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Real-time measurement of glucose using chrono-impedance technique on a second generation biosensor

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ABSTRACT

Chrono-impedance technique (CIT) was implemented as a new transduction method for real time measurement of glucose in a biosensor system based in carbon paste (CP)/Ferrocene (FC)/glucose oxidase (GOx). The system presents high selectivity because the optimal stimulation signal composed by a 165 mV DC potential and 50 mV_{RMS} AC signal at 0.4 Hz was used. The low DC potential used decreased the interfering species effect and the biosensor showed a linear impedance response toward glucose detection at concentrations from 0 mM to 20 mM,with 0.9853 and 0.9945 correlation coefficient for impedance module ($|Z|$) and phase (Φ), respectively. The results of quadruplicate sets reveal the high repeatability and reproducibility of the measurements with a relative standard deviation (RSD) less than 10%. CIT presented good accuracy (within 10% of the actual value) and precision did not exceed 15% of RSD for high concentration values and 20% for the low concentration ones. In addition, a high correlation coefficient ($R^2 = 0.9954$) between chrono-impedance and colorimetric methods was obtained. On the other hand, when two samples prepared at the same conditions were measured in parallel with both methods (the measurement was repeated four times), it should be noticed that student's *t*-test produced no difference between the two mentioned methods ($p = 1$). The biosensor system hereby presented is highly specific to glucose detection and shows a better linear range than the one reported on the previous article.

1. Introduction

Enzyme-based biosensors constitute nowadays a promising technology on several research fields, such as chemical and biomedical analysis, pollution monitoring, biotechnology, and food and agricultural product processing (Qiu et al., 2009). Amperometric enzymatic biosensors have been considered to be the most suitable ones for biochemical analysis due to their good selectivity, sensitivity, rapid response, miniaturized size, and reproducible results (Qiu et al., 2009). However, low stability and a relative low current density are its main disadvantages. Thus, this method is highly sensitive to power noise (Mayorga Martinez et al., 2010).

Chrono-impedance technique as a transduction method applied for the continuous measurement of glucose in a first generation Carbon Paste/Glucose oxidase (CP/GOx) biosensor was recently reported (Mayorga Martinez et al., 2010). This new method makes possible the measurement of glucose by using robust and low cost test systems, with a good repeatability, accuracy and precision. It presents a good correlation coefficient ($r \approx 1$) compared to the colorimetric enzymatic method commonly used to determine glucose concentration. The method is based on the application of a signal composed by a DC potential and an AC signal at constant frequency to the system (Mayorga Martinez et al., 2010).

Second generation biosensors are a modification of CP/GOx ones (Wang, 2001; Newman and Turner, 2005; Dong et al., 1992). These biosensors have an artificial mediator, besides the enzyme and the carbon paste, which shuttles electrons between the FAD center and the surface. Such mediation cycle produces a current dependence on the glucose concentration. Diffusional electron mediators, such as ferrocene derivatives, ferricyanide, conducting active organic salts, phenothiazine and phenoxazine compounds, or quinone compounds have thus been widely used to electrically contact GOx (Wang, 2008; Zhao et al., 2006; Salimi et al., 2007). By using these electro carrying mediator, measurement become largely independent of oxygen partial pressure and it can be carried out at lower potentials that prevent interfering reactions from coexisting electroactive species (dopamine, ascorbic acid, uric acid,

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etc.) (Wang, 2008). In order to effectively functionalize the system, the mediator should react rapidly with the reduced enzyme as to minimize competition with oxygen, and should present good electrochemical properties such as a low redox potential, in addition to low solubility in aqueous medium and being a non-toxic and chemically stable specie (in both reduced and oxidized forms) (Wang, 2008). The use of mediators decreases the enzyme progressive degeneration due to the application of high potentials (Bardea et al., 1999).

Second generation biosensors, composed of GOx and a derivative of ferrocene (Fc) in carbon paste electrodes, have been widely reported in the literature but by using the amperometric technique as transduction method (Razumiene et al., 2003; Gorton, 1995). The implementation and evaluation of CIT in this second generation biosensor and the corresponding determination of the optimal stimulation signal (AC and DC potentials and frequency) are presented. In addition accuracy, precision, repeatability, linearity, correlation with a reference method and interferences evaluation are also presented.

2. Materials and methods

Electrochemical measurements were performed with a Solartron 12508W impedance analyzer composed of a Solartron 1287 Electrochemical Analyzer and a Solartron 1250 Frequency Response Analyzer, commanded by the software provided by the manufacturer (ZPlot®, Scribner Associates Incorporated). A magnetic stirrer was used to provide convective transport during the impedance measurements. Data analysis and curve fitting was performed with Matlab® while statistical computations were carried out by means of GraphPad Prism version 3.00 Software.

