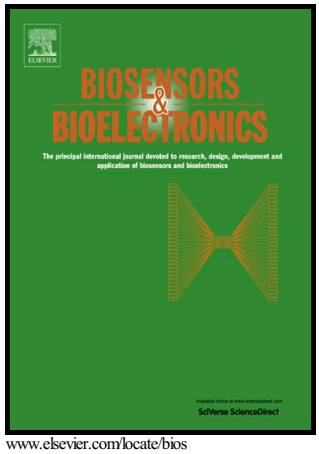
Author's Accepted Manuscript

Tear glucose detection combining microfluidic thread based device, amperometric biosensor and micro flow injection analysis

Deonir Agustini, Márcio F. Bergamini, Luiz Humberto Marcolino-Junior



www.elsevier.com/locate/bios

PII: S0956-5663(17)30410-4
DOI: http://dx.doi.org/10.1016/j.bios.2017.06.035
Reference: BIOS9805

To appear in: *Biosensors and Bioelectronic*

Received date: 3 May 2017
Revised date: 7 June 2017
Accepted date: 16 June 2017

Cite this article as: Deonir Agustini, Márcio F. Bergamini and Luiz Humberto Marcolino-Junior, Tear glucose detection combining microfluidic thread based device, amperometric biosensor and micro flow injection analysis, *Biosensors and Bioelectronic,* http://dx.doi.org/10.1016/j.bios.2017.06.035

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting galley proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

ACCEPTED MANUSCRIPT

Tear glucose detection combining microfluidic thread based device, amperometric biosensor and micro flow injection analysis

Deonir Agustini, Márcio F. Bergamini, Luiz Humberto Marcolino-Junior[1]*

Laboratório de Sensores Eletroquímicos (LabSensE), Departamento de Química, Universidade Federal do Paraná (UFPR), CEP 81.531-980, Curitiba-PR, Brazil.

luiz1berto@ufpr.br

Abstract

The tear glucose analysis is an important alternative for the indirect, simple and less invasive monitoring of blood glucose levels. However, the high cost and complex manufacturing process of tear glucose analyzers combined with the need to exchange the sensor after each analysis in the disposable tests prevent widespread application of the tear in glucose monitoring. Here, we present the integration of a biosensor made by the electropolymerization of poly(toluidine blue O) (PTB) and glucose oxidase (GOx) with an electroanalytical microfluidic device of easy assembly based on cotton threads, low cost materials and measurements by micro flow injection analysis (μFIA) through passive pumping for performing tear glucose analyses in a simple, rapid and inexpensive way. A high stability between the analyses (RSD = 2.54%) and among the different systems (RSD = 3.13%) was obtained for the determination of glucose, in addition to a wide linear range between 0.075 and 7.5 mmol L^{-1} and a limit of detection of 22.2 μmol L^{-1}. The proposed method was efficiently employed in the determination of tear glucose in non-diabetic volunteers, obtaining a close correlation with their blood glucose levels, simplifying and reducing the costs of the analyses, making the tear glucose monitoring more accessible for the population.

[1] Tel.: +55 41 3361-3177; Fax number: +55 41 3361-3186

Accepted manuscript

ACCEPTED MANUSCRIPT

Keywords: tear glucose, microfluidic device, cotton thread, micro flow injection analysis, toluidine blue O, glucose oxidase.

1. Introduction

The simplification and cost reduction related to the manufacture of systems and methods for tear glucose analysis is very important to increase the use of this body fluid for the diagnosis of diseases and evaluation of the health conditions of the people. Glucose monitoring is especially important because of diabetes mellitus, a disease that is considered a worldwide public health problem with almost epidemic rates and one of the leading causes of death and disability in the world (Dhoot and Avery, 2016).

Diabetes treatment normally includes tight control of glucose in the body with monitoring of blood levels with a portable glucose meter. This device contain an enzymatically-active disposable test strip, where is inserted a drop of blood that is obtained from a small hole in the finger (Aggidis et al., 2015). Although this method achieves accurate results, the need to perform several analyses throughout the day displeases and discourage patients from doing the suggested testing, due to pain, massive scar formation and loss of finger sensitivity that has been constantly perforated (Cha et al., 2015). In this way, tear presents as an interesting alternative for glucose monitoring, since this fluid has a good correlation with the glucose levels present in the blood and makes it possible to perform less invasive analyses on the patients (Peng et al., 2013).

The requirements for using a method in the determination of tear glucose include high selectivity, low detection limit (μmol L^{-1} to mmol L^{-1}) and analyses in a small sample volume (few μL) (Yan et al., 2011). To date, the methods used include detection with the electrochemical techniques (Chu et al., 2011), UV-vis-NIR spectroscopy (Hu et al., 2013), fluorescence spectroscopy (Badugu et al., 2004), liquid-chromatography techniques (Leblanc et al., 2005), chemiluminescent (Jang and Noh, 2015), and holographic technique (Yang et al., 2008). Recently, these methods of

Accepted manuscript

analysis have made great breakthroughs due to the use of miniature detection platforms, especially microfluidic devices (La Belle et al., 2016) and contact lenses (Yao et al., 2011).

However, while promising, many of the proposed devices present a high cost and complex manufacturing processes, which require the use of highly specialized equipment (Ascaso and Huerva, 2016; Pankratov et al., 2016). The few devices that have low cost are those based on disposable components, where the total cost becomes high when multiple analyses are required (La Belle et al., 2016).

The limitations presented by glucose analyzers can be overcome with the use of microfluidic platforms based on cotton threads, which have some characteristics that allow the construction of devices in a simple and cheap way. Cotton threads are easily found in any region of the world and for a much lower price than materials traditionally employed in miniaturized platforms (e.g. silicon, glass and polymers). In addition, the cotton threads have high surface energy, presenting a highly hydrophilic character, which added to the numerous gaps between the fibers allow the devices constructed with this material to carry the solutions without the use of any mechanical pumping system, only by capillary force (Nilghaz et al., 2013). Finally, cotton threads allow the manufacture of microfluidic devices without the need to construct hydrophobic barriers, with the solutions being confined only in the region of the thread, thus allowing the analysis of samples with small volumes (Li et al., 2010).

The microfluidic devices based on cotton threads have been mainly applied for diagnostic tests with colorimetric detection (Erenas et al., 2016; Mao et al., 2015; Ulum et al., 2016; Wu et al., 2016). Although the electrochemical techniques have interesting characteristics such as speed of analysis, high sensitivity and easy integration, they have been little used in microfluidic devices constructed with cotton threads (Glavan et al., 2016). Due to properties as high hydrophilic character, great flexibility and high mechanical strength even after wetting, another little explored possibility of cotton threads is the construction of non-disposable devices for micro flow analysis (μFIA),

Accepted manuscript

ACCEPTED MANUSCRIPT

which allows to carry out measurements for a long time, due to the constant cleaning of the device, reducing the cost of multiple analyses, in addition to simplifying and making faster the tests (Agustini et al., 2017).

