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Evaluation of chrono-impedance technique as transduction method for a carbon paste/glucose oxidase (CP/GOx) based glucose biosensor

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ABSTRACT

The chrono-impedance technique (CIT) for real time determination of glucose concentration in a first generation glucose oxidase/carbon paste electrode was implemented. The biosensor was polarized with a signal composed of 900 mV DC potential and 50 mV_{RMS} AC signal at 0.4 Hz. A frequency response analyzer was used to measure the complex impedance (magnitude $|Z|$ and phase (Φ)) of the biosensor-bulk interface. Real time measurements were performed while glucose was added to the bulk within a concentration range of 0–40 mM. The cumulative impedance dose–response curves were used to construct calibration curves, both for magnitude and phase. The best fitting was obtained with a hyperbolic equation. Four biosensors were built obtaining five calibration curves for each of them. A single test measurement (unknown glucose concentration) was also obtained after each calibration procedure. Glucose concentrations were estimated with the calibration curves and also measured by colorimetry, the latter being the reference method. Besides, one-way ANOVA test evaluated repeatability. Difference between means was not statistically significant ($p > 0.01$) for both magnitudes ($|Z|$ and Φ). The Student's t-test assessed the differences significance, which produced in all cases p levels lower or equal than 0.44. Thus, CIT was proved to be a reliable method to measure glucose concentration in real time. Moreover, it showed high repeatability and compared well against colorimetry ($r^2 = 0.98$).

1. Introduction

Chrono-amperometry is probably the most popular transduction method applied in enzymatic biosensors for on-line measurement of analytes (Janata and Josowicz, 1998). Low stability and a relative low current density are its main disadvantages. Thus, this method is highly sensitive to power noise (Shervedani et al., 2006; Ghica and Brett, 2005; Zhang et al., 2005).

On the other hand, the method proposed by Yeh (2008) – for real time measurement of glucose concentration and average refractive index using a laser interferometer – is a highly precise optical metrology system for measuring the average refractive index of a liquid solution, from which glucose concentration can be derived. The metrology system employed can measure glucose concentrations ranging from 0 to 200 g/l (Yeh, 2008). However, this technology is expensive and difficult to implement.

Lately, Electrochemical Impedance Spectroscopy (EIS) has been proposed as a transduction principle for glucose biosensor (Shervedani et al., 2006; Li et al., 2007). This method is based on the evaluation of the charge transfer resistance (R_{ct}) as function of the glucose concentration (Shervedani et al., 2006; Ghica and Brett, 2005). R_{ct} is obtained from an elemental electrical circuit fitted to a set of impedance values, which have been measured over a frequency range. The advantage of this method is its low measurement error; nevertheless it requires multiple impedance measurement to calculate a single R_{ct} value. Consequently, a sampling period of several minutes is obtained (6.23 min for a typical R_{ct} measurement in the 0.1–1000 Hz range, 10 point by decade). This time is excessively long and the method is not suitable for on-line measurements (Shervedani et al., 2006).

As an alternative, measurement time can be reduced by decreasing the number of measurements used in the model fitting procedure. If these points are decreased to one half, the measurement time only decreases to its 44%. As can be seen, this sensing strategy increases the measurement error without a significant improvement in the temporal response of the biosensor. A better

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approach could be a single-frequency analysis and, thus, removing the fitting procedure. However, optimal frequency and voltage values of the system must be previously determined.

Herein, a new methodology for the real time impedance measurement in a first generation glucose (Glu) biosensors based on carbon paste/glucose oxidase (CP/GOx) is proposed. The GOx was chosen for being an easy handling enzyme. This enzyme is widely used in biosensors for the quantitative glucose determination in biological fluids, foods, drinks and in the fermentation industry (Salimi et al., 2007; Wang, 2001). The chosen immobilization method was immobilization in volume, known as biocomposites of CP. This material has a high chemical inertness and provides a wide range of anode working potentials with low electrical resistivity (Zhang et al., 2000). It has also a very pure crystal structure that provides low residual currents and a high signal-to-noise ratio (Zhang et al., 2000; Galán-Vidal et al., 1997). Carbon provides an adequate electric media to polarize the enzyme and also allows the measurement of the current through the biosensor.