A tripolar cell was used in these impedance measurements, consisting of a working electrode (biosensor), a Ag/AgCl reference electrode and an AISI 304 stainless steel concave counter-electrode, 85 mm in diameter. Details of the mentioned homemade cell have been previously reported (Mayorga Martinez et al., 2010).

2.1. Reagents and solutions

Carbon graphite powder, glucose oxidase from Aspergillus Níger type VII (100 units/g) and Ferrocene were purchased from Sigma Aldrich. Paraffin oil was locally obtained from Palmares laboratories. D-Glucose anhydrous, potassium dihydrogen phosphate, sodium monohydrogen phosphate dehydrate and potassium chloride were purchased from Cicarelli laboratories. Enzymatic glucose kit from Wiener Lab® for colorimetric determination was used as reference method. All electrochemical experiments were carried out at room temperature (22 °C) in KH_2PO_4/Na_2HPO_4 0.07 M buffer solution pH7 (PBS) with 0.1 M KCl. All solutions were prepared with single distilled water with conductivity less than 5 μS/m.

2.2. Biosensor preparation

The biosensor was prepared by thoroughly mixing carbon graphite powder, paraffin oil, glucose oxidase and ferrocene (171 mg:71 mg:5 mg:4 mg) in an Agate mortar. A portion of the resulting paste was placed into an acrylic tube (inner diameter 8 mm and 2 mm height) in order to obtain the corresponding biosensor. Electrical contact to the paste was established by inserting a 24-carat gold disc into the plastic tube over the mixture. The front side of the CP electrode, which is exposed to the buffer-glucose mixture, was gently polished with vegetal paper and stored at 4 °C.

2.3. Electrochemical Impedance Spectroscopy (EIS) evaluation of the biosensor

Electrochemical Impedance Spectroscopy (EIS) is used to evaluate the performance of the biosensor. This technique implies the application of an alternate current (AC) rather than a continuous one (DC). To fulfill these needs, a signal composed of both (a DC and AC) was used to polarize the interface. The AC frequency range chosen was from 0.1 Hz to 65 kHz, logarithmic scale with ten points per decade. The measurement was repeated for several glucose concentrations in the 0–40 mM range.

3. Results

In order to determine the working DC potential of the CP/Fc/GOx biosensor, cyclic voltammograms (CV) experiments were carried out for different glucose concentrations. Fig. S1 shows a set of CV recorded at 20 mV/s in a PBS with 0.1 M KCl in absence and presence of different glucose concentrations. It can be noticed that glucose oxidation started at 0 mV and resulted to an ill-defined broad peak around 250 mV (vs. Ag/AgCl). In order to determine the optimum working potentials, measurements were carried out at different DC potentials within this range (0–250 mV), observing an optimal working DC potential of 165 mV.

EIS was used to evaluate the biocatalytic activity of the CP/Fc/GOx biosensor toward glucose oxidation. An AC potential in the frequency range between 0.1 Hz and 100 kHz and 50 mV amplitude is applied, since these conditions show the larger changes on the electrode-electrolyte interface impedance (EEIZ), caused by the increase of glucose concentration. Argand diagram of the biosensor in PBS with 0.1 M KCl in absence, and for different glucose concentrations is presented in Fig. S2 A.

In order to determine the working frequency, the percentage normalized modulus (PNM), defined in our previous work, was calculated by using equation 1. The measured modules without (Z_{0mM}) and with 40 mM of glucose (Z_{40mM}) in the range of 0.1–100 Hz are used for the calculation. PNM indicates the rate of impedance variation per mole of glucose added to the system (Fig. S2 B), showing a maximum value of 1.33%/mM, measured at 0.1 Hz. The impedance module was lower after glucose addition. As frequency increases, PNM continuously decreases down to approximately 0.08%/mM for frequencies higher than 1 Hz. The best measurement frequency is then, in the range of high PNM values.

$$\mathrm{PMN} = \frac{Z_{0mM} - Z_{40mM}}{Z_{0mM}} \times \frac{1}{40\,\mathrm{mM}} \times 100 \quad (1)$$

Real time determination of impedance changes ($|Z|$ and Φ in PBS during successive addition of glucose 2 mM) was carried out by using a composed signal of 165 mV DC plus a 50 mV_{RMS} AC of 0.4 Hz. This frequency was selected, because the impedance response obtained at low frequency reflects the maximum impedance variation that is the maximum electrocatalytic oxidation activity of the biosensor upon addition of glucose to the buffer solution (Fig. S2 A). Other experiments were performed at lower frequencies (data not shown), where the system shows a higher PNM, but the noise and slow response obtained impeded the real time glucose measurement.

