

## SELF-ASSEMBLED MULTILAYERS OF WATER GLUCOSE MODIFIED-CHITOSAN AND GLUCOSE OXIDASE FOR DETECTION OF GLUCOSE IN MILK SAMPLES

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### ABSTRACT

**Background:** A crucial aspect of electrochemical enzymatic biosensor development is the immobilization of the enzymes, as it directly influences the sensitivity of the bioelectrode. Among the different methods used to incorporate enzymes on the surface of the transducers, layer-by-layer (LbL) self-assembly based on electrostatic interaction with polyelectrolytes of opposite charge stands out due to its simplicity and reproducibility. **Aims:** The aim of the work was to develop an electrochemical glucose biosensor by LbL assembly of a new functionalized chitosan polycation and the enzyme glucose oxidase (GOx). **Methods:** Chitosan was chemically functionalized with glucose by the Maillard reaction. The resulting polycation, named G-Chit, is soluble in the medium compatible with the enzyme. The bioelectrode was obtained by alternating adsorption of G-Chit and GOx onto carbon paste electrodes. By selecting the number of bilayer of G-Chit/GOx, the enzyme concentration, and the pH, the electroanalytical performance of the biosensor was optimized. The electrochemical responses were characterized by cyclic voltammetry and chronoamperometry. **Results:** Under optimized experimental conditions, the biosensor exhibited a sensitivity of  $(0.81 \pm 0.03) \mu\text{A mM}^{-1}$  in a glucose concentration range of (0.18 to 1.75) mM. **Discussion:** Results indicated that catalytic response increases both with the number of G-Chit/GOx bilayers and the enzyme concentration, obtaining the best responses for 3 bilayers and  $2 \text{ mg mL}^{-1}$ , respectively, while the optimum working pH value was 7.0. **Conclusions:** The analytical response of the biosensor was tested in milk samples with negligible matrix effects, suggesting a potential application in other dairy products. Results show that G-Chit appears promising for the immobilization of enzymes.

**Keywords:** *Glucose biosensor, glucose-functionalized chitosan, carbon paste electrode.*

### 1. INTRODUCTION

Glucose quantification is of significant importance in the chemical and biological industries, in clinical analysis, as well as in food processing and fermentation (Juska and Pemble, 2020; Yusan *et al.*, 2018). In the food industry, the glucose content is associated with storage time, fermentation process, and final quality control (Paz Zanini *et al.* 2016). For those reasons, accurate glucose quantification with a quick and simple method has driven the search for new

testing devices and procedures. Various methods have been reported for GOx immobilization, such as covalent cross-linking, electrochemical polymerization, sol-gel encapsulating, and layer-by-layer (LbL) self-assembly based on the electrostatic interaction with polyelectrolytes of opposite charge (Bracamonte *et al.* 2014; Ma *et al.* 2022). This last method has attracted great interest due to its simplicity, wide variety of materials that can be used, and the precise control of the composition and thickness of the layer on the molecular level. Among other polymers, the

natural polycation chitosan (Chit), a copolymer composed of randomly distributed units of N-acetyl-D-glucosamine and D-glucosamine linked by  $\beta(1\rightarrow4)$  bonds, has been employed to immobilize enzymes (Petrucci *et al.* 2021) The biocompatibility, biodegradability, non-toxicity, and bioactivity of Chit, associated with desirable physical and mechanical properties have made this polymer interesting and promising in biosensors development. However, Chit applications are limited because of their poor solubility at neutral or basic pH. Therefore, several studies have been conducted to obtain Chit derivatives soluble in an aqueous medium at physiologic pH (Rocha Neto *et al.*, 2022). One of them involves the Maillard reaction (MR) between the amino group of Chit and the carbonyl group of reducing sugar (Arata Badano *et al.*, 2019; Hafsa *et al.*, 2021; Yang *et al.*, 2020).

In this work, we used Chit modified with glucose residues (G-Chit) synthesized through MR reaction to immobilize GOx by the LBL self-assembly technique at carbon paste electrodes (CPE). This material has advantages in its low fabrication cost, renewable or disposable electrochemical interface, and low background current (Donmez *et al.* 2017). The electrochemical properties of the bioelectrode have been characterized by chronoamperometry (CA) and cyclic voltammetry (CV). Furthermore, the electroanalytical performance of the biosensor was evaluated for the determination of glucose in standard solutions and milk samples. The presented results confirmed the suitability of this supramolecular assembled bioelectrode for the fast quantification of glucose.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Glucose Oxidase (GOx, EC 1.1.3.4 from *Aspergillus Niger* type II) lyophilized powder containing 17300 units  $g^{-1}$  solid. Reagent grade D-(+)-glucose and ferrocene methanol (FcMe) were purchased from Sigma-Aldrich SA (Buenos Aires, Argentina). Reagent-grade buffer phosphate potassium salts  $KH_2PO_4$  and  $K_2HPO_4$  were obtained from Cicarelli. Medium Mw Ch (583 kD) was from Sigma-Aldrich (MO, USA). Analytical grade acetic acid ( $CH_3COOH$ ) was purchased from Cicarelli (Buenos Aires, Argentina). Solutions were prepared with ultra-pure water.

