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# New MIP-based Electrochemical Device for On-site Detection of Glucose Oxidase

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**Abstract:** The measurement of protein concentration is a fundamental assay in diverse areas, such as medicine, environmental control and also in food industry, for monitoring enzyme content in consumer goods.

In this work, the design and synthesis of artificial receptors, based on molecular imprinting (MI) technology, is described for the development of a simple and efficient electrochemical MIP-based biosensor for detection of glucose oxidase (GOD). Disposable and cost-effective chips, consisting of carbon screen-printed electrodes (C-SPEs), were used to develop the portable electrochemical devices for on-site monitoring of GOD in industrial context.

MIP receptor films were created by electropolymerization of caffeic acid (CAF), in the presence of GOD as template biomolecule, at the carbon electrode surface, followed by enzyme wash-out from the polymeric matrix in order to create the specific binding sites. After that, specific binding properties between the MIP film surface and the template protein were observed. The developed MIP biosensor exhibited a very wide working concentration range, between $10^{-9}$ to $10^{-3}$ g $L^{-1}$, with a limit of detection (LOD) of $1.0\times10^{-10}$ g $L^{-1}$. Moreover, selectivity studies were performed against other proteins (amylase and myoglobin) revealing that the MIP surface has high specificity for GOD.

**Keywords:** Glucose oxidase (GOD), Caffeic acid (CAF), Electrochemical biosensor, Molecularly imprinted polymer (MIP), Carbon, Screen-printed electrode (SPE).

## 1. Introduction

Glucose oxidase (GOD) is a glycoprotein having a widespread use in several commercial applications [1-3]. Although it is widely known its use as catalyst in glucose sensing devices [4], it also has a very important role in food and pharmaceutical industry as (1) oxygen removal and preservative; (2) for improvement of color and flavor of food materials and; (3) as antagonist against pathogens. Thus, nowadays, the development of simple, fast and cost-effective methodologies for accurate and sensitive quantification of GOD in currently a demand in the industrial field.

Currently, most commonly used strategies to quantify large biomolecules, such as proteins and enzymes, rely on immunoassays [5-7], offering selective and specific detection through complementary interactions between the biorecognition element and the protein. However, biorecognition elements used (enzymes, nucleic acids, antibodies, etc.) makes detection expensive, having

time consuming procedures, sometimes need of labelling and carries problems related to biorecognition element instability.

To avoid some of these problems, molecular imprinting (MI) technology recently emerged in the literature [8-11] to build synthetic receptors (artificial antibodies) that can be rapidly and inexpensively fabricated through the construction of a polymeric (or inorganic) structured network that selectively binds a template biomolecule. Furthermore, molecularly imprinted polymers (MIPs) show further advantages over biological receptors, among them it's their robustness, cost-effectiveness and resistance to extreme temperature and pH conditions [8-11].

Electrochemical biosensors are powerful and versatile tools, which offer good precision, accuracy and sensitivity, with relatively low cost and simple instrumentation [12-14]. In addition, the requirement of small sample volumes and the use of disposable chips, such as the screen-printed electrodes (SPEs), allows portability, a great advantage of these biosensors that found diverse applications in diagnostics, environmental control and pharmaceutical industry fields [12-14]. Thus, the unique characteristics of MIPs, combined with electrochemical detection, makes this technology suitable for detection of GOD and this strategy was used in this work.

Although bulk polymerization is the most frequent way of MIPs preparation, electropolymerization using electrochemical techniques is a smart way to build the polymer film directly at the transducer surface, allowing simple and easy control of the thickness of the MIP receptor layer [8-11].

In this work, a new MIP-based electrochemical biosensor was developed for on-site monitoring of GOD. The MIP film surface was prepared by electropolymerization of 3,4-dihydroxyciannamic acid (also known as caffeic acid, CAF) monomer on a carbon-SPE (C-SPE) surface, in the presence of GOD enzyme as template. This natural phenol, CAF, can be easily electropolymerized on carbon electrodes by applying a constant potential to the electrochemical system (chronoamperometry) [11, 15]. Besides, the high biocompatibility of the carbon surface towards proteins (and antibodies) avoids its denaturation during the adsorption process prior to the electropolymerization process [16].