Control measurements were also carried out in order to verify if the impedance variation in real time was due to the bioelectrocatalysis of glucose. Therefore, two measurements were performed under different experimental conditions, which are shown in Fig. 1. The first one was carried out on the same biosensor with successive addition of PBS with 0.1 M KCl without glucose. In addition, CP/Fc sensor without GOx was tested upon successive addition of glucose 2 mM. Both measurement resulted in either negligible or

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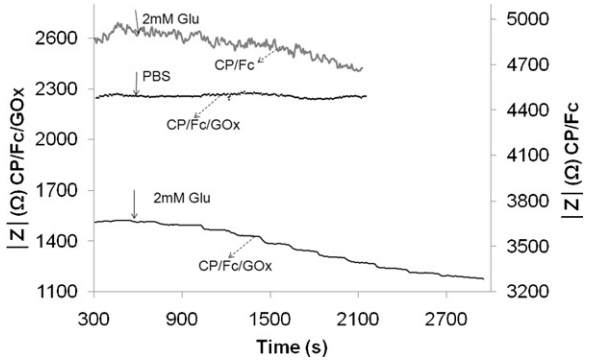

Fig. 1. On-line response of the biosensor and controls: (—) CP/Fc/GOx biosensor; (▬▬) CP/Fc without GOx both with the addition of glucose; and (…) biosensor with the addition of support electrolyte (PBS) without glucose.

no change on impedance signal, supporting the glucose bioelectrocatalytic origin of the impedance response.

Calibration curves are shown in Fig. 2 for impedance magnitude ($|Z|$) and in Fig. S3 A for phase (Φ). They are based on the average of $|Z|$ and Φ values obtained for each glucose addition as a function of glucose concentration. The obtained impedance response show a linear response with glucose concentrations up to 20 mM, with a 0.9853 and 0.9945 correlation coefficient for $|Z|$ and Φ respectively.

In order to compare the repeatability of 4 biosensors, the normalized impedance value relative to zero concentration of glucose is studied. The results of quadruplicate sets, indicated by error bars, reveal the repeatability and reproducibility (inset of Fig. 2 for $|Z|$ and Fig. S3 B for Φ) of the measurements with a relative standard deviation (RSD) less than 5% and 10% for low (2–10 mM) and high (10–20 mM) glucose concentration range, respectively.

Two samples prepared at the same conditions were measured in parallel with the chrono-impedance method and colorimetric one, used as reference method (RM). Measurement was repeated four times and Student's *t*-test presents no difference between the two methods, with a confidence interval of 0.99 ($p = 1$). Fig. S4 shows the correlation between the chrono-impedance and colorimetric methods, the latter used as reference. A high correlation coefficient was obtained ($R^2 = 0.9954$).

Method's accuracy is determined by evaluating the closeness of mean test results obtained by the chrono-impedance technique to the true value obtained by the reference one. Three concentrations were determined in the linear glucose concentration range (0–10 mM) with five measurements each one. The mean value of each measured concentration is within 10% of the values obtained with the reference method.

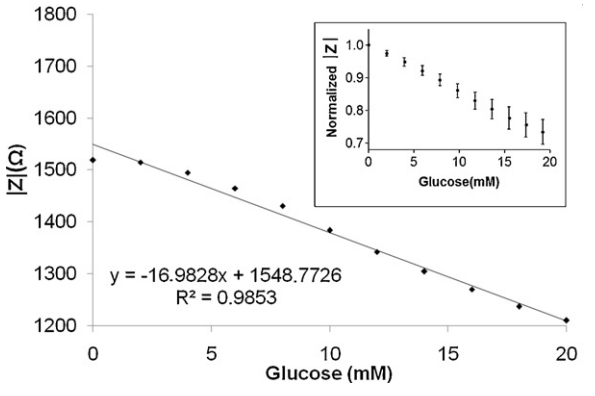

Fig. 2. Biosensor calibration. Impedance Module as a function of a glucose concentration range of 0–20 mM. Normalized impedance module vs. glucose concentration (inset).