In this paper, an impedancimetric single-frequency biosensor for the real time measurement of glucose is presented. The most important aspects and experimental conditions of the technique and sensing system are first determined. The optimal signal which stimulates the system is composed by a 900 mV DC potential superimposed with a 50 mV AC signal. This technique allows real time measurement of glucose concentration in a range of 0–40 mM. Three different fitting equations were tested, from which the hyperbolic one appeared as the most adequate for the calibration of impedance vs. glucose concentration.

2. Materials and methods

A tripolar cell composed of a CP/GOx working electrode, a Ag/AgCl reference electrode and an AISI 304 stainless steel concave counter-electrode, 85 mm in diameter, was used (Fig. 1).

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 peristaltic pump (Materflex®) was used to obtain air-saturated solutions. A magnetic stirrer provided convective transport during the measurements. Data analysis and curve fitting was performed with Matlab® while statistical computations were carried out by means of GraphPad Pism version 3.00 Software.

2.1. Reagents and solutions

Carbon graphite powder and glucose oxidase from *Aspergillus Níger* type VII (100 units/g) were purchased from Sigma–Aldrich. Paraffin oil was locally obtained from Palmares laboratories. D-Glucose anhydrous, potassium dihydrogen phosphate, sodium monohydrogen phosphate dehydrate, potassium chloride were purchased from Cicarelli. Enzymatic glucose kit from Wiener Lab® for colorimetric determination was used as the 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 with 0.1 M KCl. All solutions were prepared with single distilled water with conductivity smaller than 5 μS/m.

2.2. Electrode preparation

The CP/GOx was prepared by thoroughly mixing carbon graphite powder, paraffin oil and glucose oxidase (171 mg:71 mg:5 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 CP/GOx biosensors (Alegret et al., 2004). Electrical contact to the paste was established by inserting a 24-carat gold disc into the plastic tube over the mixture (Fig. 1). 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.

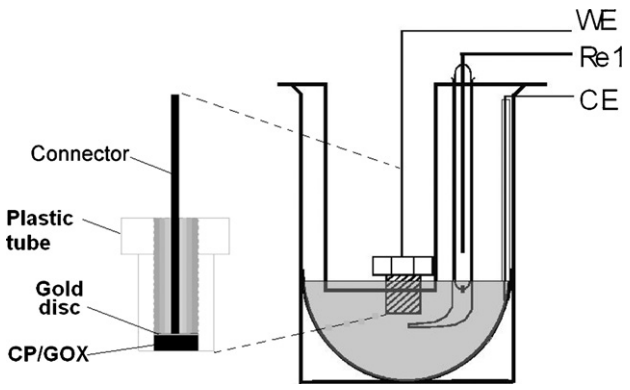

Fig. 1. Tripolar cell (WE: working electrode (biosensor); Re1: reference electrode; CE: counter-electrode). Al left the carbon paste (CP)/glucose oxidase (GOx) biosensor.

2.3. Electrochemical Impedance Spectroscopy evaluation of the biosensor

In order to assess the performance of the biosensor, Electrochemical Impedance Spectroscopy (EIS) was used. 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, DC and AC, was used to polarize the interface. The AC frequency range was 0.1 Hz to 65 kHz, logarithmic scale with 10 points per decade. The measurement was repeated for several glucose concentrations in the 0–40 mM range.

3. Results

The biocatalytic activity of the CP/GOx biosensor for oxidation of glucose was characterized by EIS. In order to find the optimum working potentials, measurements were carried out at different DC (900, 950 and 1000 mV) and AC potentials (50, 100 and 500 mV) with and without the addition of glucose. Changes of the electrode–electrolyte interface impedance (EEIZ), caused by the increase of glucose concentration, were evident at 50 mV alternating voltage superimposed on 900 mV DC. Fig. 2 shows the Argand driagrams of this biosensor in KH_2PO_4/Na_2HPO_4 0.07 M buffer solution pH7 with 0.1 M KCl in absence and for different glucose concentrations. These diagrams present a typical interface behavior. The bioelectrocatalysis of glucose is observed in the range of low frequency, where the diagrams show the greatest separation for different concentrations. It is possible to perform real time measurements of the bioelectrocatalysis of glucose by sensing impedance at certain frequency.

