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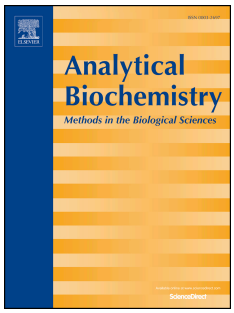

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PII: S0003-2697(18)30374-9

DOI: 10.1016/j.ab.2018.04.006

Reference: YABIO 12986

To appear in: *Analytical Biochemistry*

Received Date: 21 December 2017

Revised Date: 5 April 2018

Accepted Date: 6 April 2018

Please cite this article as: F.N. Comba, M.R. Romero, F.S. Garay, A.M. Baruzzi, Mucin and carbon nanotube-based biosensor for detection of glucose in human plasma, *Analytical Biochemistry* (2018), doi: 10.1016/j.ab.2018.04.006.

MUCIN AND CARBON NANOTUBE-BASED BIOSENSOR FOR DETECTION OF GLUCOSE IN HUMAN PLASMA

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ABSTRACT

This work reports an amperometric enzyme-electrode prepared with glucose oxidase, which have been immobilized by a cross-linking step with glutaraldehyde in a mixture containing albumin and a novel carbon nanotubes-mucin composite (CNT-muc). The obtained hydrogel matrix was trapped between two polycarbonate membranes and then fixed at the surface of a Pt working electrode. The developed biosensor was optimized by evaluating different compositions and the analytical

properties of an enzymatic matrix with CNT-muc. Then, the performance of the resulting enzymatic matrix was evaluated for direct glucose quantification in human blood plasma.

The novel CNT-muc composite provided a sensitivity of 0.44 ± 0.01 $mA.M^{-1}$ and a response time of 28 ± 2 s. These values were respectively 20% higher and 40% shorter than those obtained with a sandwich-type biosensor prepared without CNT. Additionally, CNT-muc based biosensor exhibited more than 3 orders of magnitude of linear dynamic calibration range and a detection limit of 3 µM. The short-term and long-term stabilities of the biosensors were also examined and excellent results were obtained through successive experiments performed within the first 60 days from their preparation. Finally, the storage stability was remarkable during the first 300 days.

Keywords: NOVEL HYDROGEL, CARBON NANOTUBES, MUCIN, GLUCOSE OXIDASE, BIOSENSOR, HUMAN PLASMA.

1. INTRODUCTION

The determination of glucose concentration is very important for early diagnostic and management of Diabetes. Diabetes mellitus is a pathology widely distributed around the world, with estimations of 300 million sufferers throughout the world by 2045 [1]. It is characterized by deranged blood glucose levels and metabolic abnormalities associated with numerous macro and microvascular sequelae as well as additional co-morbidities, because of insufficient or ineffective endogenous insulin. Therefore, methodologies that ensure efficient glucose quantification are highly required for early diagnosis of Diabetes as well as for its

management control in the public health system [2, 3].

The development of biosensors has potential because they are cheap, sensitive, selective, and simple operation analytical tools. Since the first enzymatic glucose biosensor, different strategies have been proposed for the development of these bio-detection platforms [4, 5]. In this sense, the electrochemical ones have demonstrated to be highly successful due to their known advantages. Among them, amperometric enzyme electrodes mainly based on glucose oxidase (GOx) have gained considerable attention. GOx catalyzes the oxidation of glucose to gluconolactone in the presence of the natural mediator, oxygen, and generates hydrogen peroxide that is then subsequently oxidized at the working electrode producing a current proportional to analyte concentration [6-9]. The amperometric determination of hydrogen peroxide has been the most commonly used in electrochemical transduction mode. The proposed mechanism involves diffusion of the glucose to GOx within the network of a hydrogel, with subsequent enzymatic reaction and production of H_2O_2 and gluconic acid. The hydrogen peroxide produced is transported by diffusion to the working Pt electrode where it is oxidized [10-15].

Selectivity, stability, and cost of amperometric enzyme biosensors are key parameters for creating effective analytical systems intended for operation with real samples. Generally, enzymes do not have long term stability in aqueous medium, thus immobilization techniques play a critical role in stabilizing GOx and other enzymes. Generally, enzymes don't have long term stability in aqueous media, thus immobilization techniques play a critical role in stabilizing GOx and other enzymes. In this sense, the enzymatic matrix determines the enzyme stability and

the overall analytical performance of an amperometric biosensor. Moreover, the possibility of avoiding these limitations will improve the useful life of a biosensor [7]. One of the most interesting materials in this regard are carbon nanotubes (CNTs). They have been extensively studied for the development of electrochemical biosensors due to their exceptional properties like high aspect ratio, adsorption of molecules, reactivity, thermal stability, flexibility, and electronic conductivity [16]–[18]. Despite of their unique and interesting characteristics, CNTs tend to aggregate in aqueous media because of their high surface area and strong π-π interactions between their aromatic rings. Nevertheless, stable aqueous suspensions of CNTs were generated by forming a supramolecular structure with the hyperbranched polymer BH40 in a previous study [19]. With a similar approach, a natural macromolecule suitable for immobilization of enzymes [12], [14], [20]. such as mucin could be used in combination with CNTs to obtain stable suspension with optimal properties as enzymatic matrix.

In this manuscript, a novel carbon nanotubes-mucin composite (CNT-muc) has been synthesized and then used to prepare the enzymatic matrix of a glucose biosensor. According to our experience, the inclusion of CNT into the enzymatic matrix would contribute to enhance the diffusion of reactive species and thus, to improve the response time of sandwich-type biosensors. Accordingly, the CNT-muc composite was mixed with albumin (alb) and GOx and then crosslinked with glutaraldehyde. After optimizing the composition of the enzymatic matrix, a biosensor with excellent analytical characteristics was obtained. The resulting biosensor was finally used for determining the glucose concentration of diverse human blood plasma samples.