### 2.2 Modification of Chitosan with glucose by Maillard Reaction

With some modifications, chitosan modification with glucose was performed following the method proposed by Abdelaal *et al.* (Abdelaal, Sobahi, and Al-Shareef 2013). Briefly, 1.5 g of native Chit were dissolved in 100 mL of 1% (V/V)  $CH_3COOH$  and then incubated at 35 °C for 4 h, respectively. The reaction medium was dialyzed with Mw cutoff 12-14 kD membrane (Sigma-Aldrich). G-Chit was spray dried with Mini Spray Dryer B-290 (BÜCHI Labortechnik AG, Switzerland).

Static Light Scattering determined the molecular weight (Mw) of G-Chit according to the methodology proposed by (Arata Badano *et al.* 2019) using a Malvern light scattering photometer (Malvern 4700 with goniometer) with a laser at 488 nm. Deacetylation degree (DD) of G-Chit was determined by proton nuclear magnetic resonance ( $^1H$ -NMR) with a Bruker UltraShield 600 Plus under a static magnetic field of 14.1 T operating at 600 MHz and equipped with an AVANCE-III console. The G-Chit obtained have a degree of deacetylation of 74.7% and an Mw ( $49 \pm 9$ ) 10 kD and a solubility of ( $0.81 \pm 0.02$ )  $gL^{-1}$ , substantially higher than medium Mw Chit ( $0.22 \pm 0.02$ )  $gL^{-1}$ .

### 2.3 Preparation of composite multilayer films

Previous to preparing the composite multilayer film, the surface of the CPE was activated by applying an anodic potential of 1.200 V for 5 min in  $Na_2CO_3$  saturated solution. The LbL film was prepared by alternating immersions of the CPE in solutions containing G-Chit ( $0.5 \text{ mg}\cdot\text{mL}^{-1}$  prepared in deionized water, pH 5) or GOx ( $2 \text{ mg}\cdot\text{mL}^{-1}$  in PBS pH 7.0). The adsorption time was 30 min for both polyelectrolytes.

### 2.4 Electrochemical measurements

Cyclic Voltammetry (CV) and chronoamperometry (CA) studies were carried out with a potentiostat /galvanostat (Teq4, Buenos Aires, Argentina). Experiments were performed in a three-compartment electrochemical cell. A large-area platinum wire and an  $Ag/AgCl/3 \text{ M NaCl}$  electrode (Model RE-5B, BAS) were used as counter and reference electrodes, respectively. The reported potentials are referred to as this reference electrode. All experiments were performed at room temperature. A 0.10 M

phosphate buffer solution (PBS) at pH 7.0 was used as a background electrolyte, with  $2.0 \times 10^{-4}$  M FcMe as an artificial mediator of the enzymatic reaction. Solutions were deoxygenated by controlled  $N_2$ -bubbling for 20 min preceding the measurements, and the gas flow was kept over the solution during the experiments. CA experiments were performed at 0.350 V under convective conditions using controlled magnetic stirring. The current response was registered as a function of time after the sequential addition of glucose aliquots of chosen concentrations.

### 3. RESULTS AND DISCUSSION:

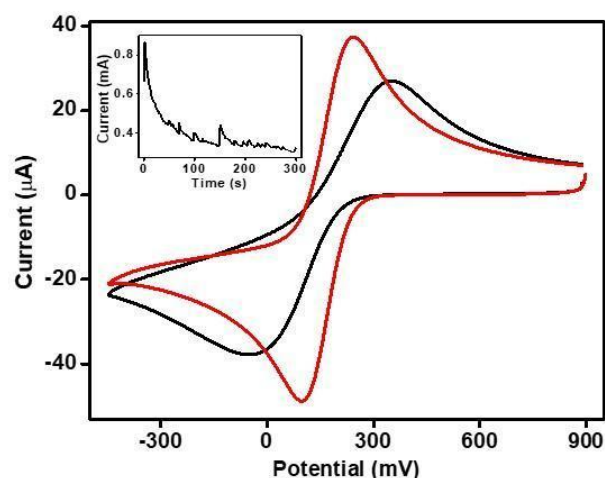
#### 3.1 Activation of the CPE surfaces and construction of the self-assembled structure

