Electrochemical synthesis and characterization of a polipyrrole/lipase composite film

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Abstract

The advantages of an irreversible immobilized enzyme for heterogeneous catalytic transformations of different substrates are numerous. In particular, since the enzyme macromolecules remains attached to the inert support it may be possible to re-use it. In some cases the immobilized molecules are more stable than the species of the solution. The kinetics of the immobilized species is likely to be influenced by the microenvironment and may be considerably altered from solution kinetics. Slow mass transfer of immobilized enzymes make possible advantageous treatment of substrate dilute solutions (waste waters etc). One of immobilization technique is entrapment in polymer matrices which cover a variety of different polymerization methods and polymer characteristics. The polymer may be an inert support (polystyrene, polyacrylamide etc) or may perform some functions itself. Conducting polymer in particular electrochemically deposited polymers have became of interest as support matrices for enzymes. Polypyrrol (Ppy) films are known to be permeable and appear to offer an ideal matrix for enzymes immobilization. Electro polymerization of pyrrole from an aqueous solution containing an enzyme (glucose oxydase) produced a Polypyrrol film containing the enzyme. The electro polymerization via a radical cation, which reacts with neighbouring pyrrolle to produce a chain that is α, α' coupled. The resulting polymer incorporates anions of supporting electrolyte and has a net positive charge. Some authors[i,ii,iii] have reported that the polypyrrole are degraded at same potential values This finding emphases that slight modifications in polymerization can alter the characteristics of the resultant polymer. Immobilization efficiency depends strongly on the composition and the structure of the entrapping polymer and this depends on the degree of cross linking and concentration of the monomer. So, it is important to manipulate the electropolymerization of pyrrole which would give a film that must be sufficiently porous to assure a maximum entrapping efficiency. The aim of this work is the study of immobilization efficiency in polypyrrole film of an acylhidrolaze (lipase) of molecular mass of 33-65 KDa obtained from Yarrowia lipolitica yeast.

Keywords: polypyrrole, lipase, yarrowia lipolytica, electropolimerisation.

Introduction

Lipases (triacylglycerol acylhydrolases - E.C. 3.1.1.3) are enzymes which catalyses the hydrolysis of esters of glycerol and other alcohols with fatty acids. The hydrolysis of ester bonds takes place at the interface between a lipid substrate phase and the aqueous phase in which the enzyme is dissolved.

Usual industrial lipases act on fats and oils and hydrolyze them gradually into di- and monoacylglycerols and finally into glycerol and fatty acids. In the absence of water, they are

capable of reversing the reaction, leading to esterification [1, 2]. Due to their ability to utilize a wide spectrum of substrates, and also to their chemo-, regio- and enantioselectivity, lipases stand amongst the most important biocatalysts used in organic synthesis for several reactions, such as hydrolysis, esterification and transesterification [3,4]. Based on these reactions, lipases have a great potential for industrial applications in food, chemical, pharmaceutical or detergent industry [5,6]. Promising fields include the biodegradation of plastics [7], such as polyhydroxyalcanoate and polycaprolactone.

Due to their biotechnological interest, many of these enzymes have been identified cloned and characterized [4]. Nevertheless, the demand for the production of highly active preparations of lipolytic enzymes has led to research on lipase producing micro-organisms and culture strategies [9,10]. Among these micro-organisms, *Yarrowia lipolytica* is one of a great interest, being able to naturally secrete several enzymes, including lipase, depending on the growth conditions [10].

Yarrowia lipolytica is the most extensively studied "non-conventional yeast", being currently used as a model for the study of protein secretion, cell dimorphism, degradation of hydrophobic substrate, including triglycerides etc [11 – 13]. Being strictly an aerobic yeast, its growth and lipase secretion are affected by the amount of oxygen available in the culture medium; therefore one of the purpose of our study was to point out the influence of aeration on the biosynthesis of lipase. Extra cellular lipase production by this micro-organism depends also on the composition of the medium, so that another purpose of our study was to establish the optimum concentration of olive oil as an inductor of the enzyme.

Lipase activity was measured by a titrimetric assay with NaOH 0,1 N, using emulsified olive oil as the substrate. 1- 5 mL of enzyme solution, 5 ml 10 mM citrate buffer pH = 7 and 2 mL $CaCl_2$ 0,6% in the above mentioned buffer solution were added to 10 ml emulsion containing 25% (vol./vol.) olive oil and 75% (vol./vol.) Arabic gum. The assay was carried out at 37°C during a 60-minute incubation. After this time interval, the reaction was stopped by adding 20 ml acetone-ethanol 1:1 (vol. /vol.) and the amount of fatty acids was then titrated.