2. EXPERIMENTAL SECTION

2.1. Reagents

Phosphate buffer solution pH 7.0 was employed as base electrolyte. This electrolyte solution (0.1 M) was prepared by mixing 0.05 M HK_2PO_4 / 0.05 M H_2KPO_4 (Merck, Germany). Then, the solution was fixed at pH 7.0 with small amounts of H_2SO_4 (Baker, USA) or KOH (Merck, Germany) and renewed weekly. Hydrogen peroxide (30% v/v aqueous solution) was purchased from (Cicarelli, Argentina). Glucose was from Merck and AA was from Sigma. GOx (Type X-S, Aspergillus Niger, EC 1.1.3.4, and 100.000 Units per gram of solid, Catalog number G-7141, Sigma, USA) was dissolved in 510 µL of base electrolyte to get a solution with 4.0 U μL^{-1} of GOx. From this solution, 5 aliquots of 20 µL were separated into vials and stored at −20 ℃. The remaining solution was further diluted to prepare aliquots of 20 µL with 20 U of GOx. These aliquots were also stored at −20 ℃. Solutions of glutaraldehyde (Backer, USA) were prepared in base electrolyte. Bovine serum albumin (Sigma, USA) was used as received. Mucin was supplied by Sigma, USA. It was dried in an oven at 37 ℃ during 24 h. After this period, it was stored at 4 ℃. CNT (Sunnano, China) with diameter that ranges between 30 and 10 nm was used as received. CNT-muc mixture was prepared weighing 10% CNT and 90% mucin. Both were put in a mortar and grinded into a fine powder by 30 minutes. The CNT-muc mixture was dried in an oven at 37 ℃ by 24 h and stored at 4 ℃ until use.

All other reagents were of analytical grade and used as received.

Polycarbonate membranes of 0.05 µm pore size (Millipore, USA) were cut in discs of 6 mm in diameter. Ultrapure water (σ =18 MΩ.cm) from a Millipore-MilliQ system was used for preparing all the solutions.

2.2. Apparatus

The electrochemical measurements were performed with an Autolab PGSTAT 30 Electrochemical Analyzer (Eco Chemie, The Netherlands). The working electrode was a 2 mm diameter Pt disk (CH Instruments, USA). Furthermore, a platinum wire and a Ag|AgCl|KCl(3M) (CH Instruments) were used as counter and reference electrodes, respectively. All potentials are referred to the latter. In all assays, a magnetic stirrer provided the convective transport during the amperometric measurements.

2.3. Preparation of the enzymatic matrix

The matrix was prepared by dissolving 6.0 mg of different mass ratios of CNT-muc and albumin in 40 µL of base electrolyte. It was mixed and sonicated for 5 and 30 seconds, respectively. All the proportions of CNT-muc and albumin resulted in a homogeneous dark black dispersion in which no type of precipitate or aggregate was observed. Then, a vial containing 20 µL of GOx was incorporated to the matrix and mixed for additional 60 seconds. The resulting volume of enzymatic matrix was mixed for extra 5 min and stored at 4 ℃ .

2.4. Construction of the enzymatic electrode

For this, a 4 µL of enzymatic matrix was mixed with 3 µL of glutaraldehyde

and placed between two membranes of polycarbonate. Subsequently, it was placed with precision tweezers at the surface of the Pt working electrode and fixed with a suitable cap. After 5 min, buffer solution was used to rinse the electrode and eliminate the excess of glutaraldehyde and other molecules that did not react with the polymeric matrix. Thus, 1.33 ± 0.01 U of GOx remains entrapped in each biosensor. This immobilization method not only produces the three-dimensional hydrogel matrix where the enzyme is trapped, but also provides an environment where the enzyme keeps most of its activity at proximity of the electrode surface.

2.5. Procedure

Amperometric measurements were conducted in a stirred (120 rpm) 0.10 M phosphate buffer solution pH 7.0 by applying the desired working potential and allowing the transient currents to decay to a steady-state value prior to the addition of the glucose and subsequent current monitoring. All measurements were performed at room temperature. After each calibration curve, the biosensor was rinsed with buffer pH 7.0.

The glucose concentration of 10 serum samples was quantified at clinical laboratory of the Medical Centre of Unión Obrera Metalúrgica, Córdoba, Argentina by the standard bi-enzymatic method. Those samples were transferred to our laboratory and analyzed within 24 hs of their reception.

For stability assays, two bioelectrodes stored at 4℃ in PBS solution pH 7.0 were analyzed. The analytical sensitivity was measured for 10 months and only decreased less 10% with respect to the original value, not shown.

3. RESULTS AND DISCUSSION

3.1 Effect of the hydrogel composition

Chronoamperometric experiments were performed to study the analytical performance of sandwich-type biosensors prepared with different enzymatic matrixes. Figure 1 shows the typical electrochemical response of a sandwich-type biosensor prepared with an enzymatic matrix composed by 1.33 U of GOx, 10/90 % w/w of CNT-muc and albumin, respectively. A 10% v/v glutaraldehyde solution was added to crosslink the enzymatic matrix. The amperometric signal was measured at +0.65V vs. Ag|AgCl|KCl (3M). Every addition of glucose increased the concentration in 0.2 mM. The current signal increased immediately after each addition and then depicted a very stable limiting current (I_{lim}) response. As it can be observed from the inset of Figure 1, there is a linear relationship between I_{lim} and glucose concentration (C_{Gluc}).

