Immobilization of proteins in new-ionic-conducting-based materials- Ion Jelly®

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Introduction

The **immobilization of biomolecules on surfaces**, such as proteins and DNA, has been extensively pursued aiming to new applications, such as nanodevices, biofuel cells and biosensors (Gilardi et al.; 2001, Murphy; 2006, Zhang et al; 2004, Lucarelli et al.; 2008). The diversity of different approaches is vast, from which it may be highlighted the physical immobilization by adsorption, entrapment on membranes or polymers and cross-linking on different beads materials (Gilardi et al; 2001, Vojinovic et al.; 2006, Liu, et al.; 2006). In particular, conducting polymers have been extensively studied due to its intrinsic properties, namely an efficient charge transfer, large active area and good stabilization of immobilized proteins (Sadik; 1999).

Ion Jelly® patented technology is based on the cross-linking of Ionic Liquids (ILs) with Gelatin that result in a viscous gel that can be molded into a film or a block, and solidifies by cooling below 35 ºC. The outcome of this combination is a transparent, light and flexible conductive polymer that adapts perfectly to a great variety of surfaces. Some of the key properties of **Ion Jelly®** are: good ionic conductivity (10^{-4} S cm^{-1}), high stability up to 180ºC, large electrochemical window, bio-compatibility and depending of the constituents it can be immiscible in alkanes, etheres or even in water (Lourenço et al; 2007).

Taking in consideration the attractive attributes that provide a stable and friendly environment for the enzymes, we decided to use this new protein-ionic-conducting-based material with tailor-made properties in biosensing applications. In the present work **Ion Jelly® technology** was applied to immobilize different proteins, such as cytochrome *c*, catalase, peroxidase, and glucose oxidase, in two studies, focusing in enzymatic activity of immobilized peroxidase and electrochemical response of immobilized citocrome *c*.

Material and Methods

The basic composition of Ion Jelly® films is an ionic liquid, 1-butyl-3-methyl-imidazolium dicyanamide [bmim][$N(CN)_2$], and gelatin. The proteins used in this work were immobilized by two methods: by co-deposition, in which it were added to the films together with the other components, or by physical adsorption from a protein solution promoted by voltammetric cycling. HRP co-deposition was performed by simply adding HRP 0.1 M phosphate buffer solution, pH 7.0, to ion jelly material at liquid state. The mixture was left to stir for 30 min., and co-deposited onto plastic substrates and left to cure for 48h at 4ºC before use.

Immobilized Horseradish Peroxidase (HRP) initial reaction rates were measured by a colorimetric method using 4-aminoantipyrine (4-AAP), phenol-4-sulphonic acid (PSA) and H_2O_2. The initial rate was determined by monitoring the increase of absorbance at 490 nm correlated with the consumption of H_2O_2 using a Hitachi U-2000 spectrophotometer (Vojinovic et al., 2004).

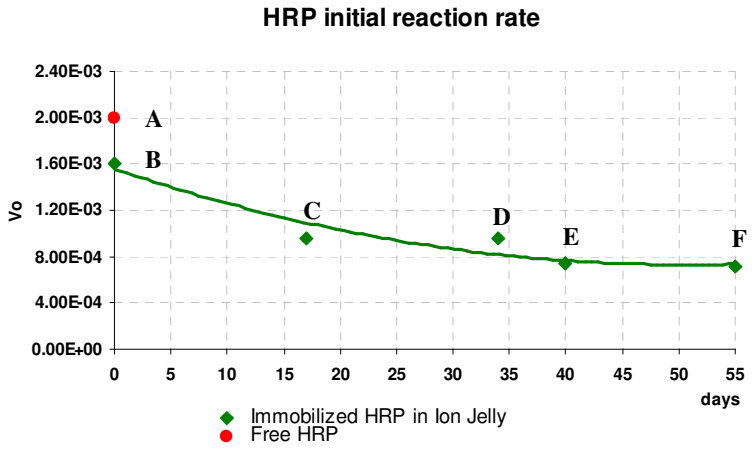
The activity assays of different samples over the time were performed by adding 25µL H_2O_2 250mM to a standard reaction mixture containing Ion Jelly® film with 2U of HRP, 475µL of 0.8mM 4-aminoantipyrine (4-AAP), 475µL of 50mM phenol-4-sulphonic acid (PSA) in 0.1 M Phosphate buffer, pH 7.0. The absorbance was monitored for 2 minutes, in a magnetically stirred quartz cell, thermostatized at 25ºC.

Electrochemical experiments were performed either in a Autolab 12 or µAutolab type III Potenciostat/Galvanostat and the data acquisition was done using GPES software. Carbon screen printed electrodes (SPE), purchased from DropSens, were used. The working electrodes diameters are 4 mm. The modified electrodes were kept at a controlled atmosphere at 4ºC. The electrochemical characterization was performed by cyclic voltammetry in 0.1 M KNO_3, 0.1 M KPB buffer, pH 7.6, or 0.010 M $K_4[Fe(CN)_6]$ / $K_3[Fe(CN)_6]$ / 0.1 M KNO_3). The proteins concentration was approximately 250 mM in 0.1 M KPB, pH 7.6. Assays were performed at air and in controlled anaerobiose conditions using an anaerobic chamber (< 10 ppm O_2).