After preparation of the sensor surfaces, a systematic study of several analytical parameters (including sensitivity, dynamic linear range and LOD) was implemented in order to evaluate the performance of the GOD electrochemical biosensor, using electrochemical impedance spectroscopy (EIS) as detection technique. The different steps for the MIP biosensor preparation and detection are schematically represented in Fig. 1.

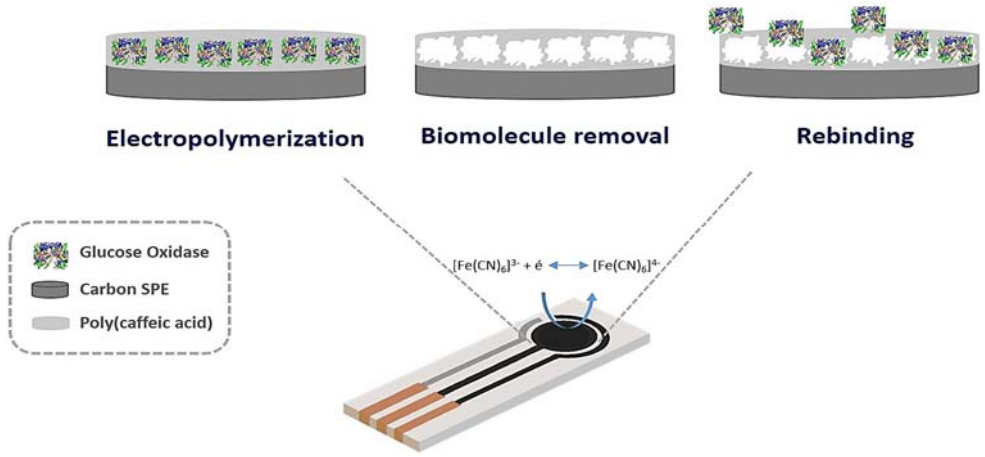



**Fig. 1.** Schematic summary of the procedures used for MIP preparation at the C-SPE surface and detection. Frist, a mixture solution of monomer and target biomolecule are subjected to a electropolymerization process aiming to create a polymer matrix containing the embedded target enzyme. Moreover, when the target biomolecule is removed from the polymeric matrix, the recognition sites are exposed and display affinity to recognize template molecules during the rebinding studies (detection). The evaluation of the receptor performance was achieved by performing ESI measurements in the presence of the well-characterized ferricyanide/ferrocyanide reporting system.

## 2. Experimental Part

### 2.1. Chemicals and Solutions

Glucose Oxidase (GOD), from Aspergillus Niger (Fluka), CAF (Sigma-Aldrich), potassium ferricyanide ($K_3[Fe(CN)_6]$, Merck), potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6].3H_2O$, Merck), sodium dihydrogen phosphate (Sigma-Aldrich), sodium hydrogen phosphate (Sigma-Aldrich) were used as received. All other chemicals were of analytical grade and were used without any further purification. All aqueous solutions were prepared using water purified with a Milli-RO3 Plus and Milli-Q purification systems (resistivity > 18 MΩ $cm^{-2}$).

Stock solutions of GOD (C = 1 mg $mL^{-1}$) were prepared in 0.1 M PBS pH 7.0. Less concentrated solutions were prepared by accurate dilution of the previous solution in the same buffer.

### 2.2. Apparatus

All the electrochemical measurements were carried out using a PGSTAT302N potenciostat/galvanostat from Metrohm Autolab, equipped with an impedance module and computer controlled with the GPES 4.9 software.