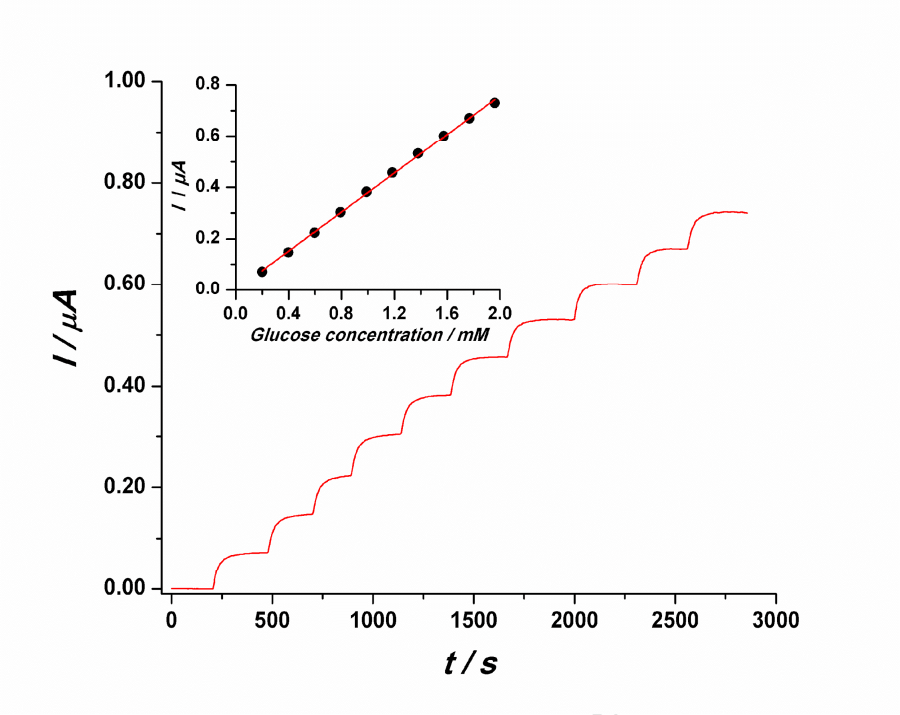


Fig. 1. Chronoamperometric curve corresponding to successive 0.2 mM injections of glucose at a Pt electrode modified with an enzymatic matrix composed by 10% CNT-muc, 90% albumin, GOx 1.33 U/sensor and glutaraldehyde diluted to 10.0 % v/v. Inset: Dependence of the limiting current observed for each standard addition of glucose.

Although the biosensor composed by 10% CNT-muc and 90% albumin presented a relatively good calibration curve, the composition of the enzymatic matrix still needs to be optimized to improve the performance of the biosensor. **Figure 2** depicts 3D plots where the proportion of CNT-muc, albumin, and concentration of crosslinker agent are varied. The composition of the enzymatic matrix that maximizes the sensitivity (**A**) and minimizes the response time (**B**) of the sensor was determined. The 3D plot shown in **Figure 2** (**A**) corresponds to

the slope of calibration curves ($I_{lim}.C_{Gluc}^{-1}$) measured with sandwich-type biosensors of different compositions, while the 3D plot of **Figure 2** (**B**) merges the values of response time measured when the current reaches 95% of I_{lim} (t95%) (**B**). All responses were obtained from amperometric experiments performed at +0.65 V for ten successive additions of glucose 0.2 mM at CNT-muc/albumin with 1.33 U GOx.

The mass ratio of CNT-muc/alb and the concentration values of glutaraldehyde that were employed for the construction of those enzymatic matrixes of biosensors were 0.0/100, 30/70, 50/50, 70/30, and 100/0 % w/w and 0.1, 1.0, 5.0, 10.0, and 25.0 % v/v, respectively. Every data point corresponds to analytical parameters averaged from at least 2 calibration curves. All biosensors presented a linear response between the steady-state current and the analyzed range of glucose concentration with squared correlation coefficient (Adj. R-Square) equal to 0.999. Adj. R-Square or R^2 can be used indifferently along of the manuscript.

Another important issue is that the inherent good hydrophilic character of these hydrogel matrixes provides a biocompatible microenvironment for maintaining the catalytic activity of enzyme. In this sense, Colombo et al. demonstrated that the concentration of GOx within the hydrogel is an important variable in the development of the optimal sandwich-type glucose biosensor [12]. Besides, they demonstrated that a matrix prepared with very high amount of enzyme would not necessarily increase the analytical signal. This is because above certain concentration of the enzyme, the sensitivity is determined by the diffusion of the analyte through the different layers of the biosensor. In this regard

they have indicated that, for a similar enzymatic matrix, the addition of more than 1.33 U of GOx per biosensor does not necessarily represent an increment on the sensitivity of the biosensor [12], [16]. Actually, this is one of the most important reasons for including CNT in the composition of the enzymatic matrix. The inclusion of CNT would decrease the viscosity of the hydrogel, enhancing the diffusion rate of reagents and products of the enzymatic reaction.

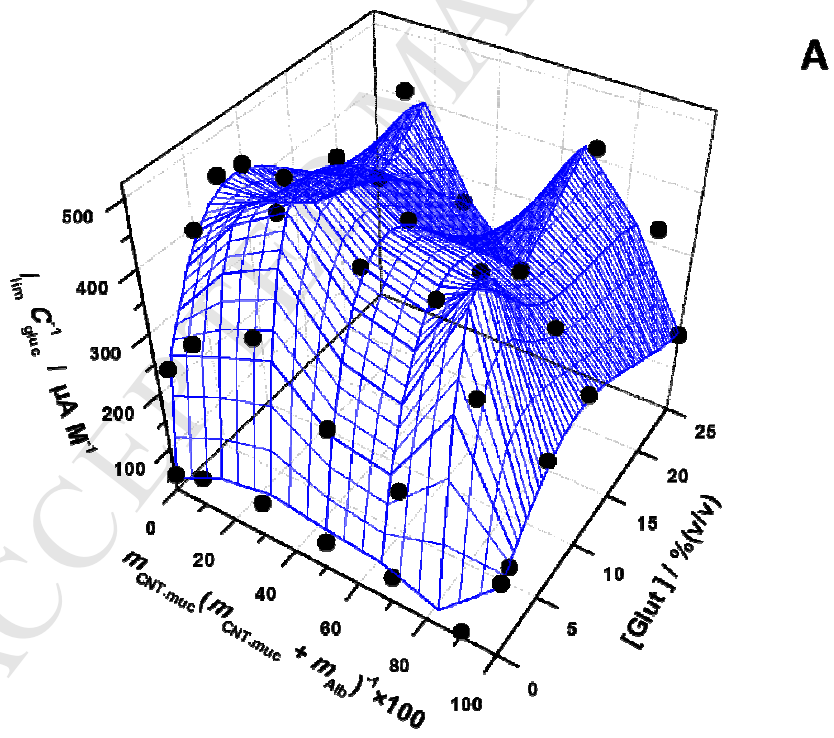
The effect of glutaraldehyde on the glucose biosensor performance is in direct relationship to the activity of covalently immobilized enzyme, **Fig. 2A**. I_{lim} is the lowest when the enzymatic matrices are prepared with 0.1 % v/v and 0.0 % v/v (not shown) of glutaraldehyde. This behavior can be explained considering that those hydrogel matrices were prepared with low amount of glutaraldehyde. Since those hydrogels were not significantly crosslinked, GOx would escape from the enzymatic matrix during the washing step and subsequent amperometric experiments. The sensitivity of the biosensor improves when the concentration of glutaraldehyde is increased from 1.0 % v/v to 5.0 % v/v, but it decreases for greater amounts of glutaraldehyde than 10.0 % v/v. This behavior demonstrates the effect of the crosslinking reaction. In this regard, a high concentration of glutaraldehyde would not only limit the diffusion of reagents, but also deactivate part of the enzyme. Since albumin is the protein with the highest amount of amino groups of the mixture its presence is essential for the elastic properties of the hydrogel. In this regard, enzymatic matrixes with less than 10.0 % w/w albumin do not have the consistence of a hydrogel irrespectively of the concentration of glutaraldehyde.