The R_{ct} was obtained by fitting the experimental data with software Zview® by using the modified Randle's model employed by Shervedani et al. (2006). When glucose was added to the buffer solution, a decrease in R_{ct} value was observed (Fig. 2A).

The EIS data were used to calculate the percent modulus normalization (PMN), which indicates the rate of impedance variation per mole of glucose added to the system. The PMN is calculated in the range of 0.1–100 Hz by applying the following equation,

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

where $Z_{0\,\mathrm{mM}}$ and $Z_{40\,\mathrm{mM}}$ stand for the impedance modulus measured at 0 and 40 mM of glucose concentration, respectively. The

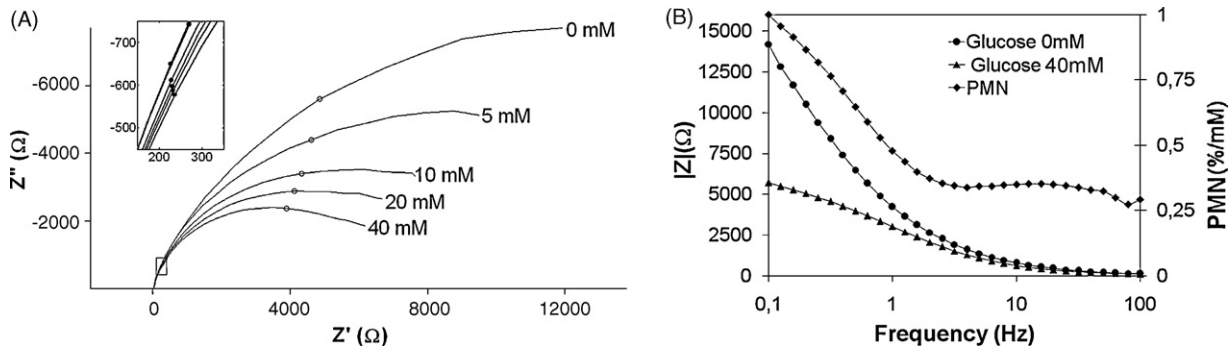

Fig. 2. EIS Characterization of the biosensor, (A) Argand Diagram, each semicircle corresponds to a glucose addition; and (B) Percent Module Normalization (PMN) of the biosensor, calculated from the impedance measured without glucose, and with 40 mM of glucose.

PMN is shown in Fig. 2B. The curve presents a maximum value of 0.99%/mM, measured at 0.1 Hz. As frequency increases, PMN continuously decreases down to approximately 0.33%/mM for frequencies higher than 1 Hz. The best measurement frequency is, then, in the range of high values of PMN.

In order to verify this assertion, two frequency values were selected from previous analyses, the first one in the high PMN range (0.4 Hz), and the second one at the low PMN range (10 Hz). The real time determination of impedance ($|Z|$ and Φ), in pH 7 phosphate buffer solutions during successive addition of glucose 5 mM, was carried out by using these two frequencies and a composed signal (900 mV DC + 50 mV_{RMS} AC). The results are shown in Fig. 3. The response obtained at 0.4 Hz appreciably reflects $|Z|$ and Φ changes, while the response at 10 Hz is less appreciated. Other experiments were performed at lower frequencies, where the system shows a higher PMN, but the noise and slow response obtained impeded the real time glucose measurement.