One unit of lipase activity was defined as the amount of enzyme that released 1 μ equivalent of carboxyl groups of fatty acid under analysis conditions (temperature = 37^0C, pH = 7, reaction time = 60 minutes). In our case, the catalytic activity of the biocomposite material was reported to the electrode surface.

Materials and Methods

Chemicals: lipase used in this work was obtained from Yarowia lipolytica Yeast and was used as received. The pyrrole and all substances utilized in this study were purchased from Aldrich Sigma Co. and were used without further purification.

Equipment: A single compartment cell, with glassy carbon and graft working electrodes ($2cm^2$), a platinum gauge auxiliary electrode and a saturated calomel reference electrode were used for all electrochemical experiments. A Gamry potentiostate /galvanostate model and Electrochem analysis software both to electrodeposit films of polypyrrole +lipase and to measure the electrochemical characteristics of the composite. All solution were purged with spectral argon during electrodeposition and were blanketed with Argon during measurement of electrochemical characteristics.

Electrosynthesys of polypyrrole and polypyrrole +lipase films

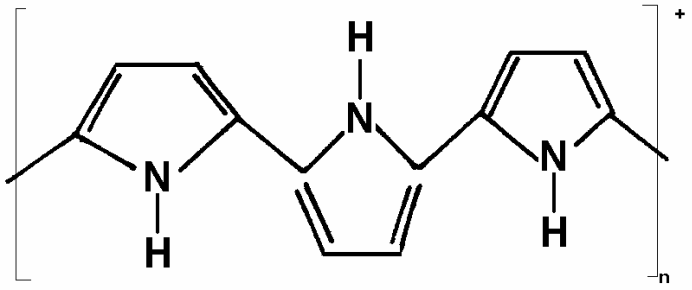
- Constant current method. Films were electrodeposited onto a carbon and glassy carbon electrodes at constant current (0.3 mA cm^{-2}) for 10 minutes prior to measurements of electrochemical characteristics. Films of Ppy and PPy doped with lipase were synthesized from an aqueous solution containing $0,1ML^{-1}$ pyrrole and 1% Lipase.
- Potential cycling method. Films were electrodeposited onto working electrodes by cycling the potential between 0.00V and 1,00V for 50 cycles with $50mVsec^{-1}$, prior to entrapped lipase by adsorption. The lipase was adsorbed from a 10% aqueous solution for 24 hour at 35,5C.

Electrochemical characterization of polypyrrole and polypyrrole +lipase films

- Open Circuit Potential measurement in neutral Na_2SO_4 0,5M solution was conducted for 300sec.
- Electrochemical impedance spectroscopy method was utilized to characterize the films in neutral Na_2SO_4 0,5 M solution at the rest potential and frequencies between 100 KHz and 0.1 Hz. The capacitance - potential dependencies were performed at different frequencies: 1KHz; 100Hz; 10Hz; 1H; 0.1Hz. For experimental data analysis was used the Electrochem Gamry software and equivalent electrical circuit editor.

Results and Discussions

The figures 1-4 present experimental results. Polypyrrolle in its conductive state is cationic with approximately one cation per 3-5 pyrrol subunits [13]:



Electrodeposition of pyrrolle in the presence of lipase results in the entrapment of the enzyme within the polymer matrix, which is electrically conductive. Entrapment of LIP within the polypyrrole matrix involve the entanglement of the two macromolecules (polypyrrole and LIP) to perform ionic interaction between anionic substitutes on LIP and the cationic backbone of conductive polypyrrole.

As results of this interaction, the electrochemical and adsorption characteristics of polymeric matrix are modified as follows:

Open Circuit Potential

The Open Circuit Potential (OCP) of polypyrrole film with and without lipase on glassy carbon and graphite in sulphate solution is presented in fig 1 and 2.