The biosensors prepared with a concentration of glutaraldehyde within the

range of 5.0 % v/v and 10.0 % v/v have practically the same sensitivity, irrespectively of the composition of the enzymatic matrix. Besides, this set of biosensors present some of the highest values of sensitivity. For instance, the sensitivity of a biosensor containing 50 % w/w CNT-muc and 50 % w/w alb (Pt-CNT-muc 50%) is practically 2.5 times larger for a sensor prepared with 5.0 % v/v of glutaraldehyde than for a similar one prepared with 25.0 % v/v of crosslinker. A high level of crosslinking will have strong impact on the biocatalytic activity of the enzyme because it would produce conformational changes in the recognition element and condition the diffusion of reagents and products through the matrix. As a consequence, not only the sensitivity will be low, but also the response-time will be high for those biosensors prepared with high amount of glutaraldehyde.

For this reason, the optimum composition of this type of biosensors was based on selecting the composition that provides not only the highest sensitivity, but also the shortest response-time when exposed to a sample with glucose. In general, sandwich-type biosensors built with CNT-muc have shown higher sensitivity than those made with mucin [12]. For example, the maximum sensitivity observed for the set of biosensors analyzed in **Figure 2**(**A**) was equal to 0.52 ± 0.04 $mA.M^{-1}$, and corresponds to the enzymatic matrix prepared with 5.0% v/v of glutaraldehyde and 30% of CNT-mucin. An equivalent study performed for a biosensor prepared without CNT showed a sensitivity of 0.38 ± 0.02 $mA.M^{-1}$ [12]. Therefore, the sensitivity of the biosensor increased more than 35% due to the inclusion of CNT into the hydrogel. With regards to the response-time of the biosensor, **Figure 2B** shows how this parameter changes with the composition of the biosensor. As a general behavior, it can be observed that the response-time of

the biosensor increases when the concentration of glutaraldehyde is changed from 1.0 to 25.0 % v/v. However, it is interesting to notice that the values of response-time depend markedly on the percentage of CNT-muc. In this regard, those matrices built with 0.0; 10.0 and 30.0% w/w CNT-muc presented the highest values of response-time, while the lowest correspond to those biosensors prepared with 50% of CNT-muc. The minimum response-time of the set of biosensors was 28 ± 2 s and it corresponds to the enzymatic matrix prepared with 50% of CNT-muc and glutaraldehyde diluted to 5.0 % v/v. This value of response-time is 25% better than that achieved with a similar biosensor, but prepared without CNT [12].



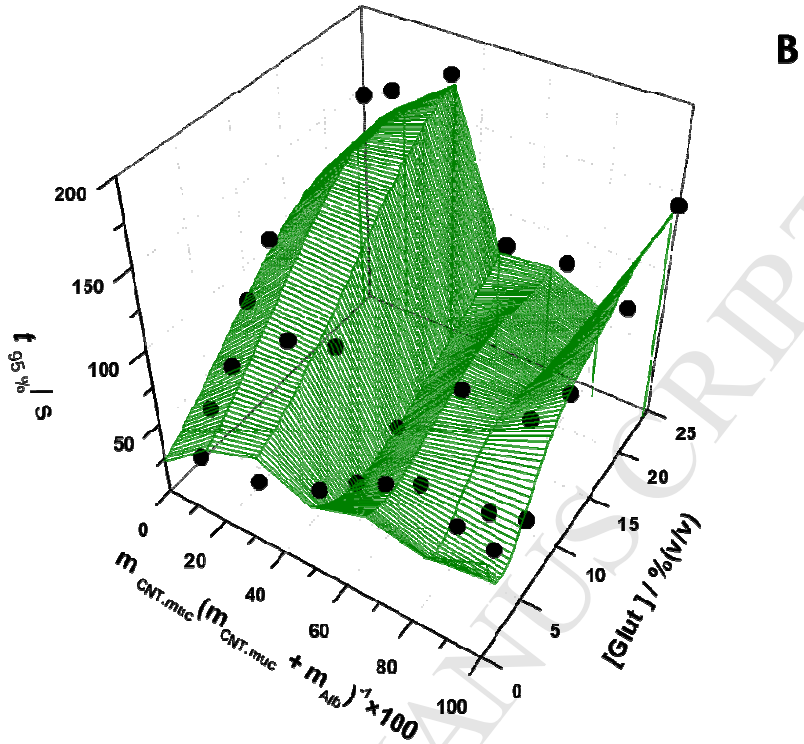


Fig. 2 Dependence of (A) sensitivity ($I_{lim}.C_{gluc}^{-1}$) and (B) response-time at 95% of I_{lim} ($t_{95\%}$) on the % of glutaraldehyde (*[Glut]*) and on the mass ratio of CNT-muc ($m_{CNT.muc}$) and albumin (m_{Alb}) employed for the enzymatic matrix. The time for the crosslinking reaction was 5 min and required 1.33 U GOx per sensor.

As it was observed, the inclusion of CNT into the hydrogel would contribute to enhance the sensitivity and reduce the response time of the biosensor. **Figure 3** exhibits the dependence of the ratio between sensitivity and response-time on the percentages of CNT-muc and glutaraldehyde used for the biosensor construction. Based on these results, a 50.0% w/w of CNT-muc and 5.0% v/v of glutaraldehyde would be the best choice for preparing a biosensor with high sensitivity and fast response-time. The sensitivity of the proposed glucose biosensor was calculated to be 0.44 ± 0.01 $mA.M^{-1}$.