The carbon screen-printed electrodes (C-SPEs, 4 mm diameter, DRP-C110), with working electrode and counter electrode made of carbon, and pseudo-reference electrode made of silver, were purchased from DropSens (Merck), as well as a switch box which allow the interface between them and the potentiostat/galvanostat.

Atomic Force microscopy (AFM) images were recorded using a Molecular Imaging PicoLe atomic force microscope and the surface topography was obtained using a silicon tip (App Nano, model ACT).

### 2.3. Electrosynthesis of Molecularly Imprinted Films and Template Protein Extraction

Prior to electropolymerization, an electrochemical cleaning was performed on the C-SPE surface using a 0.5 M $H_2SO_4$ solution. Thirty successive cycles were performed from -200 to 1000 mV at a scan rate of 50 mV $s^{-1}$.

The molecularly imprinted films were produced by covering the working area of the electrode with a mixture solution containing 1 ng $mL^{-1}$ GOD and $2\times10^{-4}$ M of CAF monomer, prepared in PBS buffer. For electropolymerization, a fixed potential of +2 V was applied to the electrochemical system for 30 s. Then, the film obtained was washed with pure water and incubated overnight in 1 M $H_2SO_4$ solution to washout the template protein. Finally, the electrode was abundantly washed with pure water to completely remove GOD residues from polymeric film. In parallel, non-imprinted polymer (NIP) surfaces were produced in a similar way as the corresponding MIP but in the absence of the template enzyme (reference system).

### 2.4. Electrochemical Measurements

EIS measurements were performed in the presence of 5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple at an open circuit potential of +0.12 V, using a sinusoidal potential perturbation with an amplitude of 0.01 V. The frequency range was from 0.1 to 100,000 Hz. Impedance data were represented as Nyquist plots and fitted to a Randles type equivalent circuit using the adequate module in FRA software.

## 3. Results and Discussion

In this work, we propose a new electrochemical biosensor based on artificial antibodies for detection of glucose oxidase (GOD), an enzyme with vast applications in industry. The MIP biosensor was prepared by electropolymerization of CAF at a C-SPE surface in the presence of GOD as template biomolecule, following by extraction of GOD for specific cavities formation (see Fig. 1). Frist, a systematic investigation of the biosensor optimization is presented. The different modification processes occurring at the MIP surface induced changes in the electron transfer the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox system that were followed by EIS measurements. Then, the biosensor performed was evaluated by analysis of GOD in buffer soliton and in commercial orange juice, using EIS technique.

### 3.1. Optimization of Electropolymerization Conditions for Creation of Binding Sites

Most of proteins and enzymes are often incompatible with organic solvents used in MIPs synthesis and the use of aqueous solutions can restrict the choice of monomers used to build the polymeric film. In this work, CAF was selected as monomer due its high stability in aqueous environment while it can be readily oxidized at the carbon surface after application of a suitable potential [11, 15].

With the goal of having a good control over the MIP-film properties, the electropolymerization of CAF the C-SPE surface was performed by chronoamperometry in the presence of different amounts of GOD. To improve the detection sensitivity of devices it is mandatory to produce an ultrathin polymer film over the transducer surface [11, 17]. Thus, in this work, the thickness of the polymer was easily controlled by adjusting both, the potential applied and time of deposition.

A typical chronoamperogram recorded during electrodepositing of CAF (C = $2\times10^{-4}$ M), in the presence of 1 ng $mL^{-1}$ GOD enzyme, both dissolved in 0.1 M PBS pH 7.0, is shown in Fig. 2. As can be seen in the figure, after the initial change in current due to a capacitive process, the current increases indicating the formation of the poly(CAF) film. After 7 s, the decrease in current indicates that the electropolymerization process is being limited by the diffusion of the monomer due the barrier caused by polymer grow. For both systems, MIP and NIP, the recorded chronoamperograms were quite similar.

