IMMOBILIZATION OF GLUCOSE OXIDASE ON POLYPYRROLE FILM VIA SUPERCRITICAL FLUID TECHNOLOGIES

Defne Kayrak-Talay, Öner Hortaçsu*, Ugur Akman

Department of Chemical Engineering, Bogaziçi University, Bebek 34342, Istanbul, Turkey
E-Mail: hortacsu@boun.edu.tr, Fax: +90 (212) 2872460

The aim of this work is to immobilize a biological component on a conducting polymer via supercritical fluid technologies for further use in the preparation of biosensors. For this purpose, glucose oxidase (GOX) is chosen as the biological component and polypyrrole (PPY) as the immobilization matrix. The effect of supercritical carbon dioxide ($scCO_2$) on electrochemically synthesized PPY are monitored by environmental scanning electron microscopy (ESEM). Supercritical Fluid Impregnation is used to immobilize the GOX onto the PPY film. Four different impregnation mediums are used, mainly as dry GOX and dry PPY (Dry), dry GOX and dry PPY with water vapor (Semi-Dry), GOX and PPY in water (Wet), and GOX and PPY in buffer (Wet-b). GOX immobilized PPY films are analyzed via ESEM and enzymatic activity methods. In Dry and Semi-Dry procedures (up to 150 bar, 40 ^{o}C and 72 hr), the activity of enzyme immobilized into or onto the PPY film was not observed, whereas films processed in Wet and Wet-b experiments enzymatic activity was observed. The maximum activity was observed for GOX immobilized PPY in the Wet-b medium (150 bar and 40 ^{o}C). In Wet medium (150 bar and 40 ^{o}C), as the impregnation time increased (5-72 hr), the amount of enzyme immobilized on the film increased hence the activity was enhanced.

INTRODUCTION

In biosensor design, the immobilization method used for the biological recognition components, i.e. the enzyme, and the immobilization matrix of the biosensor affect the biosensor performance. Various matrices have been used for the immobilization of enzymes such as polymeric films, membranes, gels, carbon graphite, silica etc. in the preparation of biosensors. Conducting polymers have attracted much interest as suitable matrices for the immobilization of enzymes in biosensors. In their review, Gerard et al. [1] state that stable immobilization of macromolecular biomolecules on conducting microsurfaces with complete retention of their biological recognition properties is a crucial problem for the commercial development of miniaturized biosensors. Additionally, they said that most of the conventional procedures for biomolecule immobilization such as cross-linking, covalent binding and entrapment in gels or membranes suffer from a low reproducibility and a poor spatially controlled deposition. As an alternative to these methods, the enzymes are entrapped in a conducting polymer during its electrochemical synthesis. However, in this one step procedure conducting polymer synthesis and immobilization conditions cannot be optimized separately. Additionally, denaturation of the enzyme may be possible during the simultaneous polymerization and the immobilization processes. A technique, which combines the retention of high enzyme activity and stability, as well as the simplicity of fabrication and

miniaturization of the biosensors has not yet been universally accepted. Investigations on new techniques and new materials are the subjects of various recent and current investigations. Therefore, in this work, as a new technique, immobilization of a biological component on a conducting polymer via supercritical fluid technologies is studied.

There are some studies utilizing supercritical fluid technologies for incorporation of biological materials into a polymer matrix [2] or microencapsulation of biological materials [3-5] by supercritical fluid technologies. In those studies mostly protein particles are used as the biological material and 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. There is no open literature study with supercritical fluid technologies on biosensor production.

The glucose biosensors have the greatest 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. Amongst the conducting polymers, PPY has been investigated the most. Therefore, GOX and PPY are chosen as the materials of this study.

MATERIALS AND METHODS

Pyrrole (98 % purity) and *p*-Toluenesulfonic acid monohydrate (PTSA, min. 98.5%) were obtained from Aldrich. Pyrrole was used without further purification. Sodium Sodecyl 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 (CO_2, 99.7 % pure), in cylinders equipped with dip tubes, was supplied by HABAS A.S. (Istanbul, Turkey).