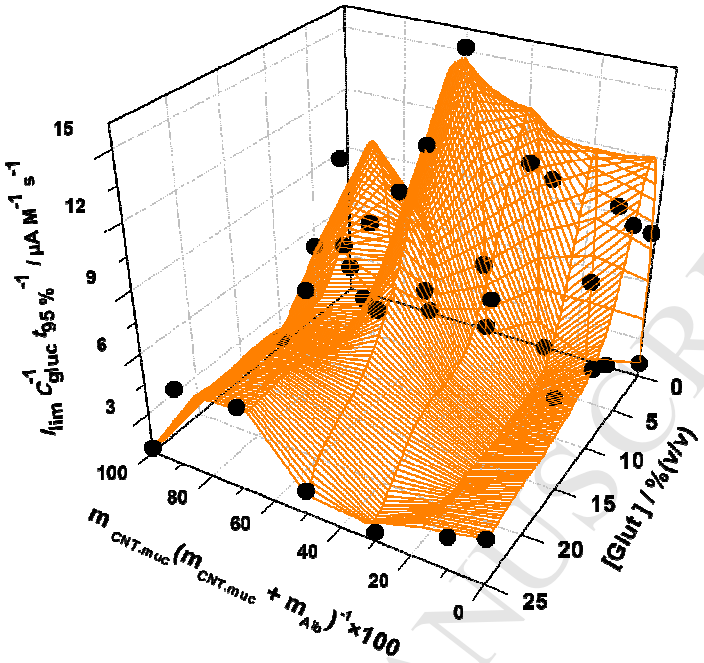


Fig. 3 Relationship between sensitivity and response-time ($I_{lim}.C_{gluc}^{-1}.t_{95\%}^{-1}$) as a function of the % of glutaraldehyde and the CNT-muc/alb mass ratio employed for the construction of the enzymatic matrix.

3.2. Detection limit and linear interval of the proposed biosensor

According to the optimal conditions found in the previous experiments, a calibration curve was measured to determine the range in which the analytical response of the biosensor presents lineal behavior. The cell was initially filled with 4.0 mL of 0.1 M phosphate buffer pH 7.0. The detection limit (LOD) and linear interval of the sensor were determined with glucose standard solutions of 1.0; 10.0 and 100.0 mM. The calibration curve involved several additions of 8 µL and 24 µL of the different standard solutions to the electrochemical cell. The LOD of a biosensor was calculated as 3 times the ratio between the standard deviation of

the background current and the slope of the calibration curve. The standard deviation of the background current was calculated from the average of ten background currents recorded 50 s before the first injection of a glucose standard.

Figure 4A shows a chronoamperometric curve corresponding to successive additions of glucose 0.002, 0.02, 0.2, and 0.6 mM for a biosensor prepared with 50.0% w/w CNT-muc, 5.0% v/v of glutaraldehyde, and 1.33 U of GOx. The calibration curve was performed in 0.1M phosphate buffer, pH 7.0. The inset displays the amperometric response of the biosensor for the lowest concentrations of glucose. **Fig. 4 B** depicts a logarithmic plot elaborated with the data of the calibration curve. From this plot it can be clearly observed that the biosensor presents a wide range of linear behavior. It is important to notice that if the results were presented in a linear plot, most of the data corresponding to the lowest concentration values would merge as a single point. The linear range of the bioelectrode goes from 0.002 to 3.2 mM with a squared correlation coefficient R^2 = 0.997. Above a glucose concentration of 3.2 mM, the bio-electrochemical response is limited by the diffusion of glucose and O_2, the natural mediator of this enzymatic reaction. Consequently, the current increases non-linearly with the glucose concentration, as expected for enzymatic reactions with a conventional ping-pong mechanism [12, 16, 20, 21].

