each data point shown are the averages of three enzymatic analysis. 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 is seen in Figure 3, as the pressure increases from 1 to 80 bar and then to 100 bar, the activity of the GOX-buffer solution slightly decreases (at most 6% decrease from the original solution) based on the average activity values. Additional increase in pressure from 100 to 150 bar shifted the activity value to original solution's level. A slight decrease and then a slight increase in activity based on the average values disappears if the standard deviations are also taken into account. Based on the average values and standard deviations of data points, it can be said that at 40 ^oC, for 24 h up to 150 bar, $scCO_2$ had no negative effect on the original enzyme activity. There is no available open literature data bringing GOX and $scCO_2$ together and investigating the effect of $scCO_2$ on the activity of GOX.

3.2 Supercritical Immobilization of Glucose Oxidase on PU/PPY Composite Foams

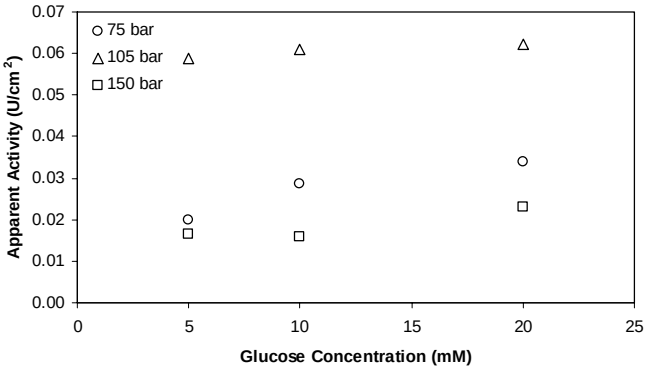


Figure 4. Apparent activity of GOX immobilized PU/PPY composite foams.

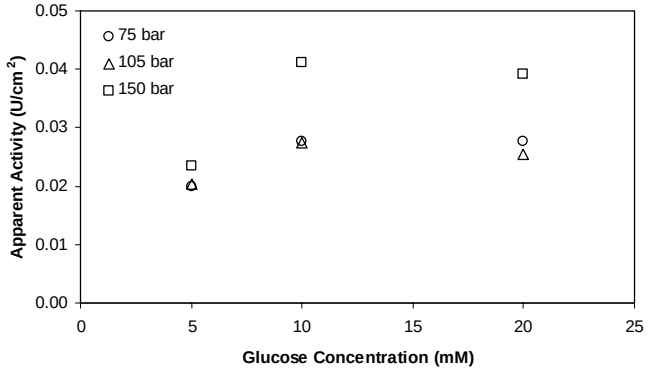


Figure 5. Apparent activity of GOX immobilized re-used PU/PPY composite foams.

Depending on previous experience on PPY films [9], GOX was immobilized on PU/PPY composite foams only in the "Wet-b" medium. The data seen in Figure 4 show the pressure dependence of the activity of GOX immobilized on PU/PPY composites. The experiments were conducted at immobilization temperature of 40 ^oC, immobilization time of 24 h and under 75, 105 and 150 bar. As it is seen from the Figure 4, 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. [11] studied the absorption of low molecular weight solutes into polyurethane $scCO_2$. At constant temperature (40 and 60 °C) the amount of solutes absorbed in PU initially increases as the pressure increased from 8 to 10 MPa and then started to decrease when the pressure increased to 12 MPa, which showed a similar trend with our case.

At the end of experiments, when the test tube, 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 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 CO_2). Therefore, it is 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. 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 $scCO_2$ which makes the monomers, dimers, trimers, or I_2, to come out of the composite more easily. Additionally, $scCO_2$ may dissolve pyrrole