The set-up used for the immobilization experiments can be seen on Figure 1. The set-up mainly consists of a pressure vessel, mounted in a constant temperature bath, having an internal diameter of 45 mm and a depth of 200 mm.

PPY films, nearly 50 μm thickness, were synthesized by electropolmerization on platinum electrodes. Platinum electrodes were in two sizes: one group has a surface area of 1.25 cm^2, and the other one has a surface area of 2.5 cm^2. Two different electrolytes were used during polymerization: SDS and PTSA.

In Dry and Semi-Dry experiments, pre-determined amount of GOX was weighed and put in a test tube and the PPY film was placed in the same test tube. Then, the test tube was put in the

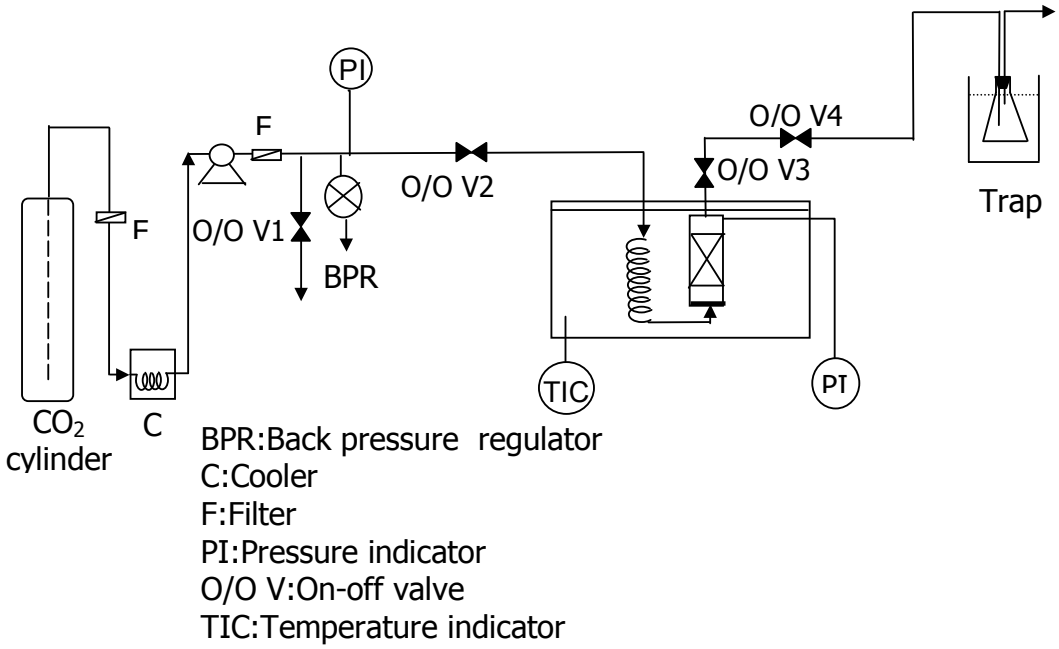


Figure 1: The experimental set-up

pressure vessel. In 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 Wet experiments, predetermined amount of GOX was dissolved in a predetermined amount of dH_2O, or pH 5.7 phosphate buffer depending on the case. Then the PPY film was placed in that solution and put in the pressure vessel. Before beginning a run, the on-off valves V1, V3 and V4 were all kept closed. After obtaining the desired temperatures throughout the system, the pressure 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 pressure cell, 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 hours. After the desired time period was over, the pressure vessel was depressurized through the on-off valves V3 and V4. After the PPY films were taken out of the pressure vessel, they were washed with dH_2O, dried and kept at +4 ^oC in test tubes covered by parafilm for enzymatic analysis. The dry GOX or the GOX solution taken out of the pressure vessel were kept at -18 ^oC.