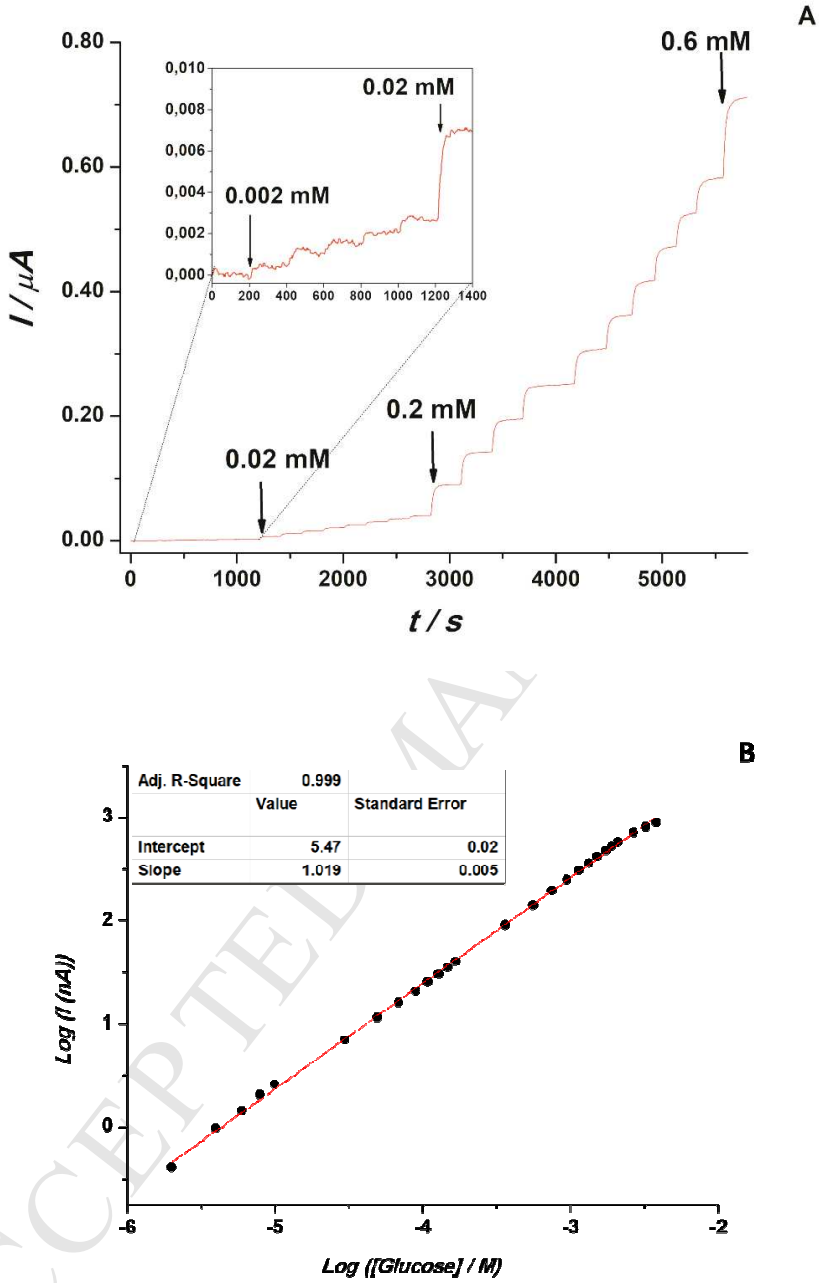


Fig. 4 (A) Chronoamperometric profile of a biosensor prepared with [Glutaraldehyde] = 5.0 % v/v, 50/50 CNT-muc/alb, C_{GOx}= 1.33 U. (B) Logarithmic dependence of I_{lim} on [Glucose].

The analytical performance of the bioelectrode was compared with other glucose biosensors prepared with GOx, **Table 1**. The LOD obtained from Pt-CNT-muc 50% was similar that of some recently reported amperometric biosensors based on the immobilization of GO*x* [12, 22–28]. It is necessary to emphasize that the biosensor reported here has excellent interval of linear behavior since the linear dependence involves more than 3 orders of magnitude. Moreover, it not only can be reused, but it also has high storage stability when compared to others [22], [25], [26], [28, [29], [34].

Table 1. Comparison of the analytical performance of glucose biosensors.

Glucose biosensor	Response time (sec)	Sensitivity ($mA.M^{-1}.cm^{-2}$)	Limit of detection (µM)	Lineal Range (mM)	AA Interference	Stability (days)	Ref.
Pt-CNT-muc 50%	29	15	3	0.002-3.2	5% [b]	300	This work
Au/Chitosan nanoparticles	2	157	1	0.001-1	Not Studied	≤ 10	[26]
Pt-muc/alb 30/70%	35	9.4	3	0.003-3.5	Not Studied	210	[12]
Fe_3O_4 nanoparticles polyvinyl alcohol	10	9360 [a]	8	0.005-30	5% [b]	30 [c]	[32]
Au–Ni noaxial nanorod array	13	770	6	0.03-26	Free	30	[27]
Au/Glutaraldehyde - dialysis membrane	1200	$1.6\ 10^{-5}$	5×10^{3}	17-444	Free	10	[28]
Pt/Nafion® - p-MAA microparticles	~30	12	1×10^{1}	0.009-8.3	Free	550	[33]
Pt/PMEH (methacrylate copolymer)	60	156	3	0.005-1	Not Studied	≤ 0,25 (Disposable)	[24]
Pt/Chitosan membrane	60	0.0019	5×10^{1}	0.01-15	Not Studied	≤ 10 [d]	[34]
SPCE/cellulose paper	?	0.002	2×10^{2}	1-5	5% [b]	120	[25]

[a] mAM^{-1}. [b] maximum physiological. [c] the sensitivity after 30 days decreased 20%. [d] the sensitivity after 30 days decreased 50%. LOD values have been expressed with one significant figure.

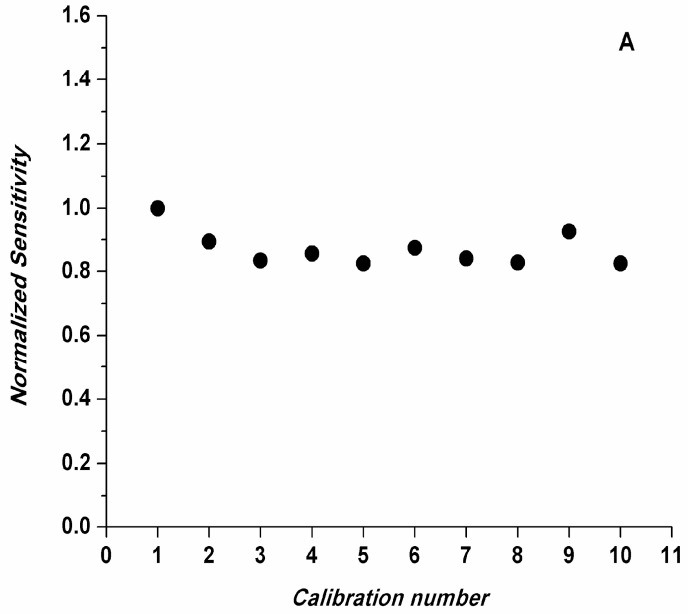
3.3. Selectivity of the proposed biosensor in presence of ascorbic acid

Ascorbic acid (AA) is a typical interfering species when the bio-electrochemical quantification of glucose, dissolved in blood plasma and other biologic fluids, is based on the oxidation of hydrogen peroxide. The effect of this potential interfering species on the response of the proposed biosensor was evaluated in 0.1 M PBS pH 7.0, when the physiological concentration of glucose found in blood human plasma (5.0 mM) is analyzed. The chronoamperometric response increased 5% after the addition of 0.1 mM of AA, not shown. The effect of AA on the biosensor selectivity was comparable to those reported for other similar glucose biosensors (5%) [25]**.** Thus, the maximum physiological concentrations of AA found in human blood plasma (0.1 mM) would not represent a relevant interference for the proposed biosensor [35], [36].

3.4. Sensor intra-day stability

The stability of the bioelectrode was evaluated by performing several chronoamperometric measurements. Considering that each calibration curve consisted in 10 standard additions of glucose and that the comparison involved the slopes of 10 calibration curves for the same biosensor, it is clear that the sensor was used for the analysis of 100 samples. Figure 5 A shows the normalized sensitivity of ten calibration curves performed with a sandwich-type biosensor prepared with an enzymatic matrix composed by 50/50 CNT-muc/alb, 1.33 U GOx, and 5.0% v/v glutaraldehyde. Every addition of glucose increased the concentration of glucose in 0.2 mM. Figure 5 B shows the corresponding average plot of the 10 calibration curves. All calibration curves showed very similar values

of sensitivity. The relative standard deviation obtained for these 10 successive calibration curves was 6.4, evidencing the excellent short-term stability of the biosensor.