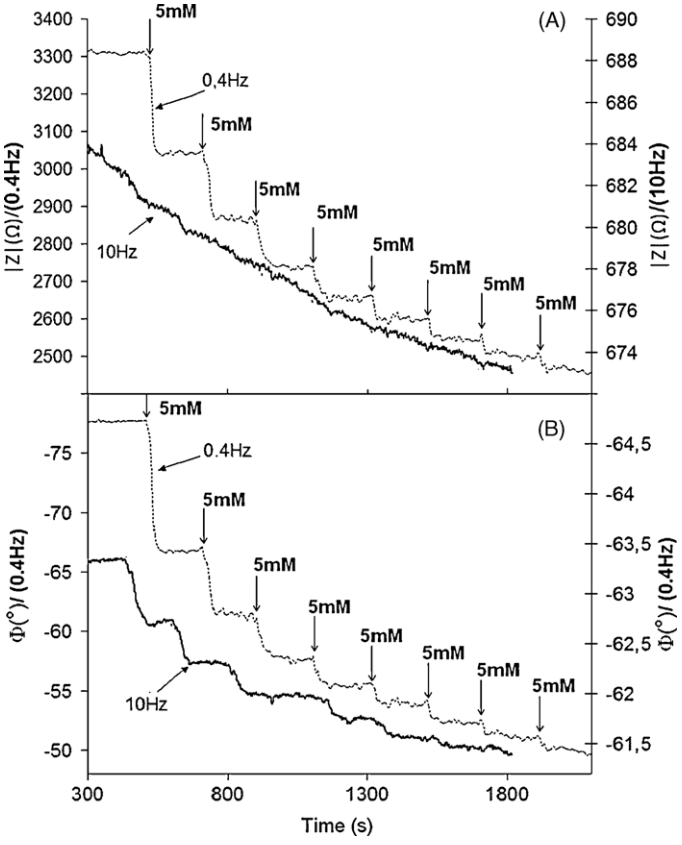

Fig. 3. On-line impedancimetric biosensor response measured with a sinusoidal of 10 Hz (—) and 0.4 Hz (···). (A) Impedance magnitude $|Z|$, (B) impedance phase Φ.

When glucose was added to the buffer solution, the impedance magnitudes obtained making use of 0.4 Hz show electrocatalytic oxidation activity of the biosensor, and these magnitudes reached steady state in 50 s.

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, three measurements were performed under different experimental conditions, which are shown in Fig. 4A and B. The first one was carried out on the biosensor with successive addition of glucose. The second one was carried out on the same biosensor with successive addition of KH_2PO_4/Na_2HPO_4 0.07 M buffer solution pH7 with 0.1 M KCl without glucose. Finally, a sensor composed only of CP without GOx was tested with glucose under the same conditions than the first one.

In the presence of oxygen, the product of main interest associated with glucose oxidation in this CP/GOx biosensor is hydrogen peroxide (H_2O_2). The impedancimetric response of the CP electrode without GOx to different concentrations of H_2O_2 was investigated in the range of 0–30 mM. The results are presented in Fig. 4C and D. The electrode clearly responds to the successive additions of 5 mM H_2O_2.

Fig. 5 shows the measurements made with the chronoamperometric method. When glucose was added to the buffer solution in the absence of the stirrer, the current time response was very slow since the substrate is not available for the enzymatic reaction. When the stirrer was switched on, the noise totally obscured the signal.

The calibration curves for $|Z|$ and Φ as a function of the glucose concentration are based on the average values obtained for each glucose addition shown in Fig. 3A and B, respectively. The modulus ($|Z|$) and phase (Φ) of complex impedance were analyzed separately. For each parameter several types of curves were fitted.

- Linear fitting,

$$P = a \times [\text{glu}] + b \quad (2)$$

- Second order polynomial fitting,

$$P = a \times [\text{glu}]^2 + b \times [\text{glu}] + c \quad (3)$$

- and hyperbolic fitting,

$$P = a + ((b \times [\text{glu}])/(c + [\text{glu}])) \quad (4)$$

where P stands for the observed magnitude ($|Z|$ or Φ) and [glu] is the glucose concentration in mM.

The values of the correlation coefficients obtained from the linear, second order polynomial and hyperbolic fitting were,