GOX immobilized PPY films were analyzed by enzymatic activity assay and characterized by Environmental Scanning Electron Microscopy (Philips XL30 ESEM-FEG). For enzymatic analysis four different β-D-glucose solutions (5, 10, 50, 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 waterbath. Enough time was given to glucose solutions to come to the reaction temperature (30 ^oC). After temperature equilibrium was established, PPY film was placed in glucose solution and incubated at 30 ^oC for different reaction times (2, 4 and 6 minutes) while the tubes are shaken. When the reaction time is over, 1 ml of the reaction solution is taken and mixed with 0.1 ml POD (1 mg POD/ml buffer) and 2.4 ml o-dianisidine (0.21mM)

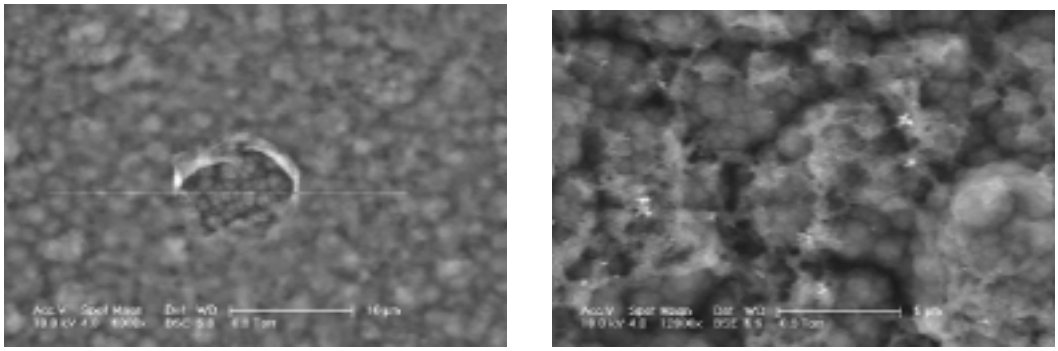
immediately. The resultant orange solution is not stable. 5ml of 5M H_2SO_4 was added in order to make it stable. The absorbance of the resultant pink solution is read at 530 nm by double-beam spectrophotometer (Analytic Jena, Specord 200). The calibration curve was prepared by different amounts of H_2O_2 (0.01mM-0.2mM). One unit 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.

RESULTS AND DISCUSSION

Initially, the effect of $scCO_2$ on PPY was investigated. The surface structures of PPY films were monitored by ESEM before and after their exposure to $scCO_2$. No clear evidence of swelling of PPY by $scCO_2$ was noticed after the experiment. But after its 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 revealed the relaxation on the PPY surface after its 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 it was synthesized, the PPY film was most probably cracked after the experiment. This may also reveal the possibility of swelling of PPY by $scCO_2$.

As it is mentioned earlier, 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 experiments no activity on PPY films was observed up to 150 bar, 40 ^oC and 72 hours impregnation time. In ESEM a very small amount of GOX particles are seen on PPY films therefore an activity was not observed by enzymatic analysis. We can only seen the particles on the surface of the PPY film by ESEM, if the GOX particles were diffused inside the polymer film, the enzymatic activity of these enzyme particles have not been observed due to a diffusion barrier and hindrance of active site. A PPY film from one of the Dry experiments showed a very low activity at 100 mM glucose solution after 60 minutes incubation time. This may due to diffusion barrier. The PPY film processed with Semi-Dry experiment showed no enzymatic activity although it has a powder like layer covering on it, even after it was washed and dried.

In Wet and Wet-b experiments, enzymatic activity was observed with the all GOX immobilized PPY films. The PPY film samples taken from the experiments Wet1 and Wet 3 had a gel like layer on it when they were wet. After the films were washed and dried, the gel like layers became powder like layers. When they were wetted, the powder again turned out to be a gel. However, the sample from experiment Wet 2 did not have a gel like cover on it like Wet 1 and Wet 3 but PPY film from experiment Wet 2 has also showed an enzymatic activity. The ESEM photographs showed that PPY film from the experiment Wet 2 had a very thin coverage on it that cannot be seen by the naked eye (Figure 2). PPY films processed with the experiments Wet-b1 and Wet-b2 did not have a gel like coverage on them but had enzymatic activity. ESEM photographs showed that PPY of Wet-b1 had a cloud small of particles bounded to its surface (Figure 2). Since the PPY film of Wet-b1 had the greatest activity amongst the PPY films from the other experiments, these small particles are thought to be GOX particles.



