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Toward continuous glucose monitoring with planar modified biosensors and microdialysis Study of temperature, oxygen dependence and in vivo experiment

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Abstract

Glucose biosensors based on the use of planar screen-printed electrodes modified with an electrochemical mediator and with glucose oxidase have been optimised for their application in the continuous glucose monitoring in diabetic patients. A full study of their operative stability and temperature dependence has been accomplished, thus giving useful information for in vivo applications. The effect of dissolved oxygen concentration in the working solution was also studied in order to evaluate its effect on the linearity of the sensors. Glucose monitoring performed with serum samples was performed to evaluate the effect of matrix components on operative stability and demonstrated an efficient behaviour for 72 h of continuous monitoring. Finally, these studies led to a sensor capable of detecting glucose at concentrations as low as 0.04 mM and with a good linearity up to 2.0 mM (at 37 °C) with an operative stability of ca. 72 h, thus demonstrating the possible application of these sensors for continuous glucose monitoring in conjunction with a microdialysis probe. Moreover, preliminary in vivo experiments for ca. 20 h have demonstrated the feasibility of this system.

Keywords: Glucose; Continuous monitoring; Screen printed; Mediator; Microdialysis

1. Introduction

Recently major efforts in diabetes diagnostic research have been dedicated to obtaining reliable analytical system for the continuous monitoring of glycaemic level. Due to the fact that glucose levels in blood can change rapidly (2.25 mg/dL min), periodic finger thick tests, largely used by diabetic patients, often fail to detect all hypoglycaemic and hyperglycaemic events. Automated and non-invasive blood glucose monitoring could then offer a valid aid to diabetes care management (Cameron and Ambler, 2004; Pickup and Alcock, 1991; Shamoon, 2000).

Some devices for continuous monitoring are on the market, e.g. the subcutaneous needle sensor of Medtronic (Minimed) having received FDA (Food and Drug Administration) approval for 3 days diabetes management or the iontophoresis based GlucoWatch of Cygnus with FDA approval for monitoring with retrospective analysis (Mastrototaro, 1999; Tamada et al., 1999; Tierney et al., 1999, 2001; Kerner, 2001).

Several attempts have been made to find a truly suitable means for a continuous monitoring of diabetes (Turner et al., 1999; Aussedat et al., 1997, 2000; Mastrototaro, 1999). Several reports describe the use of impedance-based detection systems (Caduff et al., 2003; Park et al., 2003) or near-infrared and mid-infrared spectroscopy (Khalil, 2004) for non-invasive glucose monitoring but the most popular approaches are those based on electrochemical biosensors. In this perspective there are two preferable ways: the use of implantable enzyme sensors (Koudelka et al., 1991; Pickup, 1993) and the use of a microdialysis probe coupled on-line with an electrochemical sensor modified with glucose oxidase (Moscone and Mascini, 1993; Meyerhoff et al., 1992;

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Moscone et al., 1992). This latter is less invasive, more stable, and easier to sterilise.

A wearable system (Glucoday®) based on this principle has been developed by A. Menarini Diagnostics in 2001 and is currently commercialised (Poscia et al., 2003; Varalli et al., 2003; Maran et al., 2002). The instrument is based on the use of a wall-jet cell in which a glucose biosensor is located. This biosensor is obtained by the immobilisation of glucose oxidase on a platinum electrode using a nylon membrane. The biosensor is connected to a microdialysis probe made of a hollow fibre inserted in the subcutaneous tissue. The entire system consists of a specially developed microperistaltic pump, the miniaturised wall-jet cell which contains the glucose biosensor, and the electronic circuit. The device was demonstrated to overcome the problem of sensitivity variation reported for glucose biosensors in the past and it showed an optimal stability for more than 48 h of continuous work (Poscia et al., 2003; Varalli et al., 2003).

Recently we have reported preliminary results obtained with the use of novel planar glucose biosensors as electrochemical probe to be coupled with a microdialysis fiber for continuous glucose monitoring in order to develop an improved Glucoday instrument (Ricci et al., 2005). The sensors were produced using the "screen printing" technique and showed a high degree of reproducibility together with a low cost and the possibility of mass production. Prior to enzyme immobilisation the electrodes were chemically modified with ferric hexacyanoferrate (Prussian Blue). This modification allows the detection of the hydrogen peroxide produced by the enzymatic reaction catalysed by GOD at low applied potential (−0.05 V versus Ag/AgCl), thus limiting electrochemical interferences. The layer of Prussian Blue (PB) showed high stability under the working conditions (pH 7.4). Also after 1 year of storage dry at RT no loss of activity was observed. The assembled glucose biosensors showed high sensitivity towards glucose together with a long-term operational and storage stability. On the basis of these characteristics it was then suggested that such biosensors be used in conjunction with a microdialysis probe for a continuous monitoring of glucose for clinical purposes. In the present work, the same sensor was further evaluated in terms of its eventual clinical application. Problems related to temperature and oxygen dependence were investigated and their effect on the linear range of the sensor was studied.