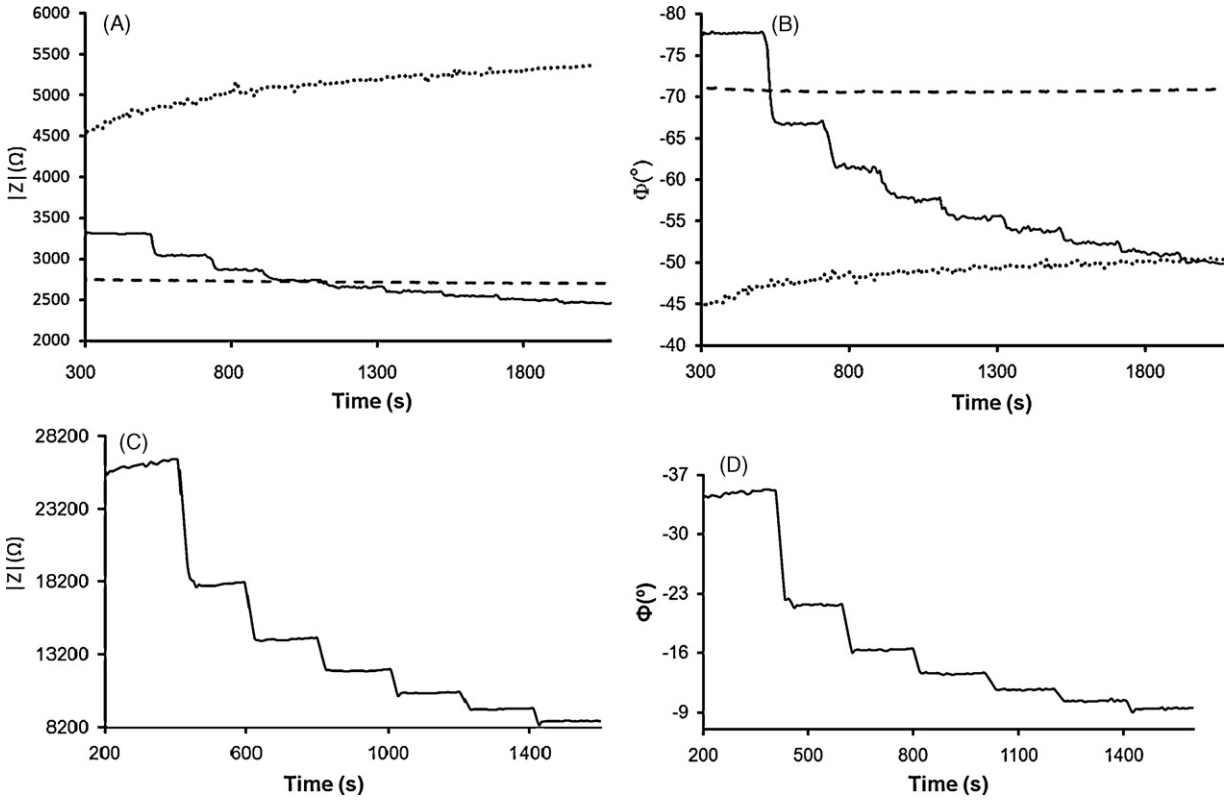

Fig. 4. On-line response of the impedancimetric biosensor and controls at 0.4 Hz: (A) impedance magnitude $|Z|$, (B) impedance phase Φ. (—) Biosensor; (···) carbon paste without GOx both with the addition of glucose; and (– – –) biosensor with the addition of support electrolyte without glucose. (C) and (D) shows the on-line response of the impedancimetric CP electrode upon successive additions of 5 mM H_2O_2 for $|Z|$ and Φ.

respectively, 0.911, 0.992 and 0.999, for $|Z|$, and 0.866, 0.983 and 0.998, respectively, for Φ. It is clearly observed that the highest correlation values correspond to the hyperbolic fitting.

The impedancimetric response of these biosensors was measured in KH_2PO_4/Na_2HPO_4 0.07 M buffer solution pH 7 with 0.1 M KCl during successive addition of glucose 5 mM (Fig. 6A, inset) and 2 mM (Fig. 6B, inset). For high range of glucose concentration (5–40 mM) the calibration curves were estimated by hyperbolic fitting using Eq. (4), while for low range (2–10 mM) the linear fitting was used (Eq. (2)). The results of quadruplicate sets indicated by error bars reveal the repeatability and reproducibility (Fig. 6C and D) of the measurements with a relative standard deviation (RSD) less than 5% for both, the high and low ranges of glucose concentration. It is important to note that, in order to compare the results, the evaluation of these parameters was made by using the normalized impedance (relative value to zero concentration of glucose).