(a) (b)

Figure 2: ESEM photographs of GOX immobilized PPY films from experiment (a) Wet 2 and (b) Wet-b1

The activities of GOX immobilized PPY films at 5-100 mM glucose concentration range are given in Figure 3. All of the experiments shown on Figure 3 were done at 150 bar and 40 ^{o}C. Other experimental conditions are given in Table 1. As it is seen from Figure 1, when the immobilization medium is dH_2O, an increase in the supercritical immobilization period caused an increase in 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 time period increased. Figure 3 reveals that pH 5.7 phosphate buffer is a better immobilization medium than dH_2O with $scCO_2$. When the $scCO_2$ meet with dH_2O, medium may be too acidic, and lowers 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 enzyme kinetic parameters K_m (Michaelis constant) and v_m (maximum velocity) were calculated and shown in Table 1. The highest v_m was calculated for the experiment Wet-b1 where maximum activity was also observed. When the results of Wet experiments are compared within themselves, it is seen that as the experiment time increased, v_m values were also increased. If the rate of reaction is assumed to be proportional to the enzyme amount present, it can be said that the enzyme amount immobilized on PPY film increased as reaction time increased. When Wet experiments' v_m values are compared within themselves, it is obvious that the increase in v_m value is higher between 5 hr and 25 hr, whereas further increase in impregnation time to 72 hr had smaller effect on v_m value.

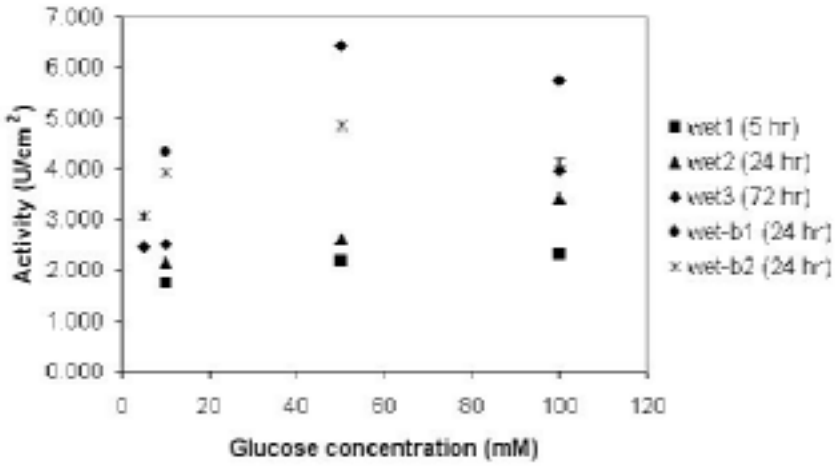


Figure 3: Activity of GOX immobilized PPY films

Table 1: Experimental conditions and v_m and K_m values of GOX immobilized PPY films

Experiment Name	**Wet 1**	**Wet 2**	**Wet 3**	**Wet-b1**	**Wet-b2**
Amount of GOX (mg)	25.0/4ml dH_2O	25.0/4ml dH_2O	12.5/2ml dH_2O	25.0/4ml buffer	25.0/4ml buffer
Duration (hr)	5	24	72	24	24
Area of PPY film	5	5	2.5	5	2.5
Type of electrolyte	SDS	SDS	SDS	SDS	PTSA
V_m (μmol/(min.cm^2))	2.358	3.221	3.714	6.472	4.695
K_m (mM)	3.575	5.156	3.028	4.819	2.526

CONCLUSIONS

In Dry and Semi-Dry procedures (up to 150 bar, 40 ^oC and 72 hr), the activity of enzyme immobilized into or onto the PPY film was not observed due to the lack of enzyme amount, diffusion problem, activity loss or active site hindrance. In Wet and Wet-b experiments (up to 150 bar, 40 ^oC), activity of GOX immobilized onto the PPY surface was evaluated. Maximum activity was obtained in buffer medium. In water medium, as impregnation time increased, the amounts of enzyme immobilized were also increased. In Wet and Wet-b experiments, there was enzyme coverage on polymer films, visible or invisible by the naked eye. There is no relation between the thickness of that enzyme coverage and the measured activity. Actually, higher activity values were obtained for thinner coverage.

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