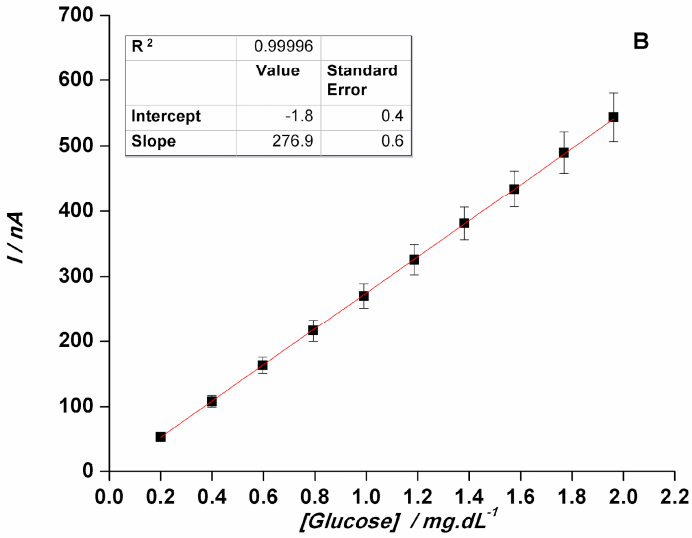


Fig. 5 A) Evolution of the sensitivity of the sensor as a function of number of calibration curves performed after its assembling. B) Average calibration curve corresponding to the ten profiles recorded after assembling the biosensor. The sensor was prepared with 1.33 U GOx, 50/50 CNT-muc/alb, 5% glutaraldehyde and stored in 0.10 M phosphate buffer pH 7.0. Additions correspond to 0.2 mM glucose.

3.5. Stability of the proposed sandwich-type biosensor

The long-term stability of the bioelectrode was analyzed by comparing the slopes of calibration curves corresponding to diverse chronoamperometric experiments performed with the same biosensor at +0.65 V. Calibration curves were collected two times per week for 2 months in order to analyze the storage stability of the biosensor. Between the measurements, the bioelectrode was stored in 0.1 M phosphate buffer pH 7.0 at 4 $^{\circ}$C. During these 60 days, calibration curves kept their linear behavior up to 1.0 mM while the LOD value remained constant. During the first 10 days, the sensitivity of the biosensor increased almost 25 % with regards to the value measured at the first day of this experiment. After the 10^{th} day the electrochemical response of the biosensor remained practically constant until the end of the study. In this regard, the average sensitivity of the biosensor was equal to 0.56 ± 0.03 $mA.M^{-1}$. After all these measurements, the bioelectrode was stored for other extra 7 months. After this period, the biosensor showed an analytical sensitivity only 10 percent lower than observed after it preparation/ first day experiment.

3.6. Real samples

Although the analysis of potential interfering species such as AA is indeed important, potential application for clinical analysis of this sandwich-type biosensor becomes evident when it is exposed to samples of blood human plasma. Real samples commonly involve several different species that limit the sensitivity and reproducibility of the proposed biosensor. As a result, a comparative analysis of the developed bioelectrode with a standard commercial method is highly desired to evaluate the real capabilities of the proposed biosensor. The samples were first analyzed with the standard bi-enzymatic method [37], [38] in the clinical laboratory of the Medical Center of Unión Obrera Metalúrgica, Córdoba, Argentina. Those samples were transferred to our laboratory and analyzed within 24 hs of their reception.

Then, the glucose concentration values corresponding to a set of samples of human blood plasma, were measured using the biosensor. The biosensor used for this study was prepared with 50% w/w CNT-muc, 50% w/w albumin, 5.0% v/v of glutaraldehyde, and 1.33 U GOx. After observing a proper background current, 40 µL of human blood plasma was added to a vial containing 4 mL of phosphate buffer pH 7.0. Figure 6A shows the chronoamperometric response of a) standard glucose (120mg.dl^{-1}), b) physiologic plasma (80 mg.dL^{-1}) and pathologic plasma (205 mg.dL^{-1}) recorded for about 400 s at +0.65 V. As seen in the image, this sensor presents an adequate response regardless of the glucose concentration and characteristics of the matrix.

Subsequently, 10 samples of human blood plasma involved glucose concentration values that ranged from physiologic 80 mg.dL^{-1} to pathologic levels

304 mg.dL^{-1} [36] were analyzed. This comparative study showed a linear relationship with a correlation factor R^2 = 0.991, which is a quite high value considering that some of these clinical samples might present different electroactive species such as uric acid or bilirubin due to their pathologic characteristics. Each value of glucose concentration informed in this plot corresponds to the average obtained from 4 repeated determinations. The developed biosensor demonstrated a 4.0 % average systematic error (% BIAS) when compared with the values determined by the standard method. The response of the sandwich-type glucose biosensor to human blood plasma was highly reproducible, providing a residual standard deviation (% RSD) of 2.2 % for the set of evaluated samples. Once this comparative analysis was finished, the extra amount of six human blood samples was stored in the fridge at -20 ℃. Also, the sandwich sandwich-type biosensor was rinsed with deionized water and stored in phosphate buffer pH 7.0 at 4 ℃. After 21 days, the comparison of the methods was repeated with the remaining six human blood samples. These samples of human blood plasma also involved physiologic and pathologic levels of glycaemia (80 to 304 mg.dL^{-1}). Under these conditions, a linear correlation was obtained with an R^2 value of 0.989. In this opportunity, the comparison with the results of the medical center showed a % BIAS of 3.7 and a % RSD of 4.7%. The comparison between the results obtained for the proposed bioelectrode and the standard spectrophotometric method indicates a quite good analytical performance of Pt-CNT-muc 50 %. Those results would also indicate that the biosensor can be used for measuring the concentration of glucose in samples of blood plasma with the usual interfering species.