It is known that electrochemical activation techniques can be used to improve the electrochemical characteristics of electrodes based on carbon materials. During electrochemical activation, the amount of a binder at the electrode-solution surface interface decreases, which produces electrochemical signal increments (Chebotarev *et al.*, 2018). Furthermore, it has been proposed that activation is accompanied by the elimination of adsorbed impurities, an increase of active sites in the edge plane, and, in some cases, the generation of surface oxides (Motta and Guadalupe 1994). Therefore, CPE was activated in an alkaline medium to optimize the electrochemical response and obtain greater reproducibility. In addition, the electrochemical response of  $[Fe(CN)_6]^{3-}$  was used to analyze the effect of the treatment. This is a redox mediator commonly used in the electrochemical characterization of electrode surfaces since its electron transfer kinetics have been extensively studied (Goldstein and Van de Mark 1982).

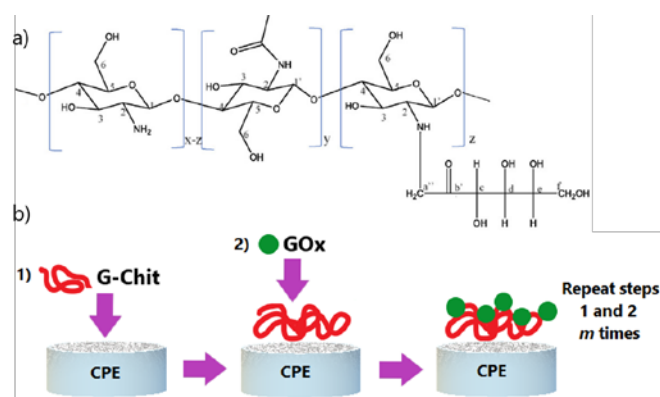
Figure 1 shows the cyclic voltammograms registered in  $[Fe(CN)_6]^{3-}$  before and after the activation procedure. For the unactivated electrode, a peak separation of 0.398 V is observed, which decreases to 0.148 V after activation treatment, demonstrating an improvement in the reversibility of the system. At the same time, a substantial increase in the peak anodic and cathodic currents are observed. These results agree with previous studies (Rice, Galus, and Adams 1983; Chi *et al.* 1997). They have been attributed to the decrease in surface hydrophobicity due to the treatment, which allows

the approximation of the ions to the surface of the electrode.

After optimizing the surface treatment, we proceeded to construct the self-assembled structure. Figure 2 shows the chemical structure of the polycation and the self-assembled procedure.



**Figure 1.** CV at CPE before (black line) and after (red line) activation procedure in 0.10 M PBS pH 7.0 containing  $5.0 \times 10^{-3}$  M  $[Fe(CN)_6]^{3-}$ . Scan rate  $100 \text{ mV}\cdot\text{s}^{-1}$ . Inset: the curve of current vs. time corresponding to the activation procedure in  $Na_2CO_3$  saturated solution.



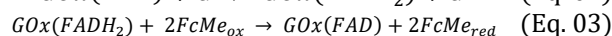
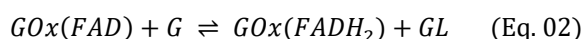
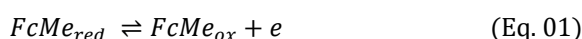
**Figure 2.** a) G-Chit structure and b) Construction of self-assembled structure at activated carbon paste electrodes.

To avoid their denaturation, the construction of multilayers that include enzymes and proteins preferably requires mild conditions such as neutral pH and room temperature. In this context, the improved water solubility of G-Chit, about Chit, makes it possible to dissolve the former in water, in the required quantity (0.5

mg·mL<sup>-1</sup>), at a difference of Chit that requires strong acid conditions (pH < pKa ~ 5.0. In an aqueous solution, the polymer has a zeta potential of (89±7) mV that allows the construction of the multilayer structure with GOx negatively charged at the pH of the assembly (the isoelectric point of the enzyme is 4.2).