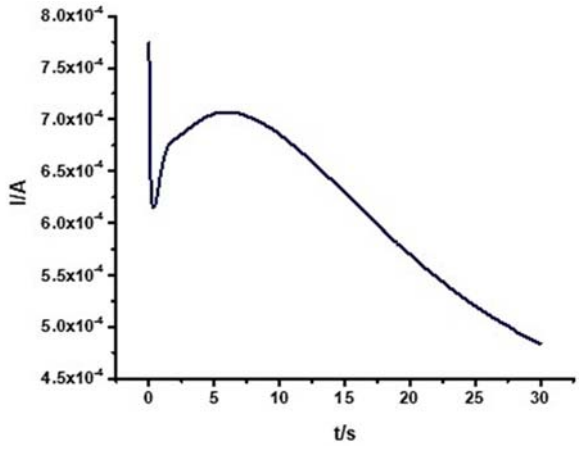



**Fig 2.** Chronoamperogram obtained during electrodeposition of CAF (C = $2\times10^{-4}$ M, in 0.1 M PBS buffer), in the presence of 1 ng $mL^{-1}$ GOD as template biomolecule, on a C-SPE surface, after an E = +2 V was applied to the electrochemical system for 30 s.

## 3.2. Protein Removal

Imprinting of large dimension biomolecules can have some difficulties associated related to its extraction from the polymer film. Therefore, effective extraction procedures are mandatory to achieved high performance biosensing devices [9]. Different strategies to remove the imprinted proteins from polymers can be used, such as chemical treatments to induce protein denaturation and amide bond cleavage [18, 19] and biochemical strategies which use enzymes to broke peptidic bonds among imprinted protein [20].

In this work, the removal of the GOD imprinted was achieved by incubating the imprinted films with a 1 M $H_2SO_4$ solution overnight to induce protein denaturation and promote the specific cavities formation. The NIP surface was also submitted to the same extraction process and no damage of the polymeric matrix was observed.

## 3.3. Process Monitoring by EIS and AFM

To monitor the fabrication of the binding sites at the chip surface, EIS was used, since this technique is able to evaluate changes in film resistance after each modification step. The Nyquist diagrams recorded at the different stages of the sensor construction are displayed in Fig. 3. The Randle's equivalent circuit was adopted to fit the experimental data.

As can be seen in impedance data presented, the freshly cleaned C-SPE surface displays the smallest semicircle. This behaviour was expected since at the bare carbon electrode the electron-transfer process is very fast and the electrochemical process is limited by diffusion (low $R_{ct}$). After the electrode surface was coated by the thin poly(CAF) film containing the entrapped imprinted enzyme, a significant increase of diagram semicircle ($R_{ct}$) was observed since the redox probe access to the electrode surface was hindered. Preliminary studies were performed in order to optimize the template concentration for improved MIP formulation. Based on the calibration plots obtained for the different concentration tested (1, 10 and 100 ng $mL^{-1}$), a GOD concentration of 1 ng $mL^{-1}$ was elected for the imprinting process due to the improved response sensitivity and linearity of the biosensor.

After protein removal, there is a significant decrease of the semicircle relatively to the obtained after polymerization. However, it still remains significantly larger than that obtained at the bare C-SPE surface due to the polymer structure still existing at the electrode surface. The recovery of some electron transfer performance proves that the extraction of the enzyme leaves empty binding sites in the polymeric film facilitating the access of the electrochemical probe to the electrode surface.

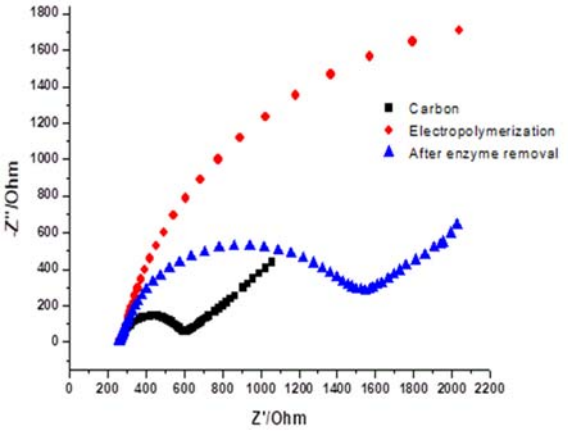



**Fig. 3.** Representative EIS diagrams of MIP film preparation, obtained in the presence of 5.0 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$, (black) before and (red) after electropolymerization of CAF, in the presence of 1 ng $mL^{-1}$ of GOD, at the C-SPE surface, followed by (blue) biomolecule extraction.