Thus, in this work we present the development of a simple and inexpensive method for the determination of tear glucose by μFIA in a microfluidic system that has a unique configuration by the integration of a microfluidic thread-based electroanalytical device (μTED) with an amperometric biosensor constructed with poly(toluidine blue O) (PTB) and glucose oxidase (GOx) (PTB-GOx biosensor). Tear samples were collected from non-diabetic volunteers, as well as blood glucose tests performed on the same volunteers with a portable glucose meter, in order to evaluate the correlation between tear and blood glucose levels.

2. Materials and methods

2.1. Chemicals and solutions

GOx (150 U mg^{-1}, purified from Aspergillus niger) and D - (+) - glucose anhydrous were purchased from Sigma-Aldrich (Brazil). Toluidine blue O (TB) was purchased from EMS (USA). All others reagents employed in the work were analytical grade and were used without prior purification. The solutions were prepared daily using ultrapure water (specific resistivity > 18 MΩ cm) obtained with a water purification system (Millipore Corporation, USA). Sodium phosphate monobasic monohydrate and potassium phosphate dibasic anhydrous (Synth, Brazil) were used to prepare the phosphate-buffered saline (PBS, 0.10 mol L^{-1}, pH 7.40) solution.

2.2. Instruments

Micrographs with secondary electrons were obtained of the PTB-GOx biosensor surface with a scanning electron microscope (SEM) (Tescan a.s., Czech Republic), in addition to elemental analysis and chemical mapping carried out by energy-dispersive X-ray spectroscopy (EDS) (Oxford Instruments, England). Using SEM images and the

Accepted manuscript

ACCEPTED MANUSCRIPT

principle of stereoscopy, 3D reconstructions of the topography and roughness information of the electrodes surface were obtained with MeX 5.1 software (Alicona Imaging GmbH, Austria). Raman characterizations (spectrum and mapping) of the PTB-GOx film on the electrode surface were performed with a confocal Raman microscope (WITec GmbH, Gernany), with helium-neon laser (wavelength of 633 nm). All electrochemical measurements were conducted with the potentiostat/galvanostat μAutolab type III (Metrohm Autolab B.V., Netherlands) in the proposed microfluidic system at 20° C, where the pseudo reference and counter electrode were composed of pencil leads (graphite) with 0.5 and 0.7 mm in diameter, respectively, in addition to the working electrode that was made from a pencil lead of 0.5 diameter containing a PTB-GOx film. Blood glucose levels of the volunteers were obtained with a portable glucose meter (LifeScan Inc., USA).

2.3. Preparation of the μTED and PTB-GOx biosensor

The manufacturing process of the μTED is based on the components and dimensions described and optimized in previous works (Agustini et al., 2016, 2017), which employs materials with low cost and great availability. In this work, the base and the detection zone of the device were prepared using as substrate glass plates with 25 mm width, 6.5 mm thickness and 25, 55 and 85 mm lengths (Fig. 1A). In the highest region of the substrate was placed a double-sided tape, three pencil leads that function as a three-electrode system for the electrochemical analyses and two pieces of double-sided tape in order to delimit the area of the detection zone. Before the addition of the pencil lead corresponding to the working electrode, it was gently sanded for the removal of the outer layer of resin that is present in this material. Subsequently, the preparation of the PTB-GOx biosensor was performed (Fig. 1B) with the addition of 50 μL of a buffer solution containing TB and GOx in the detection zone. Cyclic voltammetry (CV) was performed from -0.60 to +0.70 V vs graphite at a scan rate of 50 mV s^{-1} for the immobilization of GOx on the surface of the working electrode due to

Accepted manuscript

ACCEPTED MANUSCRIPT

electropolymerization of TB. The solution was then removed and the detection zone was washed with 3 aliquots of 50 µL of buffer solution in order to remove the species not immobilized. Finally, the assembly of the microfluidic device was completed (Fig. 1C) with the placement of two polymeric reservoirs, one fixed at the upper end of the device (inlet reservoir) and the other at the lower end (outlet reservoir), followed by the addition of a piece of double-sided tape near the inlet reservoir, introduction of the microchannel (9 threads of hydrophilic cotton) from the inlet to the outlet reservoir and placing a small pieces of cotton threads over the region of the electrodes in order to maintain a minimum amount of solution for the electrochemical analyses. The transport of the solutions in the µTED was performed by passive pumping through the capillary and gravitational forces generated in the cotton threads without the need of any mechanical pumping system.

Figure 1

2.4. Electrochemical analysis procedure for the glucose

Glucose analyses were performed by µFIA with the PTB-GOx biosensor integrated to the µTED and detection by chronoamperometry employing an interval time of 100 ms for sampling of signals. PBS pH 7.4 was used as supporting electrolyte, which was added in the inlet reservoir and had its flow maintained in the system throughout the entire analysis time. Using a micropipette, the glucose additions (injection volume of 2.0 µL) were performed directly in the microfluidic device channel 5 mm from the detection zone (Fig. 1D). The determination of glucose in the system was based on the glucose reaction with the GOx without oxygen participation, with the PTB conductive film acts as mediator between the GOx and the electrode surface, where the chronoamperometric response for glucose detection is obtained in the form of a variation for less negative values of the cathodic current of PTB film obtained in the presence of glucose (Wang et al., 2015; Yao and Shiu, 2007), as shown in the reactions below:

Accepted manuscript

ACCEPTED MANUSCRIPT

$$\text{Glucose} + \text{GOx(ox)} \rightarrow \text{Gluconolactone} + \text{GOx(red)} \quad (1)$$

$$\text{GOx(red)} + \text{PTB(ox)} \rightarrow \text{GOx(ox)} + \text{PTB(red)} \quad (2)$$

$$\text{PTB(red)} \rightarrow \text{PTB(ox)} + ne^{-} \quad (3)$$

Some parameters of the proposed method were evaluated and optimized during the work, such as pH of the TB and GOx solution, amount of GOx, number of cycles for electropolymerization and potential applied in the chronoamperometric glucose analyses.

2.5. Detection of tear and blood glucose levels

Tear and blood samples were collected from 10 healthy volunteers of both sexes, in the age group between 20 and 45 years, without medical history of hyper or hypoglycemia, after an 8-hour fast. In order to stimulate the production of tears in a simple and risk-free way for the eyes of the volunteers, macerated raw onion (often employed in domestic preparations) was used, which was placed close to the volunteers for a period of 2 minutes. Tears were collected on the face of the volunteers using a disposable pasteur pipette and then transferred and stored in polymeric tubes. The glucose determinations in the collected tear samples were made using the proposed method with the tears being analysed (sample injections of 2.0 µL) as they were collected, without any purification step. The quantification of the tear glucose levels was done by the standard addition method. At the time of tear collection, the blood glucose levels of the volunteers were also evaluated using a portable glucose meter. Finally, the data obtained with tear and blood glucose determinations were used to evaluate the correlation between the two body fluids.