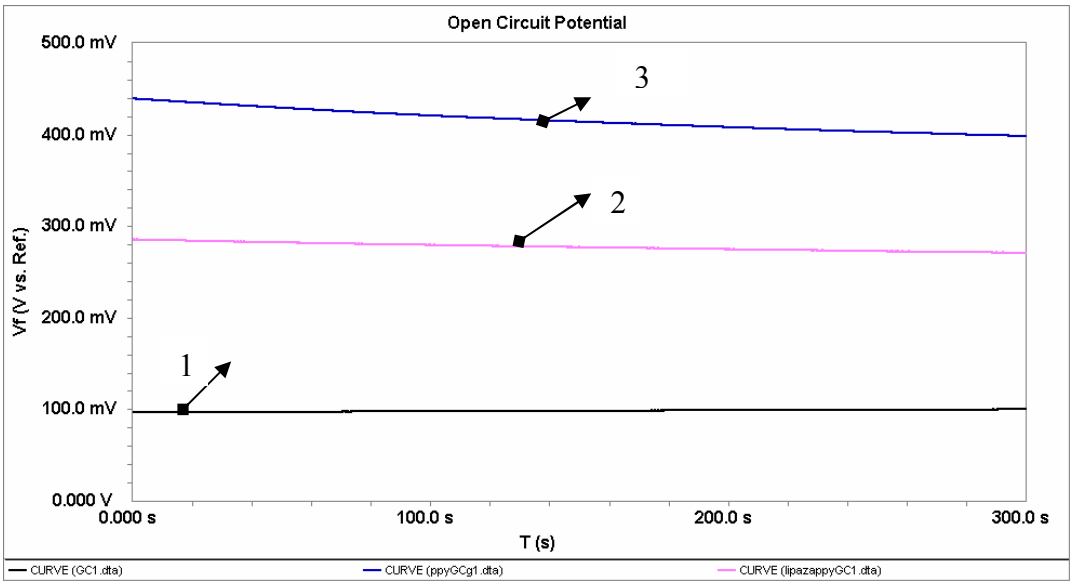


Figure 1. The Open Circuit Potential (OCP) of polypyrrole film with and without lipase on glassy carbon and in sulphate solution. 1-glassy carbon electrode, 2- glassy carbon + Polypyrrole + lipase, 3- glassy carbon + Polypyrrole

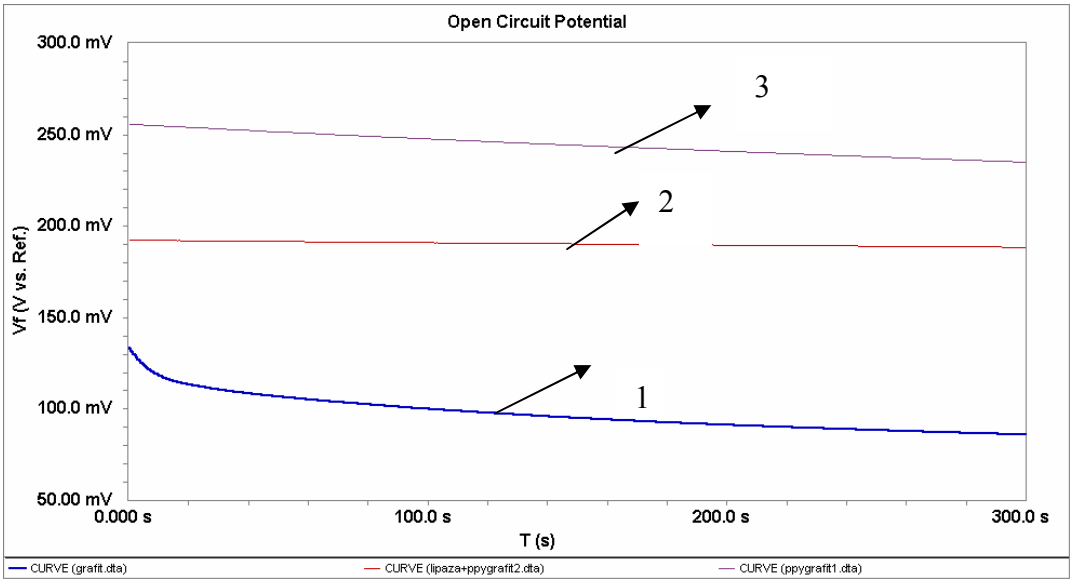


Figure 2 The influence of immobilized lipase in polymeric film on OCP in sulphate solution. 1-graphite 2-graphite + Polypyrrole + lipase, 3- graphite + Polypyrrole

From the **figure 1** can be seen the influence of ionic interaction between PPy and LIP on values of OCP which is changed from 420mV for Ppy conducting film to 290 mV for Ppy + lipase film with ΔV=130 mV. For the same system but on graphite electrode fig.2, ΔV=65 mV. The experiments reported here detect an effect of LIP adsorbed to the polypyrrole matrix. This effect is alteration in the voltage dependence on the surface concentration of the adsorbed YLIP [4]. The magnitude of the effect and the sign of the voltage which induce it both depend on the LIP. When Lipase is added to the aqueous phase, the voltage dependant capacitance changes in a very specific way. The changes depend on both, voltage and enzyme charge sign.

The Ppy membrane surface charge change due to adsorption of Lipaze

$$q_o = zF\Gamma$$

where q_0 is membrane charge cm^{-2}; z is LIP charge; f =Faraday number and Γ is the surface concentration of LIP.

At the given concentration of enzyme in aqueous phase, the change in the capacitance due to changes in adsorbed LIP can be written in the form:

$$\Delta C = \frac{\Delta q_0}{\alpha \Delta V} \quad (2)$$

Where Δq_0 = charge change due to applied potential ΔV and α is a proportionality factor.