AFM was also used to investigate the morphology of the electrode surface after surface modification. The obtained AFM images are shown in Fig. 4. The bare C-SPE surface (Fig. 4, top), displays the surface roughness typical of the carbon ink films used in the fabrication of C-SPEs with a root mean square (RMS) surface roughness of 680 nm. After electropolymerization, the MIP film appear denser, compact and containing larger agglomerates (probably corresponding to GOD). The RMS value decreased to 174 nm (Fig. 4, middle) which can be interpreted by the clogging of the electrode imperfections by the formation of the polymeric network at the electrode surface. When the enzyme is removed, the RMS surface roughness increased to 579 nm (Fig. 4, bottom), indicating that GOD removal from the polymeric network induced an increase in film roughness.

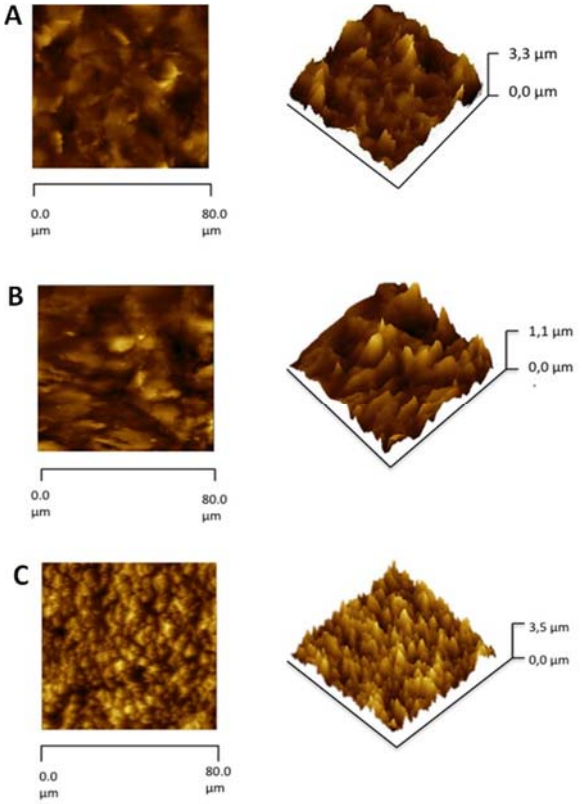



**Fig. 4.** AFM images in 2D and 3D corresponding to the different modification steps occurring at the electrode surface: A) clean C-SPE surface, B) electrode surface after electropolymerization of CAF in the presence of GOD and C) electrode surface after enzyme removal from the polymer.

### 3.4. Analytical Performance of MIP Biosensor

After optimization of experimental conditions, rebinding studies were performed by incubating the MIP sensor surface with standard solutions of GOD (concentration range between $1\times10^{-11}$ to $1\times10^{-3}$ g $L^{-1}$) for 30 min, following EIS measurement in the presence $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox pair. The calibration curve obtained is shown in Fig. 5. As can be seen in the figure, the increase of GOD concentration leads to an increase of the charge transfer resistance due to the systematic occupation of the imprinted protein. Two linear response ranges were obtained. Concerning the working response range, a linear pattern of $R_{ct}$ against GOD concentration logarithm was observed for concentrations ranging from $1\times10^{-9}$ to $1\times10^{-3}$ g $L^{-1}$, with a correlation coefficient of 0.9932. The LOD obtained was 1.0×10-10 g L-1 and was estimated according to Recommendations of IUPAC for ion-selective electrodes [21].