3. Results and discussion

3.1. Electropolymerization of PTB and GOx on the electrode surface

Accepted manuscript

ACCEPTED MANUSCRIPT

For the PTB-GOx biosensor preparation, the electropolymerization process was performed by CV of a PBS solution pH 7.4 containing 5 mmol L^{-1} TB and 10 mg mL^{-1} GOx. A pair of reversible redox peaks (a) (Fig. 2) at -0.314 V (anodic peak) and -0.373 V (cathodic peak) vs. graphite was observed, which corresponds to the redox reaction of TB monomers (structures I and II). When the scanning of potential by CV in the anodic direction reaches +0.550 V, an abrupt increase in current (b) was observed, corresponding to the formation of cations radical (structures III and III') associated with one-electron oxidation of the amino group which is required for the subsequent electropolymerization of TB (Cai and Xue, 1998). No peak was observed in the cathodic direction, indicating that the cation radical had undergone the following chemical reaction. Then, a new pair of redox peaks (c) was observed at more positive potentials than for the TB monomer peaks, with potential values of -0.150 V in the cathodic direction and -0.120 V in the anodic direction. These peaks correspond to the structures IV and V formed due to the radical dimerization process of the monomers through carbon-nitrogen coupling reactions yielding to PTB film (Zhou et al., 1998). It was observed that the current values related to the pair of redox peaks c increase with the number of cycles, indicating the continuation of electropolymerization process of PTB on the electrode surface.

Figure 2

Finally, after the preparation of the biosensor, CVs analyses were carried out in PBS pH 7.4 with scan rates between 10 and 250 mV s^{-1}. It was observed that the current values for the anodic peak of the PTB had a proportional increase to the scan rate (Fig. S1), indicating that the process is adsorptive-controlled attributed to PTB on the surface of the biosensor (Moon et al., 2017; Wang and Hu, 2006).

3.2. Surface characterization of the PTB-GOx biosensor

Biosensor surface analyses were performed to evaluate the morphology and homogeneity of the PTB-GOx film. Through the SEM micrograph (Fig. 3A) and the 3D

Accepted manuscript

ACCEPTED MANUSCRIPT

reconstructions, it was observed that the pencil lead surface before electropolymerization of PTB and GOx presented an irregular topography (Fig. 3B) and a high roughness (Fig. 3C), with an average value (Ra) of 1.13 µm, which indicates a large surface area. After the electropolymerization step, a more flat morphology was observed with the SEM micrograph (Fig. 3D) and 3D topographic image (Fig. 3E), besides a decrease in the roughness of the PTB-GOx biosensor surface (Fig. 3F), with a Ra of 0.62 µm, indicating a good incorporation of PTB and GOx in the pencil lead surface. In addition, through the chemical mapping performed with EDS (Fig. S2), the presence of N and S was detected along the entire surface of the biosensor, showing that the electropolymerization of PTB and GOx occurred homogeneously on the pencil lead.

Figure 3

In order to complement the information obtained with SEM and EDS, Raman analyses confirmed the presence of electropolymerized PTB and GOx on the biosensor surface, according to the additional peaks observed in the Raman spectrum for the PTB-GOx biosensor when compared with the pencil lead electrode (Fig. 3G) and the assignments of the vibrational bands corresponding to these species (Mažeikienė et al., 2008a; Mažeikienė et al., 2008b; Navratil et al., 2016; Qi et al., 2016) (Table S1). The presence of GOx on the biosensor surface was also confirmed by the high background observed in the Raman spectrum for the PTB-GOx biosensor, which is due to the fluorescence effect generated by flavin belonging to flavin-adenine dinucleotide (FAD), responsible for redox activity of GOx (Cerdeira Ferreira et al., 2013). Finally, a uniform coating of the biosensor surface was observed in the Raman mapping of the band between 440-515 cm^{-1} (Fig. 3H), confirming the efficiency and homogeneity of the immobilization of PTB and GOx on the pencil lead surface.

3.3. Optimization of experimental conditions

Accepted manuscript

ACCEPTED MANUSCRIPT

The analytical parameters related to the preparation of the PTB-GOx biosensor and the detection technique were evaluated and selected in order to obtain a good analytical performance with high sensitivity, speed and stability in the measurements for the glucose detection. For the study and selection of the best experimental conditions, the response to a 2.5 mmol L^{-1} glucose solution was monitored in all optimization analyses with a PTB-GOx biosensor coupled to µTED, employing the chronoamperometric detection and µFIA.

Initially, the influence of the potential applied for the glucose determination was investigated, using biosensors prepared by the electropolymerization of a PBS pH 7.4 solution containing 5 mmol L^{-1} TB and 10 mg mL^{-1} GOx. A progressive increase in both the transient signal current corresponding to glucose and the noise of baseline was observed when the detection potential was changed to more negative values. Therefore, for the selection of the best detection potential, the signal-to-noise (S/N) ratio was employed and not the total current values. Thus, a maximum value was observed at -0.55 V (Fig. 4A), which was chosen for the next steps of the work.

Figure 4

Subsequently, the effect of the amount of GOx used in PTB-GOx biosensor preparation was evaluated using a solution containing 5 mmol L^{-1} TB and amounts of GOx varying between 1 and 40 mg mL^{-1}. There was an improvement in the electrochemical response to glucose with the increase in the amount of GOx used, being observed a maximum current with 10 mg mL^{-1} GOx (Fig. 4B) and a decrease in biosensor response to larger amounts of GOx (20 and 40 mg mL^{-1}). This behavior is explained by a greater decrease in the electrical conductivity of the biosensor surface when large amounts of GOx are immobilized. Therefore, the amount of 10 mg mL^{-1} GOx was used to prepare the PTB-GOx biosensors employed for the further studies, which is a value commonly used in other works (Li et al., 2016; Zhang et al., 2014).

Another parameter studied and optimized was the pH of the TB and GOx solution used in the electropolymerization step. Biosensors were prepared from

Accepted manuscript

ACCEPTED MANUSCRIPT

solutions containing 5 mmol L^{-1} TB and 10 mg mL^{-1} GOx adjusted in pH values varying from 4 to 8. A constant increase in the current values was observed for the glucose analyses using biosensors prepared at pH values between 4 and 7.4 (Fig. 4C) and a decrease in the signal for the biosensor manufactured at pH 8. Thus, for the subsequent studies, a TB and GOx solution at pH 7.4 was selected for biosensor preparation, value of the physiological pH of the human body and that preserves the activity of GOx (Bankar et al., 2009).

Finally, the influence of the thickness of the electropolymerized PTB-GOx film was evaluated, which is controlled by the number of voltammetric cycles applied during the electropolymerization step. In this way, biosensors containing PTB-GOx films electropolymerized between 1 and 20 cycles were used. A rapid increase in glucose current was observed between 1 and 5 cycles (Fig. 4D), and a decrease in response of biosensors prepared with 10 and 20 cycles, which was explained by the difficulty in the diffusion of the species on the electrode surface because of the large film thickness (Yao and Shiu, 2007). Thus, due to better response and fast preparation, for the work sequence, 5 voltammetric cycles were adopted for the electropolymerization of PTB and GOx in the biosensor.