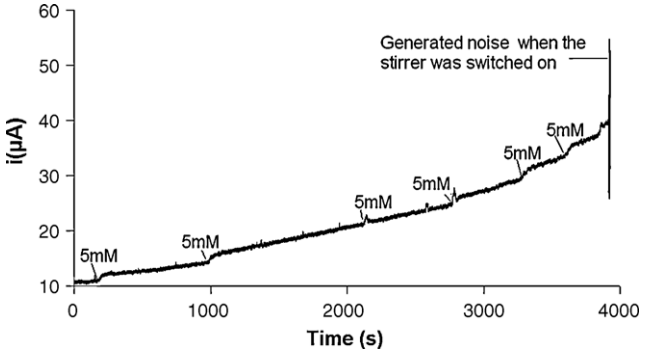

Fig. 5. Current–time response of CP/GOx biosensors, upon successive additions of 0.5 mM glucose by applying a DC potential of 900 mV.

Another method used to study the repeatability of this impedancimetric method was the measurement of a test sample, which was prepared with a known volume of glucose. The corresponding glucose concentration was calculated by means of the calibration curve. Four CP/GOx biosensors were independently evaluated. The measurement was repeated five times for each biosensor. Applying the ANOVA one-way statistical test, with a confidence interval of 0.99, the mean values of the measured glucose concentrations were not different ($p = 0.32$ for $|Z|$ and 0.40 for Φ).

Fig. 6E shows the correlation between the chrono-impedance and colorimetric methods, the latter used as reference. A high correlation was obtained ($r^2 = 0.98$).

Two samples prepared at the same conditions were measured in parallel with the chrono-impedancimetric method and the colorimetric one. The measurement was repeated four times. The Student's *t*-test produced no difference between the two methods, with a confidence interval of 0.99 ($p = 0.44$).

The 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 (colorimetric). Three concentrations were measured in the linear range (0–10 mM) with five determinations of each one. The mean value of each measured concentration is within 8% of the values measured with the reference method.

Precision was evaluated by using three concentrations in the linear range (0–10 mM) with five determinations per concentration. The RSD were respectively 12%, 9% and 19.6% from high to low concentrations.

4. Discussion

The working DC potential is normally identified by means of a voltamperometric assay. On the other hand, the bibliography