Taking in consideration the change in surface charge density with potential, produce an effective capacity given by:

$$C = 2F\Gamma \frac{d\Theta}{dV} \quad (3)$$

Which represents so called pseudo capacitance [8].

Electrically adsorption pseudo capacitance (C) can be represented as a voltage dependent capacitor. The charging rate of this capacitor is proportional to the rate of adsorption of lipase to the polypyrrol matrix.

To demonstrate this behaviour of Polypyrrol +lipase enzyme electrode we studied by electrochemical impedance spectroscopy method and especially Niquist plots and capacitance potential plot the electrochemical characteristics of composite film.

The Niquist plots for Polypyrrole and Polypyrrole lipase films are presented in the **figure 3**.

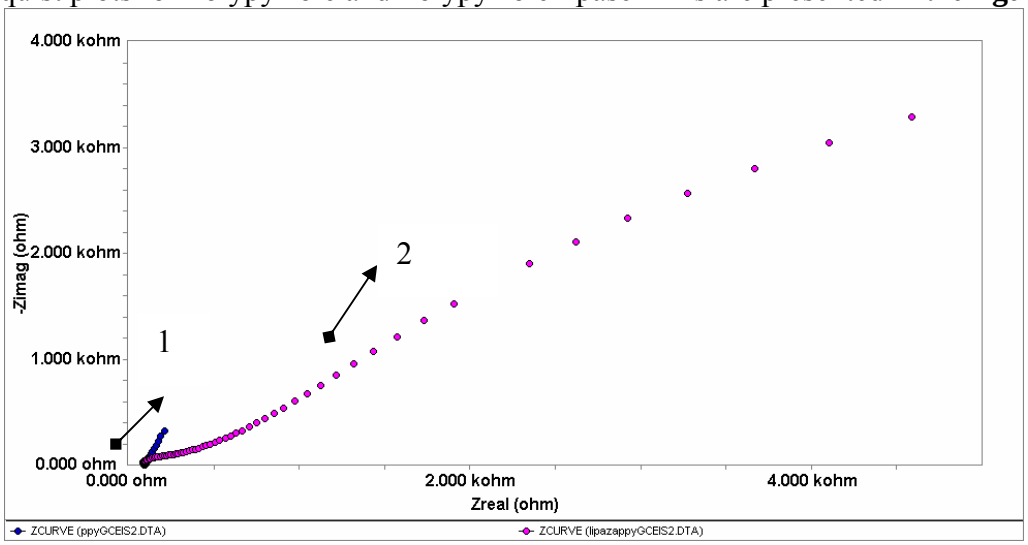


Figure 3 The Niquist curves of Polypyrrole (1) and Polipyrrole with lipase (2) films in 0,5 M Na_2SO_4 solution obtained between 100 KHz and 0,01Hz frequencies

The Niquist plot shows the frequency dependence of impedance implicitly as the imaginary impedance on the real impedance as it results from the known equivalent electric circuit:

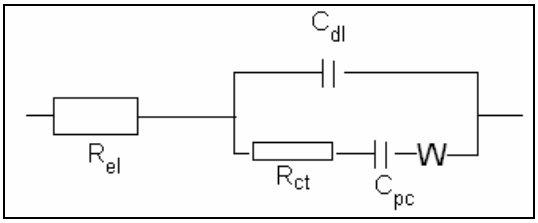


Figure 4 The equivalent electrical circuit for adsorbed charged species on the polymeric film

The dependence on potential of the equivalent circuit parameters and especially the pseudo capacitance is a sensitive indicator of the changes on surface of the composite electrode.

In the **table 1** we present same values of the pseudo capacitance and conductivity both polypyrrole matrix film and of composite polypyrrole +lipase film.
The pseudocapacitance was obtained at open circuit potential values.

Table 1 The influence of entrapped enzyme on the electrical characteristics of Polypyrrole films in Sulphate solution

	PPy	PPy +lipase
Capacity $\mu F/cm^2$	259	274
Conductivity mS/cm^2	50	44

From these data we conclude that entrapment of lipase in the matrix of polypirrole depend on changes in the pseudocapacitance and conductivity of the film.
The catalytic activity of this composite material is expressed in activity units of lipase (about 8 unit cm^{-2}).

Conclusions

- The entrapping efficiency of lipase in polypirrole film was studied by Open Circuit potential and EIS techniquies. It was found that the amount of entrapped enzyme is proportional with the decrease of OCP, increase of pseudocapacitance and decrease of conductivity of film.
- The catalytic activity of the composite biopolymer tested by titration of hydrolysis product of olives oil is 8 unit cm^{-2}.

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