3.4. Analytical performance

Under selected conditions, the analytical characteristics of the proposed method were evaluated by obtaining a calibration curve with injections (in triplicate) of standard glucose solutions (Fig. 5A). A linear response (I (nA) = 57.21 (nA) + 421.16 $C_{Glucose}$ (mmol L^{-1})) was observed with a coefficient of variation of 5.27% for the range of 0.075 to 7.5 mmol L^{-1}, which comprises the glucose levels commonly found in the tear of both non-diabetic and diabetic people. An excellent correlation (99.92%) between the sensitivity of the increasing (421.33 nA L $mmol^{-1}$) and decreasing (421.00 nA L $mmol^{-1}$) glucose concentrations curves was observed (Fig. 5B), indicating the non-leaching of the PTB-GOx film or the loss of enzymatic activity during the analysis. A limit of

Accepted manuscript

ACCEPTED MANUSCRIPT

detection (LOD) of 22.2 µmol L^{-1} (3*SD/Slope of the analytical curve, where SD is the standard deviation of baseline noise) and a limit of quantitation (LOQ) of 74.0 µmol L^{-1} (10*SD/Slope of the analytical curve) were calculated. In addition, the transient signals showed a rapid response to glucose and a short cleaning time, allowing a further injection of the sample every 30 s (analytical frequency of 120 injections per hour).

Figure 5

In order to evaluate the reaction kinetics of immobilized GOx on the biosensor surface, a double reciprocal plot (Fig. 5C) of the analytical curve was constructed to obtain the Michaelis-Menten constant for the biosensor by the Lineweaver-Burk equation:

$$\frac{1}{I_{ss}} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max}} \frac{1}{C_{glucose}} \quad (4)$$

where I_{ss} is the µFIA current reached after glucose addition, I_{max} is the maximum µFIA current obtained for glucose, K_m^{app} is the apparent Michaelis-Menten constant of the PTB-GOx biosensor and $C_{glucose}$ is the injected glucose concentration. A K_m^{app} of 3.64 mmol L^{-1} was obtained, confirming the efficient immobilization of GOx on the PTB-GOx biosensor surface and the high affinity of this enzyme for glucose. Thus, the analytical performance of the proposed method showed values of linear range, LOD and K_m^{app} similar or even better when compared to other methods also based on biosensors and that used miniature devices for the amperometric determination of glucose (Deng et al., 2014; Gangadharan et al., 2011; Gu et al., 2014; Qin et al., 2016; Rama et al., 2017; Wang et al., 2010; Yan et al., 2011; Yang et al., 2014) (Table S2).

3.5. Repeatability, reproducibility and stability studies

For the repeatability and reproducibility assays, µFIA analyses of a 2.5 mmol L^{-1} glucose solution were performed employing the µTED with the PTB-GOx biosensor. A relative standard deviation (RSD) of 2.60% was observed for 20 consecutive glucose

Accepted manuscript

ACCEPTED MANUSCRIPT

injections performed in the same device (repeatability) (Fig. S3A), and a RSD of 3.24% for analyses performed on 6 different devices (reproducibility) (Fig. S3B). Thus, the low RSD values showed the absence of oscillations in the μFIA measurements and a good control in the preparation of the PTB-GOx biosensor and μTED assembly process.

In order to evaluate the long-term stability of the PTB-GOx biosensor, μFIA measurements of a 1 mmol L^{-1} glucose solution were performed for 31 days, using the system for 1 h during each day of analysis and storing with PBS at 4 ° C between the tests. A slight and progressive decrease in the glucose response during the evaluation days (Fig. S4) was observed, obtaining 70% of the initial value on the 31st day of measurement. This stability indicated that the electropolymerization of PTB reduces the denaturation of GOx and prevents the loss of the immobilized film on the biosensor surface during the flow analyses. Thus, even with a loss of 30% in the response over 1 month, the sensitivity presented by the system allows its use for long periods, which requires only a previous calibration.

3.6. Selectivity studies of the PTB-GOx biosensor

For accurate determination of tear glucose levels, it is necessary that the other species present in this fluid do not promote any influence on the results. Therefore, the PTB-GOx biosensor selectivity was evaluated in the determination of 0.5 mmol L^{-1} glucose solutions containing 10 mmol L^{-1} lactate, 10 mmol L^{-1} urea and/or 50 μmol L^{-1} ascorbic acid, which are the main potentially interfering species found in the tear (Yao et al., 2011). According to the results, no significant interferences were observed in the PTB-GOx biosensor response to glucose (variation less than ± 5%), both in the individual and joint evaluation of potentially interfering species for glucose analysis (Fig. S5). In this way, the PTB-GOx biosensor showed a high selectivity for glucose and potential to be employed in the determination of this biomarker in the tear.

3.7. Real samples analyses

Accepted manuscript

ACCEPTED MANUSCRIPT

The measurements of tear glucose were performed according to the proposed method, employing the μTED integrated to the PTB-GOx biosensor for the realization of μFIA with chronoamperometric detection. Tear samples from 10 non-diabetic volunteers were analysed and obtained glucose concentrations between 0,358 and 0.593 mmol L^{-1} (coefficient of variation of 2.58%), values that agree with tear glucose levels (between 0.1 and 0.6 mmol L^{-1}) normally reported (Witkowska Nery et al., 2016). In addition, the blood glucose levels of the volunteers were determined with a portable glucose meter in order to correlate with the tear glucose concentrations observed through the proposed method. A correlation plot was obtained with the results of blood and tear glucose analyses (Fig. 6), in addition to a linear regression of the data following the equation $C_{tear\ glucose}$ (mg dL^{-1}) = -0.727 + 0.0961 $C_{blood\ glucose}$ (mg dL^{-1})) was achieved. An R^2-value of 0.961 and a mean tear/blood ratio of 0.0961 were found for the glucose levels present in these fluids, showing a strong correlation between blood and tear glucose values and a similar ratio of the values described in the literature, which reports glucose levels of 10 times lower in tear than in blood (Hansen et al., 2012; Tehrani et al., 2015).

Figure 6

4. Conclusions

In this work the development of a simple, inexpensive and rapid method for the determination of tear glucose was performed by integrating a PTB-GOx amperometric biosensor, a μTED and measurements by μFIA. The biosensor preparation was done in a simple and fast way, with a single step of 5 voltammetric cycles. Due to the efficient and homogeneous immobilization of PTB and GOx on the biosensor surface, absence of film leaching during flow analysis and slow fall in enzymatic activity over the days was observed, with the PTB-GOx biosensor showing a long shelf life, maintaining 70% of the response after 31 days of analysis. The μTED was composed by inexpensive and highly available materials, simple manufacturing process and

Accepted manuscript

ACCEPTED MANUSCRIPT

excellent reproducibility of response between the devices (RSD = 3.13%). The μFIA technique made possible a new injection of the sample every 30 s without the need to exchange any component of the system, due to the continuous transport of solutions in the system, rapid cleaning of the biosensor surface and high stability between injections (RSD = 2.54%). Thus, the proposed method allowed glucose analyses in tear samples of non-diabetic volunteers without any purification step and in a small volume (few μL). Finally, the results obtained with the measurements of tear glucose reached a close correlation with the blood glucose levels, showing a great potential for a simple, cheap and less invasive monitoring of blood glucose levels through of indirect determination by tear. In order to improve the proposed method, the future work includes the developing and integrating in the analysis system of a miniaturized battery-based electronic device and a smartphone app to replace the conventional potentiostat and microcomputer and make the analysis system totally portable. In addition, other enzymes will be immobilized in the PTB film to explore the versatility of this redox mediator and allow the selective analysis of other biomarkers present in the tear.