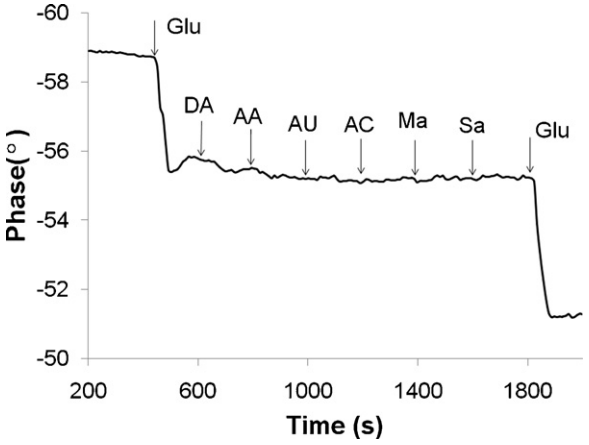

Fig. 3. CP/GOx/Fc biosensor response upon successive addition of 5 mM of glucose, 0.2 mM of DA, 0.2 mM AA, 0.2 mM AU, 1 mM AC, 11 mM Ma and 5 mM Sa.

Precision was evaluated by using three concentrations within the linear range (0–10 mM), with five measurements per each concentration. The RSD obtained from high to low concentrations were 11.97%, 14.65% and 16.71%, respectively.

Fig. 3 shows the electrochemical response toward several interfering species: dopamine (DA), ascorbic acid (AA) and uric acid (UA). This study is of special importance; as it considers commonly interfering species in human blood. The concentration of these interfering species is considered at least 30 times lower than glucose concentration in human blood (Jiang and Zhang, 2010; Lee and Park, 2010). On the other hand, this biosensor can be used for industrial process control. In this case the interfering species considered will be citric acid CA (preservative) and Mannitol Ma (sweetener), chemicals commonly used in food and pharmaceutical industries. The experiments for interference effect evaluation were carried out by successive injection of glucose 5 mM, Saccharose (Sa) 5 mM and interfering species (DA 0.2 mM, AA 0.2 mM, UA 0.2 mM, AC 1 mM, Ma 11 mM). It can be concluded that small amount of DA, AA, and other compounds (such as UA, CA), Ma and Sa can be neglected.

4. Discussion

Amperometric measurements in second generation biosensors are generally performed at 205–400 mV AC vs. Ag/AgCl reference electrode (Alegret et al., 2004; Qiu et al., 2007; Qiua et al., 2008; Razumiene et al., 2003; Chaubey and Malhotra, 2002). In the present paper, a DC voltage of 165 mV and 50 mV AC is used for EIS, as to avoid exceeding the optimum DC voltage (205 mV). In first generation biosensors, the AC voltage amplitude of 50 mV is not significant compared to the 900 mV DC, but for these second generation biosensors, 50 mV could lead to overcoming the optimum working potential. This lower DC potential also prevents the occurrence of other reactions (usually of interfering for glucose measurement) that occur at higher potentials, thus reducing its influence. In addition, the DC potential used in this second generation biosensor is lower than the potential reported in the bibliography (Alegret et al., 2004; Qiu et al., 2007; Qiua et al., 2008; Razumiene et al., 2003; Chaubey and Malhotra, 2002). As for first generation biosensors, the optimum working frequency is in the low frequency range (0.4 Hz), as it can be calculated from EIS measurements and the percentage normalized modulus (Fig. S2 A and B).

In our specific case the optimum measurement range is up to 20 mM, which is the concentration range usually used for glucose quantification in blood (in diabetic patients the range is between

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4 and 8 mM). On the other hand, the linear range obtained for this second generation biosensor is higher to the previously reported one (Mayorga Martinez et al., 2010). In addition, low potential used in the experiments probably avoid enzyme degradation. The low DC potential used and the evaluation of interfering species normally found in food and biological samples, indicates that the CP/Fc/GOx biosensor is highly specific to glucose. For this reason it is possible to use this system for glucose detection in real samples.

5. Conclusions

In this paper, we successfully demonstrated that chrono-impedance technique can be applied to sense glucose in a second-generation biosensor. In this case, the optimal signal which stimulates the system is composed by a 165 mV DC potential superimposed with 50 mV AC at a single frequency of 0.4 Hz. This biosensor uses a very low DC potential. This contributes to decrease the effect of the interferences and enzyme degradation, increasing at the same time, the linear range for glucose detection with respect to the first generation biosensor. In our specific case, transduction method presents high repeatability and a good correlation with the colorimetric method used as reference. Therefore, the developed biosensor is accurate and precise toward glucose detection.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2011.08.018.

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