Introduction
Over the past few years interest has been increasing in the application of simple, rapid, inexpensive and disposable biosensors in clinical, environmental or industrial analysis.

The most common disposable biosensors are those produced by thick-film technology. A thick-film biosensor configuration is normally considered to be one which comprises layers of special inks (or pastes) deposited sequentially onto an insulating support or substrate. Screen printing seems to be one of the most promising technologies allowing biosensor to be placed large-scale on the market in the near future because of advantages such as miniaturisation, versatility at low cost and also particularly the possibility of mass productions. The use of thick-film technology for the production of sensor systems is an emerging field. The most critical point in manufacturing thick film biosensors is the sensitive or active membrane and its adhesion to the transducer layer [1,2,3].

The aim of the present work is activation and derivatization of carbon powders to be used in screen printed electrode preparation. The oxidizing treatment of carbon powder with the aqueous H2O2 solution introduces mainly carboxylic and phenolic groups on the carbon surface after that as well as derivatization, it is easy to use this groups in covalent immobilization of biomolecules [4]. Glucose oxidase was used as a model enzyme to test the usage of the different covalent immobilization methods on the treated carbon surfaces.

Material and Methods

'Immobilization of enzyme: binding of the enzyme were performed on (1) EDC activated, (2) CDI activated carbon and/or 10% rhodium graphite powder (Aldrich 20.016-4), or dextran (25%) or lysine coupled carbon.'

Preparation of the carbon based inks for printing working electrodes: Inks for printing working electrodes were prepared by mixing a commercially available carbon ink (Du Pont 7101) with oxidised carbon and/or 10% rhodium graphite powder (Aldrich 20.016-4), or dextran (25%) or lysine coupled carbon.

Printed Electrodes: Printed electrodes were fabricated by depositing several layers of inks on a PVC substrate (Fig 1). The conducting paths and pads (Fig.1a) were deposited directly on the PVC sheets using Ag/Pd ink (DuPont, 5025). Then, an Ag/AgCl ink was deposited to obtain the reference electrode (Fig.1b). Different oxidized carbon powders were printed to obtain the working electrodes (Fig.1c). Finally, an insulator layer was placed over the conducting paths (Fig.1d). After each printing step, the pads were treated at 60 oC for 60 min.

Preparation of Enzyme Electrodes Different techniques were used for the covalent immobilization of enzyme: binding of the enzyme were performed on (1) EDC activated, (2) CDI activated carbon surfaces and also, (3) Lysine coupled surfaces using EDC activation., respectively.

(1) Electrode surfaces were treated with EDC [1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide] 0.1 mol/L in NaH2PO4 (pH 4.75, 0.1 mol/L) solution for 45 min, washed with distilled water and 10 mmol/L PBS (phosphate buffer saline, pH 7.5). Then, 10 µl of enzyme solution (1 µg of enzyme in 10 µl phosphate buffer, 50 mmol/L, pH 7.5) was spread on the surface and allowed to stand at 4 oC overnight [6].

(2) The procedure was similar with the previous one except CDI (N,N-carbonyldimidazole) was used instead of EDC for the activation of surface [7].

(3) Before printing of the oxidized carbon powder, EDC activation was performed as described before and then, instead of enzyme, carbon was treated with O.1 M of lysine coupled graphite. After printing of the lysine coupled powder, the procedure 1 was performed.

Results and Discussion

The use of commercially available or home made inks for electrode printing and the wide range of possibilities with respect to the substrate material allow the development of low cost/high performances transducers which can be largely applied for biosensing in any field of analytical determinations [8]. The oxidizing treatment does modify the surface chemistry of the electrode, by creating surface oxygen complexes that make it more acidic. Scanning electron microscopy shows in good evidence that the porous structure of the carbonaceous support was not affected (Fig 2) by the treatment.

Furthermore, the pH value of the carbon slurry was found to be changed from 6.5 to 4.4 after oxidizing step because of the presence of acidic groups on the surface. Our results are well in agreement with [5]. On the other hand, as well as the covalent immobilization by using the introduced acidic groups, negatively charged functional groups on the electrode surfaces could provide useful matrices for ionic binding of proteins which could strongly interact in aqueous solution.

In this study, different oxidised carbon matrices (graphite-rhodium, carbon, carbon-dextran and lysine coupled carbon) were prepared to be printed as a working electrode. Afterwards, covalent immobilisation of glucose oxidase was performed. Both features of sensors towards to glucose and hydrogen peroxide are shown in Table 1.

<table>
<thead>
<tr>
<th>Glc</th>
<th>O2</th>
<th>Linearity</th>
<th>Sensitivity (nA/L mmol)</th>
<th>C.V. (%)</th>
<th>Correlation coefficient (R²)</th>
<th>Sensitivity (nA/L mmol)</th>
<th>C.V. (%)</th>
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<td>0.25</td>
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<td>0.76</td>
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As it can be seen in the last column of Table 1, in some cases, covalent immobilization of the enzyme directly on the electrode surfaces caused higher biosensor responses in comparison to hydrogen peroxide. This behaviour could be due to diffusion effects through the enzyme layer toward the electrode surface. This behaviour was never observed with biosensors using membranes for enzyme immobilization because diffusion of both glucose and hydrogen peroxide is greatly controlled by the membrane properties, especially the thickness.

The observed behaviour could be explained with a better diffusion of the hydrogen peroxide produced by the enzyme catalysed reaction from the active site of the enzyme toward the very close electrode surface. It was observed only with EDC, not with CDI, probably because the enzyme is bound on the electrode surface in a different site. With the dextran-EDC matrix the effect was not observed probably because the dextran acts as a barrier and limits diffusion of reagent and product in the same way of a membrane.

Moreover, storage stabilities at 4 oC were investigated. GOXs both, immobilized on carbon-dextran mixture by EDC and CDI activations completely lost their activities after 30 days. However, activity of immobilized GOX on graphite-rhodium by EDC and CDI activations were found to be 92.7% and 67.3%, respectively 60 days after. GOXs immobilized on EDC activated carbon and lysine coupled carbon were found to have 80% and 30% of their activities after 60 days.

Conclusions

Activation and derivatization of carbon powders to be used in screen printed electrode preparation were performed for obtaining easier enzyme covalent immobilization. The oxidizing treatment of carbon powder with an aqueous H2O2 solution introduced mainly carboxylic and phenolic groups on the carbon surface which were used for further covalent immobilization of biomolecules. Glucose oxidase was used as a model enzyme to test the usage of the different covalent immobilization methods on the treated carbon surfaces obtaining very good sensitivity and storage stability.

References