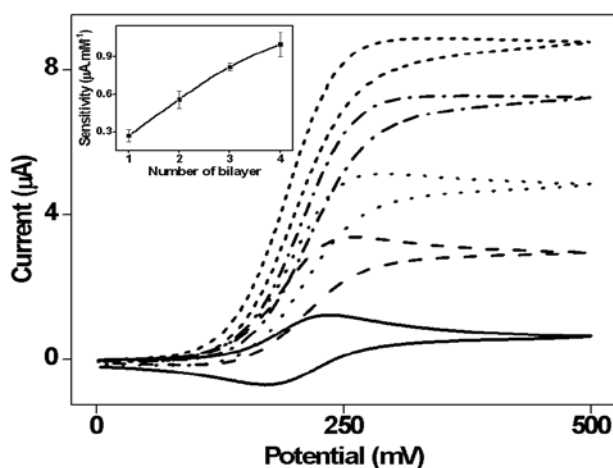
### 3.2 Evaluation of the enzymatic response

The enzymatic response at CPE-modified electrodes was analyzed by CV. Figure 3 shows cyclic voltammograms of CPE/(G-Chit/GOx)<sub>m</sub>, where m corresponds to the number of bilayers (m=1,2,3,4) in an N<sub>2</sub>-saturated solution. In the absence of glucose, reversible redox waves of FcMe are observed. After the addition of 0.050 M glucose, a well-defined sigmoidal voltammetric profile is noticed due to the GOx catalytic reaction at the bioelectrode, according to Equations 1 to 3.



G = Glucose; GL = Gluconolactone

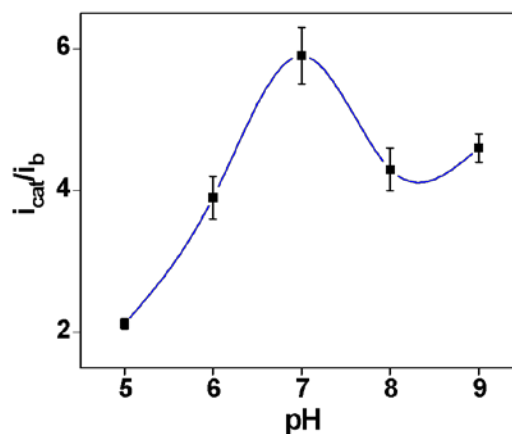
This result suggested that the enzyme has successfully assembled on the electrode surface and remains active. Moreover, the catalytic current, *i*<sub>cat</sub>, increases proportionally with the number of G-Chit/GOx bilayers in the range evaluated up to m=4.



**Figure 3.** CV at CPE/(G-Chit-GOx)<sub>m</sub> in 0.10 M phosphate buffer solution pH 7.0 + 2.0 × 10<sup>-4</sup> M FcMe in absence (solid line) and presence of 0.050 M of glucose. Scan rate 5 mV·s<sup>-1</sup>. Inset: Variation of sensitivity as a function of bilayer number.

The influence of enzyme concentration used in the construction of CPE/(G-Chit-GOx)<sub>3</sub> was evaluated by analyzing the sensitivity obtained in CA experiments from the fit of the linear range in the calibration plot. The values obtained are (0.44 ± 0.06), (0.82 ± 0.03), and (0.8 ± 0.1) µA·mM<sup>-1</sup> for 1, 2, and 5 mg·mL<sup>-1</sup> of the enzyme, respectively. The sensitivity value duplicates when the enzyme concentration increases from 1 to 2 mg mL<sup>-1</sup> and then remains unchanged, indicating that the maximal amount of enzyme adsorbed is reached under this condition.

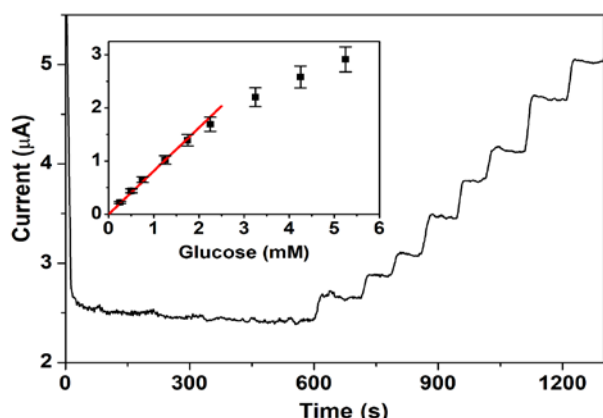
The ionization state of the amino acids in the active site can affect enzyme activity (Grahame *et al.*, 2015). Thus, pH has a significant function in maintaining the proper conformation of the active site in the enzyme. The pH effect on the biosensor response was evaluated by CV in the range from 5.0 to 9.0, adding 0.050 M glucose to the cell. The ratio between the *i*<sub>cat</sub>, and the current in the absence of glucose, *i*<sub>b</sub>, were calculated. The maximum response, *i*<sub>cat</sub>/*i*<sub>b</sub>, was obtained at pH 7.0 (Figure 4); hence, it was chosen as the optimum pH for further studies. The results are an average of five measurements.