Accepted manuscript

Acknowledgements

The authors thank the volunteers for the tear and blood samples used in the glucose analyses.

Supplementary material available: Five figures with surface characterizations of PTB-GOx biosensor and data regarding the stability and specificity of the system for the glucose detection. Two tables with information of the assignment of Raman vibration bands for the biosensor before and after the electrodeposition of the PTB-GOx film, besides of the comparison on the analytical performance of the proposed method with other studies for the determination of glucose.

ACCEPTED MANUSCRIPT

References

Aggidis, A.G., Newman, J.D., Aggidis, G.A., 2015. Biosens. Bioelectron. 74, 243-262.

Agustini, D., Bergamini, M.F., Marcolino-Junior, L.H., 2016. Lab Chip 16, 345-352.

Agustini, D., Bergamini, M.F., Marcolino-Junior, L.H., 2017. Anal. Chim. Acta 951, 108-115.

Ascaso, F.J., Huerva, V., 2016. Optometry and Vision Science 93, 426-434.

Badugu, R., Lakowicz, J.R., Geddes, C.D., 2004. Anal. Chem. 76, 610-618.

Bankar, S.B., Bule, M.V., Singhal, R.S., Ananthanarayan, L., 2009. Biotechnol. Adv. 27, 489-501.

Cai, C.X., Xue, K.H., 1998. Talanta 47, 1107-1119.

Cerdeira Ferreira, L.M., da Costa, E.T., do Lago, C.L., Angnes, L., 2013. Biosens. Bioelectron. 47, 539-544.

Cha, K.H., Qin, Y., Meyerhoff, M.E., 2015. Electroanalysis 27, 670-676.

Chu, M.X., Miyajima, K., Takahashi, D., Arakawa, T., Sano, K., Sawada, S., Kudo, H., Iwasaki, Y., Akiyoshi, K., Mochizuki, M., Mitsubayashi, K., 2011. Talanta 83, 960-965.

Deng, H., Teo, A.K.L., Gao, Z., 2014. Sens. Actuators, B 191, 522-528.

Dhoot, D.S., Avery, R.L., 2016. Curr. Diabetes Rep. 16, 122.

Erenas, M.M., de Orbe-Paya, I., Capitan-Vallvey, L.F., 2016. Anal. Chem. 88, 5331-5337.

Gangadharan, R., Anandan, V., Zhang, A., Drwiega, J.C., Zhang, G., 2011. Sens. Actuators, B 160, 991-998.

Glavan, A.C., Ainla, A., Hamedi, M.M., Fernandez-Abedul, M.T., Whitesides, G.M., 2016. Lab Chip 16, 112-119.

Gu, S., Lu, Y., Ding, Y., Li, L., Song, H., Wang, J., Wu, Q., 2014. Biosens. Bioelectron. 55, 106-112.

Hansen, J.S., Christensen, J.B., Petersen, J.F., Hoeg-Jensen, T., Norrild, J.C., 2012. Sens. Actuators, B 161, 45-79.

Hu, Y., Jiang, X., Zhang, L., Fan, J., Wu, W., 2013. Biosens. Bioelectron. 48, 94-99.

Jang, H., Noh, H., 2015. Macromol. Res. 23, 493-495.

La Belle, J.T., Adams, A., Lin, C.E., Engelschall, E., Pratt, B., Cook, C.B., 2016. Chem. Commun. 52, 9197-9204.

Leblanc, J.M., Haas, C.E., Vicente, G., Colon, L.A., 2005. Intensive Care Med. 31, 1442-1445.

Li, J., Koinkar, P., Fuchiwaki, Y., Yasuzawa, M., 2016. Biosens. Bioelectron. 86, 90-94.

Li, X., Tian, J., Shen, W., 2010. ACS Appl. Mater. Interfaces 2, 1-6.

Mao, X., Du, T.E., Wang, Y., Meng, L., 2015. Biosens. Bioelectron. 65, 390-396.

Mažeikienė, R., Niaura, G., Eicher-Lorka, O., Malinauskas, A., 2008a. Vib. Spectrosc. 47, 105-112.

Mažeikienė, R., Niaura, G., Malinauskas, A., 2008b. Electrochim. Acta 53, 7736-7743.

Moon, J.M., Kim, D.M., Kim, M.H., Han, J.Y., Jung, D.K., Shim, Y.B., 2017. Biosens. Bioelectron. 91, 128-135.

Navratil, R., Kotzianova, A., Halouzka, V., Opletal, T., Triskova, I., Trnkova, L., Hrbac, J., 2016. J. Electroanal. Chem. 783, 152-160.

Accepted manuscript

ACCEPTED MANUSCRIPT

Nilghaz, A., Ballerini, D.R., Shen, W., 2013. Biomicrofluidics 7, 51501.

Pankratov, D., González-Arribas, E., Blum, Z., Shleev, S., 2016. Electroanalysis 28, 1250-1266.

Peng, B., Lu, J., Balijepalli, A.S., Major, T.C., Cohan, B.E., Meyerhoff, M.E., 2013. Biosens. Bioelectron. 49, 204-209.

Qi, G., Wang, Y., Zhang, B., Sun, D., Fu, C., Xu, W., Xu, S., 2016. Anal. Bioanal. Chem. 408, 7513-7520.

Qin, Y., Cui, J., Zhang, Y., Wang, Y., Zhang, X., Zheng, H., Shu, X., Fu, B., Wu, Y., 2016. Sci. Bull. 61, 473-480.

Rama, E.C., Costa-Garcia, A., Fernandez-Abedul, M.T., 2017. Biosens. Bioelectron. 88, 34-40.

Tehrani, F., Reiner, L., Bavarian, B., 2015. PloS One 10, e0145036.

Ulum, M.F., Maylina, L., Noviana, D., Wicaksono, D.H., 2016. Lab Chip 16, 1492-1504.

Wang, F., Gong, W., Wang, L., Chen, Z., 2015. Microchim. Acta 182, 1949-1956.

Wang, Y., He, Q., Dong, Y., Chen, H., 2010. Sens. Actuators, B 145, 553-560.

Wang, Y., Hu, S., 2006. Biosens. Bioelectron. 22, 10-17.

Witkowska Nery, E., Kundys, M., Jelen, P.S., Jonsson-Niedziolka, M., 2016. Anal. Chem. 88, 11271-11282.

Wu, T., Xu, T., Xu, L.P., Huang, Y., Shi, W., Wen, Y., Zhang, X., 2016. Biosens. Bioelectron. 86, 951-957.