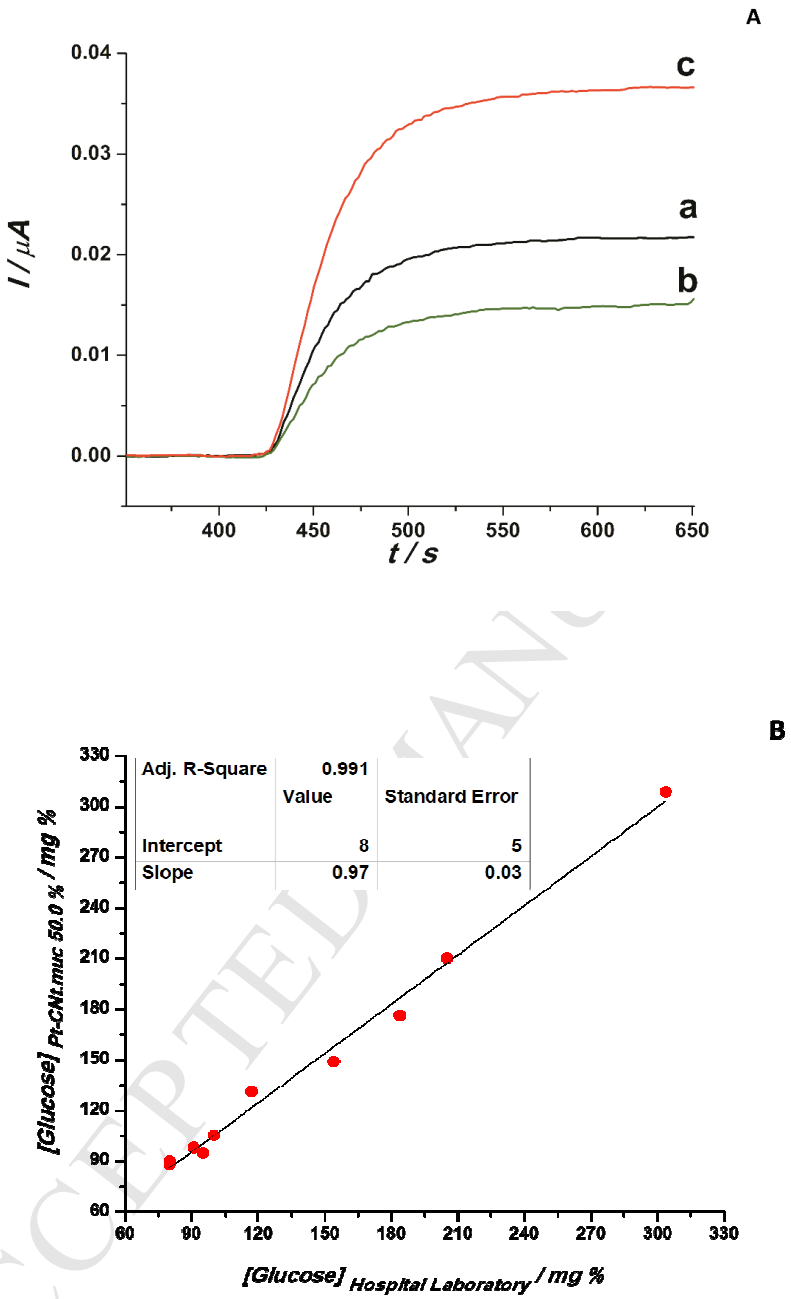


Fig. 6 A) Amperometric response of a biosensor prepared with 1.33 U GOx, 50/50 CNT-muc/alb, 5% glutaraldehyde after addition of a) standard glucose (120mg.dl^{-1}), b) physiologic plasma (80 mg.dL^{-1}) and pathologic plasma

(205 mg.dL^{-1}) B) Comparison of the plasma glucose measurement performed with the biosensor and the standard method used in a clinical laboratory.

4. CONCLUSIONS

In this manuscript, the development and optimization of an electrochemical sandwich-type glucose biosensor has been presented. In this opportunity, a blend of CNT with mucin and albumin was prepared as the scaffold of the enzymatic matrix. The analytical performance of biosensor depends on the ratio of CNT-muc/alb as well as on the amount of crosslinker employed for the enzymatic matrix. The best relationship between sensitivity and response-time of the different biosensors corresponds to the enzymatic matrix composed by 50% w/w of CNT-muc, 50% w/w albumin, 1.33 U GOx per biosensor, and 5.0 % v/v of glutaraldehyde.

The developed sandwich-type glucose biosensor showed higher sensitivity and lower response-time than a previous biosensor prepared in our group without CNT. The incorporation of CNT to the enzymatic matrix would reduce the viscosity of the enzymatic matrix and thus, the diffusion coefficients of reagents and products of the enzymatic reaction would be higher than those of an enzymatic matrix without CNT. As a result of this, the concentration of glucose would increase inside the enzymatic matrix, providing higher analytical response to the biosensor. This hypothesis is also consistent with the diminution of the response-time of the biosensor.

The linear response of the sensor engages solutions with glucose concentrations that range from micro to millimolar. Considering that most

intermittent use biosensors commonly involve the dilution of samples, between 20 and 200 times, the dynamic range of the biosensor widely encompasses the feasible glucose concentrations of human blood plasma corresponding to healthy and diabetic patients. Moreover, this biosensor has not only demonstrated good repeatability, reproducibility, and intra-day stability in presence of standard solutions of glucose, but also for the analysis of real samples of blood plasma.

The very good long-term stability observed under storage and intermittent use conditions points out that the developed biosensor can be used for systematic quantification of glucose in real biological system. These results make this biosensor in a suitable alternative for glucose determination in diabetic patients, since it represents a very economical, robust, and highly sensitive platform for the quantification of glucose in complex samples. In this sense, it would be also quite simple to adapt this analytical methodology for measuring the concentration of glucose in other samples such as fruit and vegetables. This is also desirable for diabetic patients, because the fruit-ripening process is closely associated with an increasing glucose level.

ACKNOWLEDGEMENTS

The authors thank the financial support from CONICET, FONCYT and Secyt-UNC. M. Romero, A. Baruzzi, and F. Garay are permanent research fellows of CONICET. F. Comba acknowledges to CONICET for the fellowship received.

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