The large number of functional groups of proteins greatly increases the possibility of non-specific interactions with the MIP film, which can decrease the selectivity of the binding process. Thus, rebinding experiments were also performed at the NIP surface. The NIP sensor displayed a random and inconsistent response over the concentration range tested, indicating that specific interactions occurs between the enzyme and MIP polymer film.

### 3.5. Study of Selectivity

A selectivity study was carried out at the MIP platform aiming to evaluate the performance of the biosensor in the presence of co-existing species in real samples. Thus, myoglobin, amylase and glucose were chosen as interfering species for the studies.

EIS measurements were performed after incubation of the MIP film surface with the pure interferent solutions at different concentration levels. Results obtained show no significant changes in charge transfer resistance for the three different interferents tested, suggesting that the prepared MIP surface has high specificity against GOD.

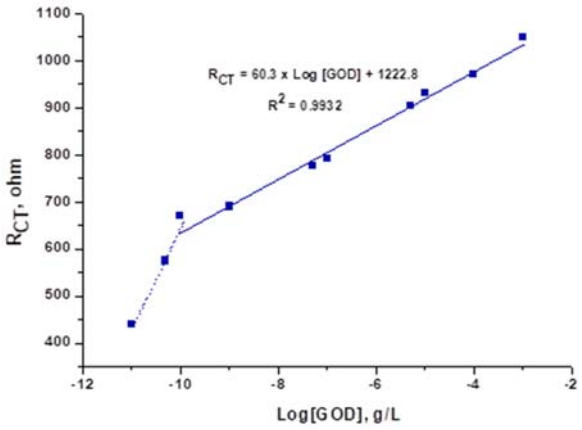



**Fig. 5.** Calibration curve obtained at the MIP film surface, by performing EIS measurements in the presence of 5 mM $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ redox couple.

### 3.6. Analytical Application Feasibility

Recovery is a very important parameter to access the accuracy of (bio)sensing devices. Recovery studies were performed in buffer solution and commercial orange juice spiked with known amounts of GOD. The concentration values found, calculated from the standard calibration curve obtained in the samples tested.

For the determination of GOD in buffer solution, a good agreement was found between added and found amounts of GOD. Overall, recoveries ranged from 87.9 to 117.6 % with an average relative error of 10.5 % (relative errors between -13.7 to +15.0 %) suggesting a good response of the developed biosensor in buffer solution.

Relatively to the experiments performed in juice, the relation between the concentration found and the concentration added suggests that the juice matrix have some affect on the biosensor performance (errors

affecting the concentration found ranges from -23.0 to +23.1 %). However, these error levels were considered to be acceptable due to the very large working concentration range of the MIP biosensor when compared to the slow variation of the electroanalytical response.

## 4. Conclusions

In this work, the design and synthesis of artificial receptors at the carbon sensor surface was able to recognise GOD in order to collect valuable proteomic results in food industry context.

A simple, efficient and cost-effective strategy to develop the electrochemical devices is reported. MIP receptor films were created by electropolymerization of CAF, in the presence of GOD, at the C-SPE surface, followed by enzyme wash-out from the polymeric matrix in order to create the specific binding sites. The simplicity of MIP preparation procedures shows unequivocally that this approach can be easily implemented to develop biosensors for other relevant enzymes. In fact, this strategy was applied by our research group for the development of a new electrochemical device for detection of stress biomarker α-amylase in point-of-care.

The evaluation of MIP receptor film performance was achieved by EIS measurements in the presence of the well-characterized ferricyanide/ferrocyanide reporting system. The prepared MIP biosensor exhibited ability for protein rebinding through specific interactions. A very wide working concentration range was obtained, between $1\times10^{-9}$ to $1\times10^{-3}$ g $L^{-1}$, allowing the easy application of the MIP biosensor as valuable tool for on-site screening of GOD in food products. Acceptable results were obtained for detection of GOD in orange juice samples.

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