Yan, Q., Peng, B., Su, G., Cohan, B.E., Major, T.C., Meyerhoff, M.E., 2011. Anal. Chem. 83, 8341-8346.

Yang, J., Nam, Y.-G., Lee, S.-K., Kim, C.-S., Koo, Y.-M., Chang, W.-J., Gunasekaran, S., 2014. Sens. Actuators, B 203, 44-53.

Yang, X., Pan, X., Blyth, J., Lowe, C.R., 2008. Biosens. Bioelectron. 23, 899-905.

Yao, H., Shum, A.J., Cowan, M., Lahdesmaki, I., Parviz, B.A., 2011. Biosens. Bioelectron. 26, 3290-3296.

Yao, Y.-L., Shiu, K.-K., 2007. Electrochim. Acta 53, 278-284.

Zhang, Y., Li, Y., Wu, W., Jiang, Y., Hu, B., 2014. Biosens. Bioelectron. 60, 271-276.

Zhou, D., Sun, J., Chen, H., Fang, H., 1998. Electrochim. Acta 43, 1803-1809.

Accepted manuscript

Fig. 1. (A) Assembly of the substrate and the detection zone of the μTED. (B) Preparation of PTB-GOx biosensor by cyclic voltammetry electropolymerization of 50 μL of a PBS pH 7.4 solution containing TB and GOx. (C) Completion of the μTED assembly with addition of inlet and outlet reservoir and microchannel (cotton threads). (D) Procedure for chronoamperometric determination of glucose, with PBS pH 7.4 as

supporting electrolyte, sample injection volume of 2.0 µL and detection by µFIA in the µTED containing the PTB-GOx biosensor.

Fig. 2. Cyclic voltammograms obtained during the electropolymerization of 5 mmol L^{-1} TB and 10 mg mL^{-1} GOx on the pencil lead electrode with the respective reactions (a, b and c) and species generated during the electropolymerization process. Scan rate: 50 mV s^{-1}; supporting electrolyte: PBS 0.1 mol L^{-1} pH 7.40.

Fig. 3. SEM characterizations including micrographs, 3D reconstructions of topography and color images of roughness (with average roughness - Ra) of the pencil lead electrode (A-C) and PTB-GOx biosensor (D-F). (G) Raman spectra obtained on the surface of the electrodes before and after the electrodeposition of the PTB-GOx film. (H) Raman mapping of the PTB-GOx biosensor surface using the band between 440-515 cm^{-1}.

Fig. 4. Effect of the applied potential (A), amount of GOx (B), pH of the electropolymerization solution (C) and number of voltammetric cycles (D) for the PTB-GOx biosensor preparation on the chronoamperometric response of 2.5 mmol L^{-1} glucose. Supporting electrolyte: PBS (0.10 mol L^{-1} pH 7.40).

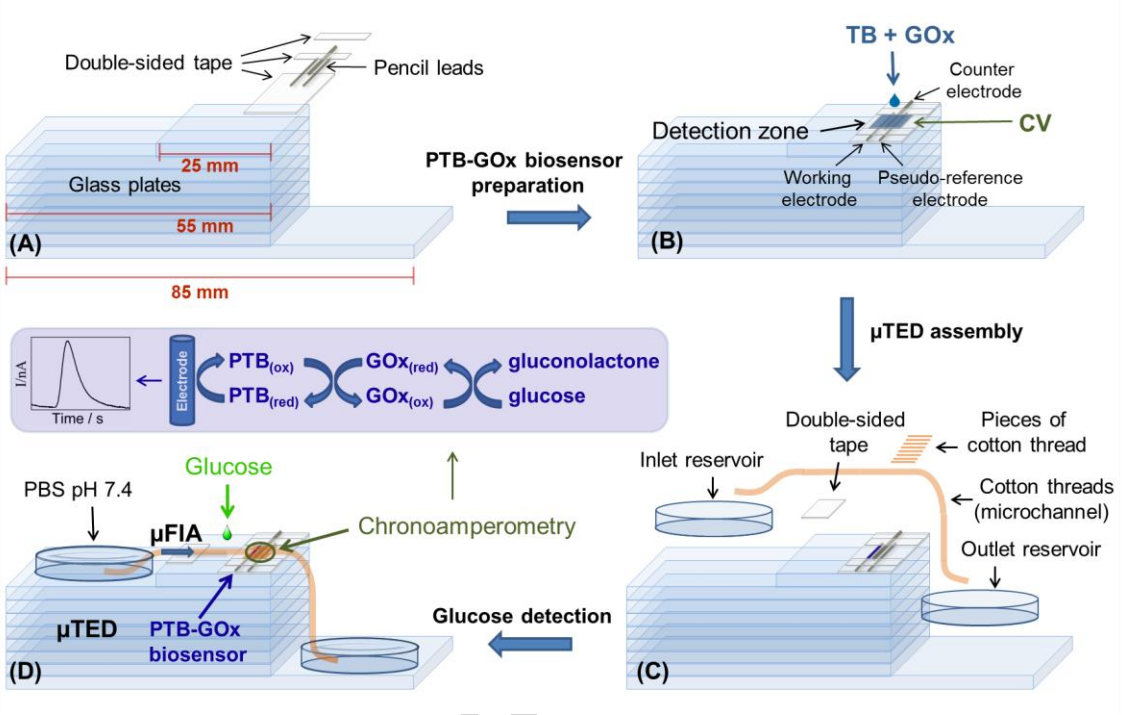
Fig. 5. (A) Chronoamperometric responses obtained for injections (n = 3) of glucose solutions with concentrations between 0.075 and 7.50 mmol L^{-1} using the proposed system. Potential applied: -0.55 V; interval time: 100 ms; supporting electrolyte: PBS (0.10 mol L^{-1} pH 7.40); injection volume: 2.0 µL. (B) Calibration curve constructed with the current values obtained from chronoamperometric responses. (C) Lineweaver-Burk plot obtained for the determination of the Michaelis-Menten constant.