monomers, dimers, trimers or I_2 and take them out of the composite. The chemicals coming into the GOX solution can decrease the enzyme activity. Therefore, the composites taken out of pressure were first enzymatically analyzed and then immobilization process was repeated at the same experimental conditions after two months. The results show that the activities of the re-used polymer samples processed at 150 bar increased between 42-160% for three different glucose concentrations compared to its first usage. However, the activities of the re-used polymer samples processed at 75 bar show a slight decrease and the activities of the re-used polymer samples processed at 105 bar show a sharp decrease compared to their first usage. 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 their re-use, it is more logical to say that except at 150 bar, the re-use of polymers do not enhance the immobilization process. Figure 5 demonstrates the activities of the re-used polymer samples. Polymers were re-used after two months, during this period there are certain losses in the activities of the samples. After the first time usage 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 activities of the re-used samples were lower than their initial (first usage) activities at 75 and 105 bar, they were higher than the activities measured after 15 days storage following up the first usage. At the first usage the highest activity values were obtained at 105 bar, and among the re-used polymers the highest activity values were obtained at 150 bar. However, these activity values (2^{nd} use, 150 bar) are still lower than the values measured after the first usage at 105 bar (Figures 4 and 5). All these results may reveal that, at the first usage at 105 bar, most of the available sites for enzyme immobilization were occupied by GOX particles, whereas at 150 bar there were still unoccupied sites on polymer after first usage and during the second usage of the polymer some of these vacancies were also occupied. 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 equivalent amounts of enzyme on each polymer sample are given in Table 1.

Table 1. v_m and K_m values of GOX immobilized PU/PPY composites (Immobilization T: 40 ^oC, and time: 24 h)

Experiment Name	Pressure (bar)	v_m μmol/(min.cm^2)	K_m (mM)	Equivalent Enzyme Amount (μg)
PU/PPY-Wet-b1	75	0.045	6.3	0.30
PU/PPY-Wet-b2	105	0.063	0.4	1.05
PU/PPY-Wet-b3	150	0.022	2.1	0.14
PU/PPY-Wet-b1^{2nd}	75	0.034	12.5	0.27
PU/PPY-Wet-b2^{2nd}	105	0.030	2.3	0.26
PU/PPY-Wet-b3^{2nd}	150	0.061	7.7	0.44

Figure 4 shows 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. However, the reacted amount of glucose decreases with time even though it must increase as it is the case for the samples impregnated at supercritical conditions. The decrease in the reacted glucose amount with time may be explained by the loss of GOX particles from the polymer matrix. Since the reacted glucose amount decreases with time, activity cannot be calculated. The results may reveal that, at atmospheric pressure, GOX makes looser bonds with the PU/PPY composite and GOX particles fall into the solution. On the other hand, the supercritical immobilization process gives stable immobilization. In Table 1, the v_m and K_m values of the PU/PPY composite samples are given.

In order to investigate a suitable storing medium for GOX immobilized PU/PPY composites, the samples were kept in buffer solution at +4 ^oC and at room temperature in a dessicator. After fifteen days storage, it was seen that the decrease in the activity value of the composite kept at room temperature in a dessicator (%45) was less than the one kept in buffer solution at +4 ^oC (%70).

4 CONCLUSIONS

In this study, initially, the enzymatic activities of the GOX-buffer solution for pressure in $scCO_2$ and temperature effects were tested. The results showed that there was no negative effect in activity up to 150 bar and 50 ^oC. However decrease in activity above 50 ^oC suggested that further work for enzyme immobilization should be performed under 50 ^oC.

GOX immobilization on PU/PPY composites were done via Supercritical Fluid Immobilization (SFI) in “Wet-b” medium at 40 ^oC, for 24 h, at 75, 105 and 150 bar. Immobilized GOX activity increased significantly as the immobilization pressure increased from 75 to 105 bar, whereas further pressure increase from 105 to 150 bar caused a

decrease in the activity. The activity values at 150 bar decreased to even lower values than measured at 75 bar. The PU/PPY composite samples were re-used for immobilization pressures of 75, 105, and 150 bar. Only at 150 bar, the re-used sample activity exceeded the first usage activity, yet it was still lower than the activity measured after the first usage at 105 bar.

Immobilization of GOX into PU/PPY composite was successfully done by SFI. The immobilization mechanism was suggested to be a combination of adsorption and impregnation since PPY surfaces are able to adsorb proteins. The procedure followed at atmospheric pressure without $scCO_2$, which actually facilitated adsorption only, did not yield satisfactory results and the procedure was concluded to be infeasible. Therefore it can be said that $scCO_2$ improves immobilization of GOX into PU/PPY composite. In our previous study [9], GOX was immobilized on PPY film. When the activity values for PPY films and PU/PPY composites measured and calculated in U/cm^2, are compared, it was seen that PU/PPY composite foam had higher activity values than PPY films. Therefore, it can be said that the PU/PPY composite is a better immobilization matrix than the PPY film if SFI is to be utilized.

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