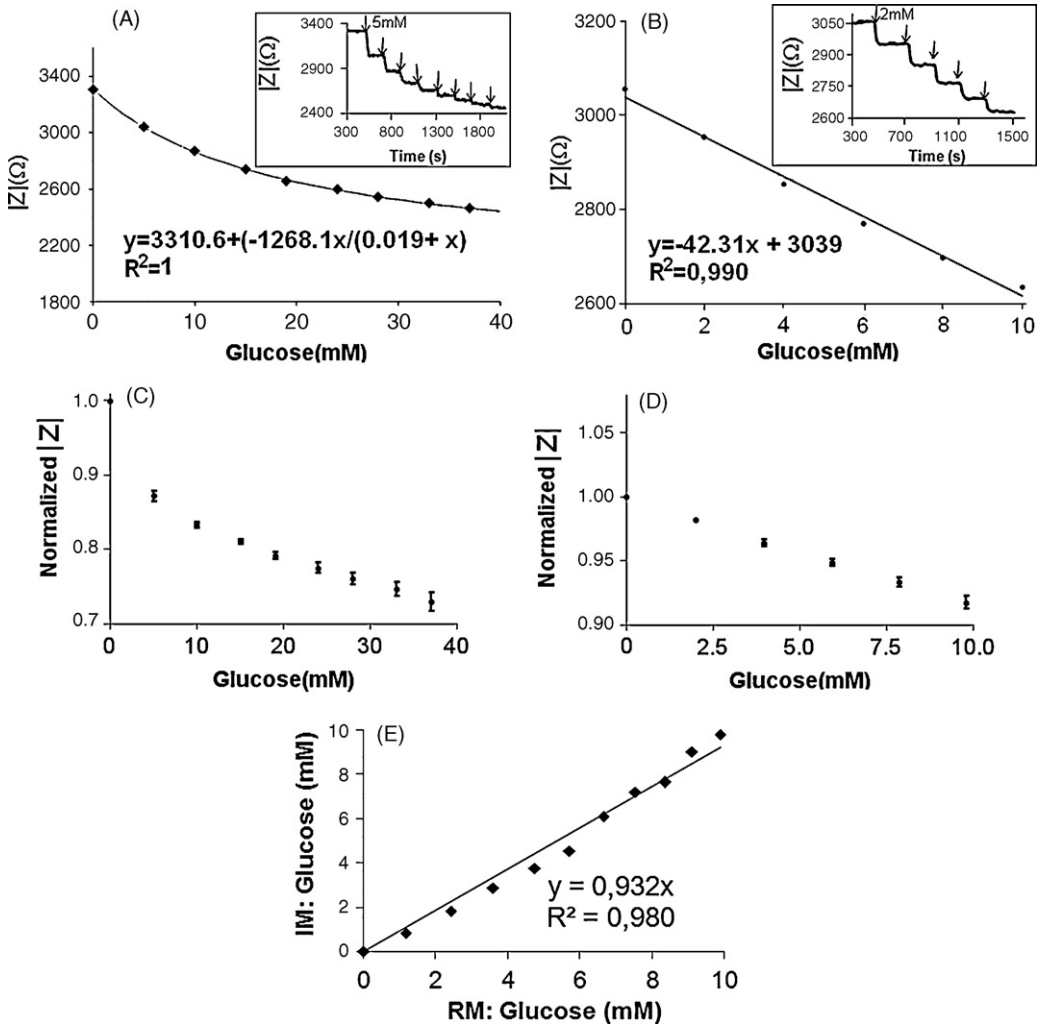

Fig. 6. Biosensor calibration. (A) Glucose concentration range of 0–40 mM as a function of the Impedance magnitude. (B) Glucose concentration range of 0–10 mM as a function the impedance magnitude. (C) and (D) Normalized impedance magnitude vs. glucose concentration for high and low concentration range, respectively. (E) Correlation between chrono-impedancimetric and colorimetric methods.

describes that potentials over 900 mV applied to carbon electrodes electrooxidate the hydrogen peroxide (Alegret et al., 2004). A similar potential was tested with a voltamperogram, finding that 900 mV was the most adequate DC potential. The determination of the AC potential was empirical, finding that 50 mV was the best choice. This small signal does not significantly modify the potential at which the biosensor works.

Fig. 2A shows a typical electrode–electrolyte interface (EEI) behavior. A decrease in R_{ct} was evident. In an EEI an electric current flows due to the charge transfer of electrochemical reactions occurring there. In the present case, the electrochemical reaction on the biosensor surface can be described by the following mechanism:

$$\text{Glucose} + O_2 \overset{\text{GOx}}{\longleftrightarrow} \text{Gluconic acid} + H_2O_2 \quad \text{(i)}$$

$$H_2O_2 \overset{900\,\text{mV}}{\longleftrightarrow} O_2 + 2H^+ + 2e^- \quad \text{(ii)}$$

Reaction (ii) shows electron generation, which increase the interface electric charge. This fact may explain the decrease in R_{ct}. This reaction was also verified with the impedancimetric response of the CP electrode upon successive additions of H_2O_2 (Fig. 4C and D).

On the other hand, if the EEI is polarized with a high anodic overpotential, the energy barrier that must be overcome for the charge transfer reaction to occur is lower, leading to a lower R_{ct}. The higher the glucose concentration, the higher the current and this contributes to diminish R_{ct}.

An electronic circuit commonly used to fit these data includes a constant phase element (CPE or also constant phase angle, CPA) (Jorcin et al., 2006) from which R_{ct} can be calculated. Its inverse value R_{ct}^{-1} has been proposed as an estimator of glucose concentration in self-assembly monolayers (SAM) biosensors (Shervedani et al., 2006). This model is not quite adequate, however, since CPE is an empiric description of an electronic component, which does not have an actual electronic equivalent. CPE is used because the electrode–electrolyte impedance frequently shows frequency dispersions that cannot be described with simple circuit elements (Jorcin et al., 2006).

Accurate glucose estimations involve many impedance measurements, especially those carried out at low frequencies. The time consumed in such estimation can be as long as 15–20 min (Shervedani et al., 2006; Ghica and Brett, 2005; Bardea et al., 1999) and the technique, finally, is not suitable for real time glucose measurements. The method herein presented operates at a single frequency and the measurement corresponds to a three point averaged value.