Results and discussion

Stability of immobilized HRP in Ion Jelly® film

The study of the stability of immobilized Horseradish Peroxidase (HRP) in Ion Jelly® film will be evaluated for a period of time of six months in order to verify the viability of this immobilization. The results for the first two months are represented in the following graph.



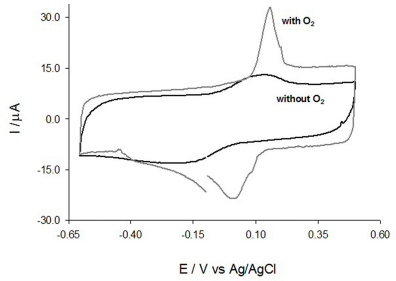
Graph 1. HRP initial reaction rate.
A- Free HRP; B- Immobilized HRP in Ion Jelly at liquid state; C- Immobilized HRP in Ion Jelly film after 17 days stored at 4ºC; D- Immobilized HRP in Ion Jelly film after 34 days stored at 4ºC; E- Immobilized HRP in Ion Jelly film after 40 days stored at 4ºC; F- Immobilized HRP in Ion Jelly film after 55 days stored at 4ºC.

The highest initial reaction rate was obtained for free HRP (2.0E-3 mM/s) and show to be around twice the initial reaction rate obtained when immobilized in Ion Jelly® (9.6E-4 mM/s) after 31 days stored at 4ºC. After 40, 55 days it was observed a slightly decrease in initial reaction rate (7.4E-4 mM/s), however seem to be constant over the time. This figures indicates good enzymatic stability for this simple immobilization, however, it was observed some diffusional issues that need to be circumvent in order to increase the enzymatic activity.

Ion Jelly® redox behaviour

The Ion Jelly® films present **complex electrochemical behaviour** with several redox processes that evolve with continuous potential cycling. In the presence of a solution containing oxygen there is a first stage with the development of sharp current peaks. After **multiple cycling** these processes start to decrease its intensity and the films behaviour become stable. Experiments performed in an **anaerobic chamber**, in conditions of absence of atmospheric oxygen and using solutions with and without dissolved oxygen (figure 1), have shown that these processes are related with the oxygen content in film or in the characterization solution and may be related with the formation of **hydroxide** species in the interfacial region between the film and the carbon electrode.

Figure 1. Ion Jelly® films redox behaviour in 100mM KPB buffer, on air and under anaerobiose conditions.



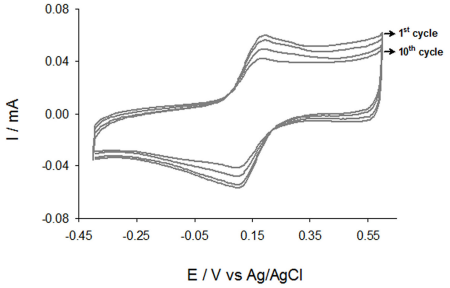
Ferrocyanide diffusion coefficient

The potassium ferrocyanide diffusion coefficients were estimated by cyclic voltammetry, using the Randles-Sevcick equation. The **values of D**, at room temperature, seem very **dependent on the water content** of the films, as already mentioned by other authors (Schröder et al.; 2000). The D values increase two orders of magnitude, from $1.7x10^{-7}$ cm^2s^{-1} (older and dryer films) up to a maximum of $9.8x10^{-5}$ cm^2s^{-1} for fully water saturated films. The D values found are unusual high for the normal diffusion coefficient found for this species, and should be the subject of further studies.

Ferrocyanide incorporation

Ferrocyanide can be incorporated into the Ion Jelly® film by multiple cycling assays. After three times multiple potential cycles followed by washing thoroughly with Millipore water, the films have shown to **retain the ferrocyanide ions**. The current intensity of the redox processes decrease with the number of cycles, indicating progressive wash out of the redox species (figure 2).

Figure 3. Ion Jelly® films (deposited on carbon SPE, configuration (2) multiple cyclic voltammograms on 0.1 M KNO_3, showing the ferrocyanide characteristic current peaks.

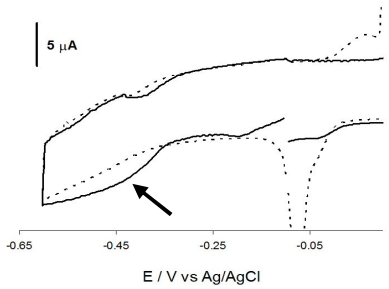


Immobilized protein response

Cytochrome *c* was immobilized by multiple cycling from a solution of 250 mM cyt *c* / 100 mM KPB, pH 7.6, at slow scan rate. After, the films were washed with Millipore water and characterised in the same solution in the absence of the protein (figure 3). A new cathodic process, indicated in the figure, becomes visible at approximately -0.430 V vs Ag/AgCl, indicating that the **protein has**

become immobilized on the film and that **direct electron transfer** between the film and the cyt *c* is achieved.

Figure 4. Ion Jelly® films on a solution of 100 mM KPB, pH 7.6, after cytochrome *c* immobilization by physical adorption The arrow indicates the new cathodic features due to the cyt *c*.



Conclusions

The preliminary results are of the significant importance in the area of immobilization of proteins in conducting matrix, representing a very simple route to obtain such materials through the simple combination of gelatin with ILs.
Ion Jelly® films present good electronic conductivity, dependent of the oxygen and water content in the films. The incorporation of a redox inorganic compound and the immobilization and direct electron transfer of proteins on the films were successful.

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Acknowledgments
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