**Figure 4.** Effect of pH on the response of the CPE/(G-Chit-GOx)<sub>1</sub> at 0.486 V in 0.10 M phosphate buffer at different pH + 2 × 10<sup>-4</sup> M FcMe and 0.050 M of glucose.

The electroanalytical properties of the bioelectrode immediately after preparation for the optimized experimental conditions: m=3, GOx 2 mg·mL<sup>-1</sup>, and pH 7.0 were analyzed. Figure 5 shows the current-time recorded and the inset corresponding calibration plot. The detection limit (LOD) and LOQ were calculated using the criterion of 3 × SD/s or 10 × SD/s, respectively, where SD is the standard deviation of the background current and s the sensitivity (Paz Zanini *et al.*

2016). The linear range of operation is (0.18-1.55) mM, while the values obtained for LOD, LOQ, and sensitivity obtained are 60  $\mu\text{M}$ , 180  $\mu\text{M}$ , and (0.82  $\pm$  0.03)  $\mu\text{A}\cdot\text{mM}^{-1}$ , respectively.



**Figure 5.** The current vs time plot and calibration curve were obtained at CPE/(G-Chit-GOx)<sub>3</sub> at 0.486 V in 0.10 M phosphate buffer plus 2.0 x 10<sup>-4</sup> M FcMe.

There is little information reported in the literature about multilayer systems built on CPE that include GOx so, it is very difficult to compare the analytical parameters obtained with those of other biosensors of similar composition.

Therefore, and to highlight the analytical characteristics of the developed biosensor, we compare it with others built in CPE, which use enzymatic mediators derived from ferrocene. In this context, we find that the sensitivity obtained is similar to that of a CPE modified with functional GOx/silica/lignin hybrid material, 0.78  $\mu\text{A}\cdot\text{mM}^{-1}$  (Jędrzak *et al.* 2018), and significantly higher than that of a Cellulose Acetate/GOx modified CPE, 0.290  $\mu\text{A}\cdot\text{mM}^{-1}$  (Sanjaya *et al.* 2021).

The bioelectrode was tested in real samples of commercial milk La Serenisima®. The obtained value for the seven consecutive determinations was (2.2  $\pm$  0.2) g·L<sup>-1</sup>. According to a t-test at 95% confidence level, these values agree with the manufacturer-reported value, e.g., 2 g·L<sup>-1</sup>. In addition, to check possible matrix effects, the percentage of relative recovery (RA%) of the current signal produced by an aliquot of 0.25 mmol L<sup>-1</sup> glucose before and after adding 10  $\mu\text{L}$  of the sample was measured and calculated by Equation 4.

$$RA(\%) = \frac{i_2}{i_1} \times 100\% \quad (\text{Eq. 04})$$

Where  $i_1$  and  $i_2$  are the amperometric signals

before and after adding the sample. (Burns, Danzer, and Townshend 2003). The sample volume used produced %RA values ranging 62-85%.

## 4. CONCLUSIONS:

Multilayer films made of G-Chit and GOx were successfully built onto CPE. The best responses were obtained with 2 mg·mL<sup>-1</sup> of enzyme, 3 bilayers of polyelectrolytes, and working at pH 7.0. Under optimized experimental conditions, the biosensor exhibits a sensitivity of (0.81  $\pm$  0.03)  $\mu\text{A}\cdot\text{mM}^{-1}$  and a linear range of (0.18 to 1.75) mmol·L<sup>-1</sup>.

Although obtaining high sensitivity values is one of the most desired characteristics in developing a biosensor, it is also important to achieve devices that involve a simple preparation procedure. In our case, we can claim that the developed biosensor combines both characteristics as a consequence of the ease and economy in the construction of a CPE and the versatility of the layer-by-layer assembly.

The analytical response of the biosensor was tested in milk samples with negligible matrix effects. Results show that G-Chit appears promising for the immobilization of enzymes.

## 5. DECLARATIONS

### 5.1. Study Limitations

The study is limited to the samples analyzed.

### 5.2. Acknowledgements

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#### 5.4. Competing Interests

The authors declare that they don't have any conflict of interest that exists in this publication.

#### 5.5. Open Access

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