The glucose-related change of impedance was observed in the low-frequency range (0.1–1 Hz, Fig. 2B) and the analysis of PMN corroborated this finding. Furthermore, the rate of impedance

change was much greater for low-frequency signals (0.4 Hz) than for the higher frequency ones (10 Hz). At higher frequencies, the capacitive impedance is so low that the variations are negligible, and the impedance is the purely resistive bulk one.

Two different measurements (without glucose and without GOx) were carried out as a control of the electrochemical process. Both resulted in either negligible or no change at all (Fig. 4A and B), supporting the glucose bioelectrocatalytic origin of the impedancimetric response. Linear and polinomic fitting did not provide correlation values as good as the hyperbolic one did.

The equation of the hyperbolic fit (Eq. (4)) is similar to Michaelis–Menten equation:

$$V = \frac{V_{\max}[c]}{K_M + [c]} \tag{5}$$

where V is the initial reaction rate, c is the substrate concentration and $V_{\max}$ the maximum rate. Comparing Eqs. (4) and (5), the new equation results as:

$$P_{ss} = P_{ini} + \left(\frac{P_{\max} \times [\text{glu}]}{K_M^{app} + [\text{glu}]}\right) \tag{6}$$

where P_{ss} is the steady-state value of the measured magnitude ($|Z|$ or Φ), [glu] is the glucose concentration, $P_{\max}$ is the maximum value of the magnitude when the solution is saturated and K_M^{app} is the apparent Michaelis–Menten constant. P_{ini} was added because the point at which the line crosses the y-axis is not zero.

Normally, the hyperbolic fit is used to evaluate the Michaelis–Menten enzymatic kinetics in amperometric biosensors. The apparent K_m is an indirect measure of enzyme affinity for the substrate (Dui Dong et al., 1997). In the present case, the same characteristic can be observed as the biosensor calibration equation resembles the Michaelis–Menten one.

The measurements of an unknown sample with the four biosensors tested were not statistically different. This proves that this impedancimetric method was very repeatable. This method also shows an excellent correspondence and correlation with the measurement made with the colorimetric one. Accuracy is within 8% of the actual value, and precision does not exceed 15% of RSD for high concentration values and 20% for low concentration. All this suggests its potential applicability for real time glucose measurements.

This method may be called chrono-impedance technique, since it allows glucose dosage based on real time impedance measurement. Compared to classical chrono-amperometric technique, chrono-impedance showed a very stable and noise-free response of the tested system (measurement cell). This is mainly due to the digital correlation technique implemented in the Solartron 1250FRA analyzer. The digital correlation takes into account only the operating frequency in each case (whether 0.4 or 10 Hz) and any other frequency component contributes to the impedance measurement.

Classical chrono-amperometric test was also carried out by operating the SI1287 as potentiostat with no sinusoidal signal. The low amplitude current sensed (microampers) was strongly obscured by electromagnetic interference. The main source of interference was the magnetic stirrer. The chrono-amperometric measurement could finally be accomplished when the stirrer was turned off (Fig. 5). In this case, the signal still remained noisy and the steady-state response of the system was reached in a longer time. The AC current measured in the impedancimetric test ranged from 0.1 to 0.4 mA, thus providing acceptable signal-to-noise relationship.

5. Conclusions

This paper presents a new method applicable to biosensors. It proves that the chrono-impedance technique (or chrono-impedancimetry) is a reliable method for real time determinations of glucose concentrations, making use of a simple experimental model of a known glucose first generation biosensor. This technique allows continuous and rapid time response measurements in a low cost experimental system, and it is only limited by the transient response of the biosensor. The use of EIS to evaluate the electrocatalysis of glucose showed advantages when compared to the classical amperometric method commonly used in biosensors. First, by adding an alternate voltage, it is possible to measure the interface impedance and to evaluate R_{ct}. In addition, the SNR is improved and the measurement is less sensitive to electromagnetic interference. Second, the use of a single-frequency signal allows online continuous measurements that cannot be accomplished with the multi-frequency fitting-based EIS methodology. This method is supposed to be applicable to any other biosensor system assuming that the optimum working conditions are previously determined for each case, that is, the AC and DC values, and mainly the frequency. The statistical one-way ANOVA test confirmed the high repeatability of the chrono-impedancimetric method. Its measurements were very similar and correlative with the colorimetric ones. This method is also very precise and accurate.

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