Accepted manuscript

ACCEPTED MANUSCRIPT

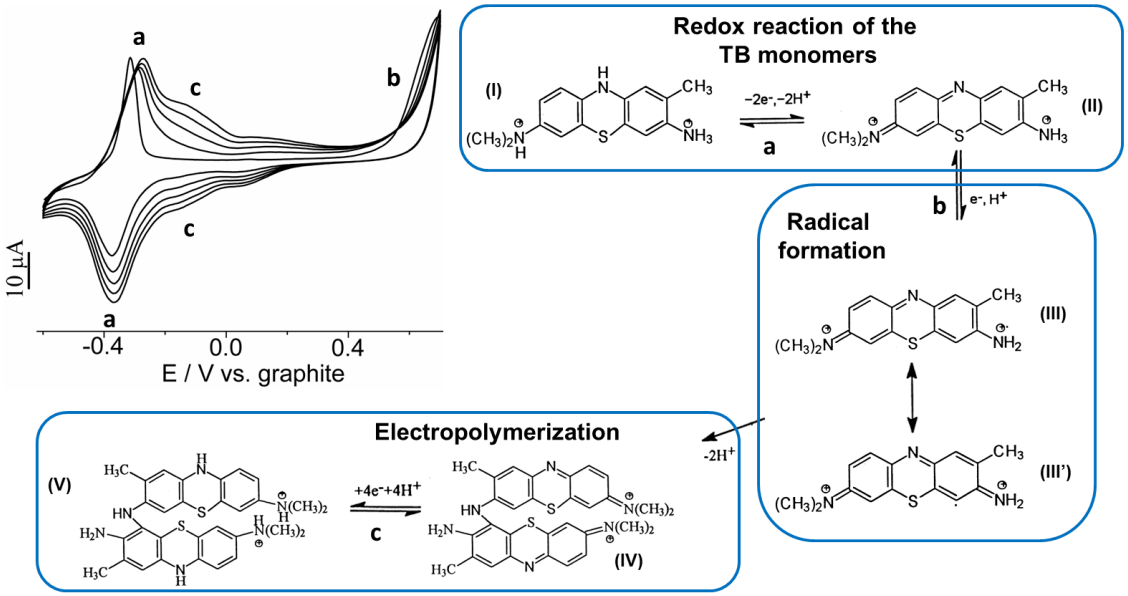
Fig. 6. Correlation plot between tear and blood glucose levels of 10 non-diabetic volunteers.

Figure 1



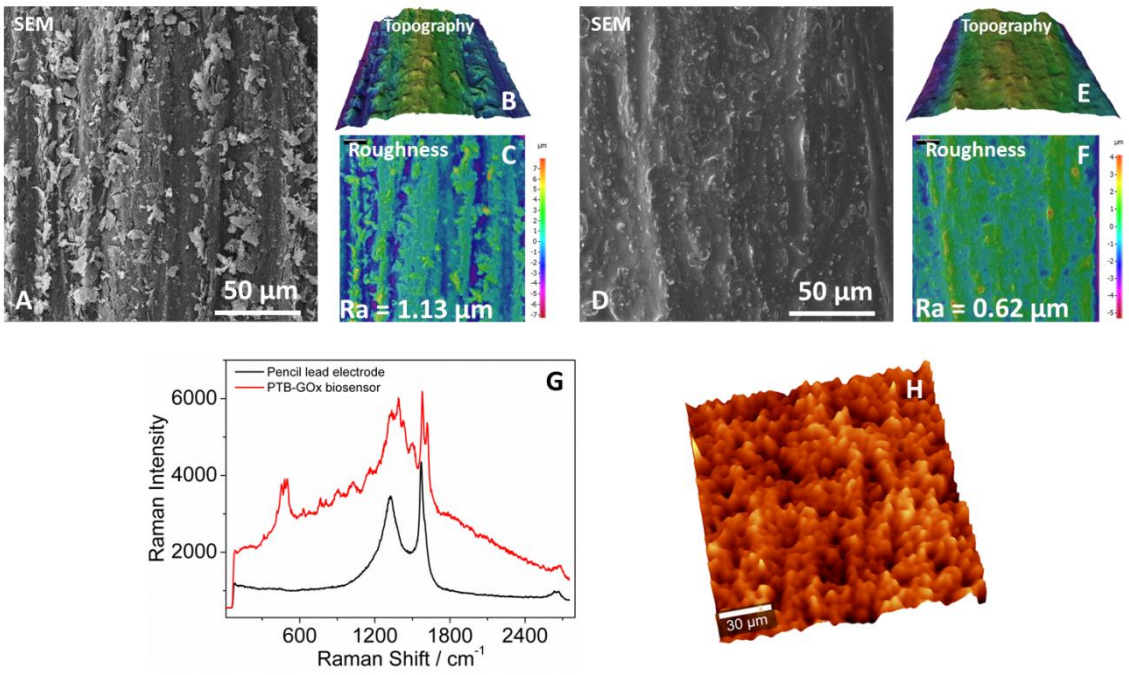
Accepted

Figure 2



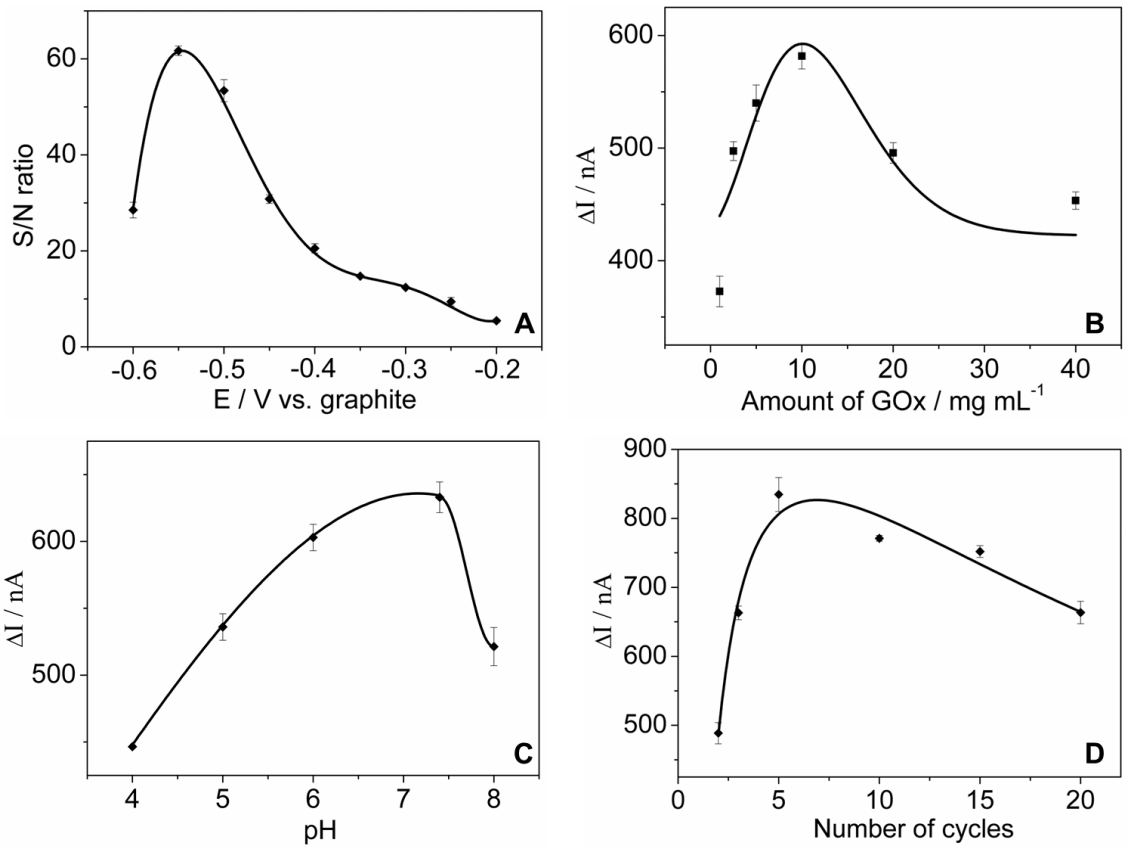
Accepted manuscript

Figure 3



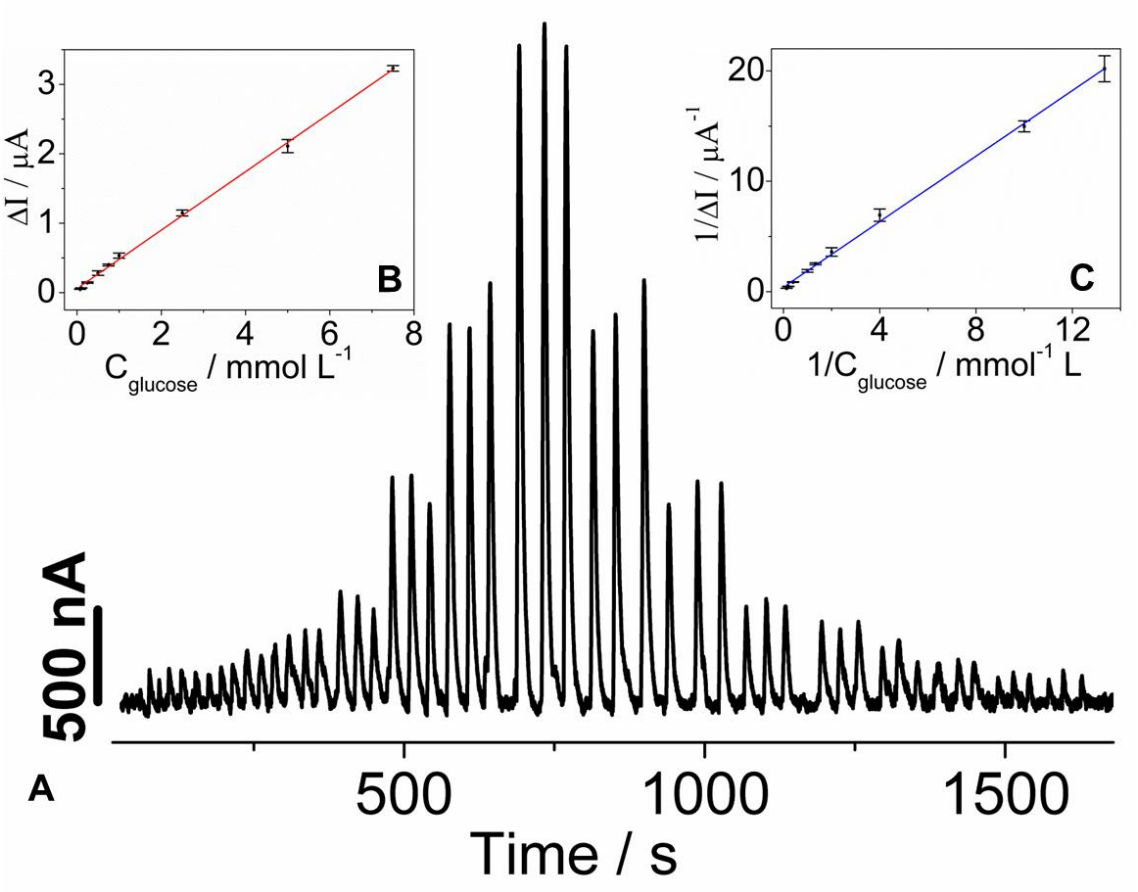
Accepted manuscript

Figure 4



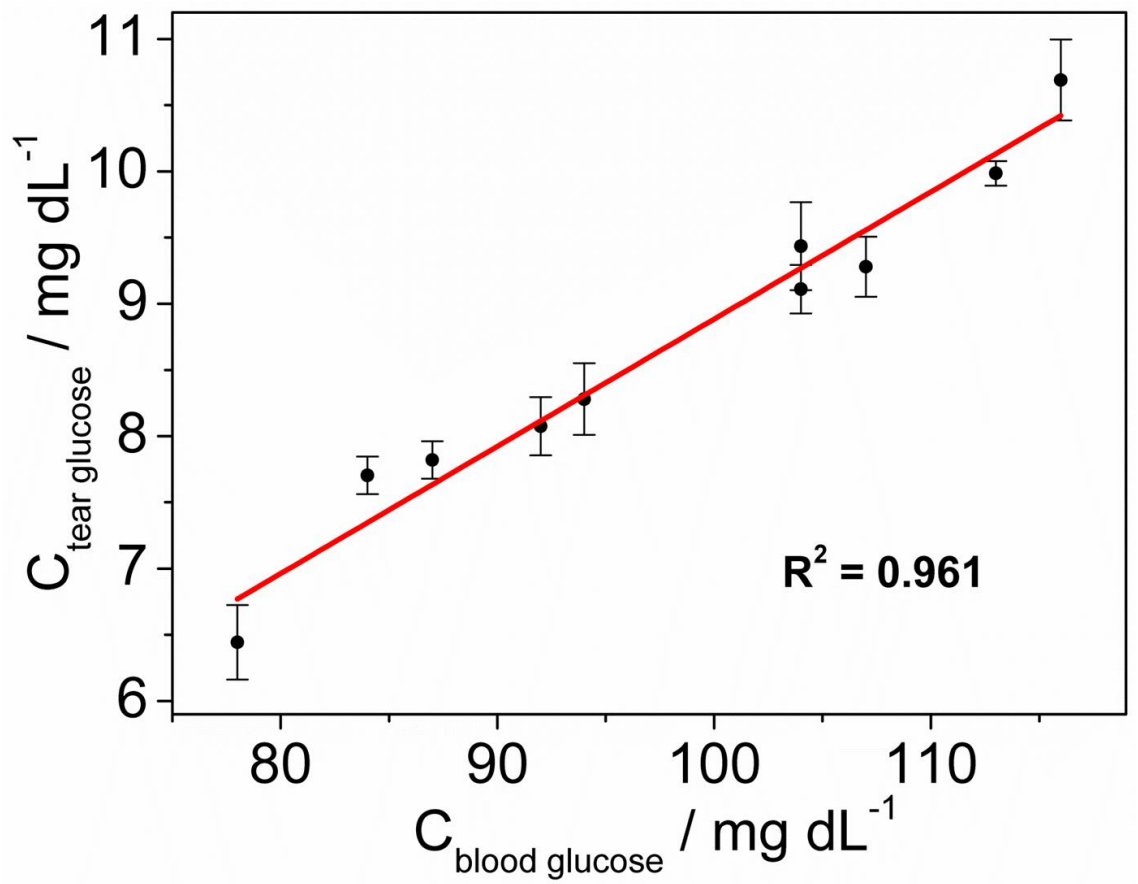
Accepted ma

Figure 5



Accepted m

Figure 6



Highlights

- A simple and inexpensive method for detecting tear glucose was developed.
- Excellent analytical performance was observed with sample injections every 20 s.
- Tear glucose detection were performed without purification in a small sample volume.
- A good correlation was found between the tear and blood glucose levels of healthy volunteers.

Accepted m