Finally, the sensors were tested in conjunction with a microdialysis probe, with serum samples and in animals testing, to illustrate the positive and encouraging results.

2. Materials and methods

2.1. Materials and reagents

The perfusion solution (i.e. buffer solution) was prepared by adding 1 g/L of sodium benzoate to a Dulbecco's physiological buffer (NaCl 136.9 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, Na_2PO_4 8.1 mM, pH 7.4).

The glucose test solution (i.e. control solution) was prepared by using the same perfusion solution with a concentration of 5×10^{-4} M of glucose and 0.1% of Kathon added as microbial preservative. Other glucose solutions were prepared by adding different amounts of glucose to the perfusion solution.

All the chemicals were of analytical grade. Ferric chloride, potassium ferricyanide, glutaraldheyde, hydrogen peroxide and β-D-glucose were obtained from Sigma.

Glucose oxidase from *Aspergillus niger* (181 U/mg) was obtained from Sigma.

The human serum, obtained from A. Menarini Diagnostics, was a lyophilised human-based control serum usually used for quality control in clinical chemistry. The serum was reconstituted with distilled water prior to use.

2.2. Glucose biosensor

Screen-printed electrodes (SPEs) were home produced with a 245 DEK (Weymouth, England) screen printing machine. Graphite-based ink was used to print the working electrode, while a silver ink was used for the reference and counter electrodes. The substrate was a flexible polyester film obtained from Autotype Italia (Milan, Italy). The working electrode (diameter 0.2 cm) was modified with Prussian Blue using a chemical procedure already optimised in a previous work (Ricci et al., 2003).

Glucose biosensors were obtained by immobilising glucose oxidase onto the Prussian Blue modified electrode surface. The procedure used consisted is a cross-linking method employing glutaraldheyde and nafion (Ricci et al., 2005).

The biosensors were inserted into a wall-jet cell and connected with a prototype portable instrument capable of applying a constant potential (−50 mV) and also controlling a peristaltic pump with a flow rate of 10 μL/min.

2.3. Instruments

A prototype portable instrument was made available by A. Menarini Diagnostics. The portable system consists of a potentiostat with a fixed applied potential of −50 mV that is connected to the screen printed electrode (glucose sensor) inserted into the cell. The potentiostat is able to continuously record the current produced in the cell and to store current values. A programme allows the downloading of all the stored values in a PC. Moreover the system controls a microperistaltic pump which continuously pumps a solution to the measuring cell at 10 μL/min.

2.4. Microdialysis probe

Custom made poly-sulphone one-way probes were expressly assembled by the supplier (MicroBiothec AB, Stocholm, Sweden) using 0.5 cm of hollow fiber. For in vivo use the probes were sterilised with ethylene-oxide gas.

2.5. Procedures

2.5.1. Operational stability study

The operational stability of the glucose biosensors was evaluated by the following procedure: the biosensors were inserted in the wall-jet cell and a perfusion solution was driven into the

cell by use of a peristaltic pump. Once a stable baseline was obtained (ca. 3 min), the perfusion solution was replaced by a solution of glucose at a fixed concentration and the signal due to glucose was continuously monitored. All the current values recorded by the instrument were automatically converted into glucose concentration values using the following formula:

$$\frac{[i]_c - B}{[G]_c} = F_c, \qquad [G]_n = \frac{[i]_n - B}{F_c} \tag{1}$$

where $[i]_c$ is the current at the time of calibration, $[G]_c$ the glucose concentration present at the time of calibration, B the background current (calculated at the beginning of the monitoring), $[i]_n$ the measured current, and $[G]_n$ is the calculated glucose concentration. This calibration was usually performed at the beginning of each monitoring session. In this case, all the successive points could be automatically converted by the instrument by use of the simple mathematical proportion.

When two calibration points were used, taken at the beginning and at the end of the experimental session respectively, the instrument was programmed to use a weighted calibration factor which varied linearly between the initial and final value.

2.5.2. Temperature monitoring

To evaluate the temperature dependence of the glucose biosensor, a concurrent record of the perfusion temperature was produced using a separate portable instrument that could store values of temperature every three minutes and allows to download the recorded values in an electronic format.

2.5.3. Operational stability with serum sample

To evaluate the stability of the glucose biosensor with biological samples, the continuous flow system was connected to the microdialysis probe and it was perfused with the physiological solution (perfusion solution). At the beginning of the experiment the probe was immersed in a perfusion solution to evaluate the background current. The microdialysis probe was then placed in stirred human serum samples. Moreover, Kathon (0.1%) was included as preservative to avoid the decrease of glucose concentration in the serum due to bacteria growth.

2.5.4. In vivo experiments

The performance of the glucose biosensor used in conjunction with the microdialysis probe was tested by in vivo experiments with dogs (at Research Technology Center, Pomezia, Rome). A sterilised microdialysis fiber was inserted subcutaneously through the skin by use of a specific needle. The fiber was inserted through a Teflon guide and connectors placed on the back of the animal to avoid involuntary muscle contractions near to the sampling point or eventual disconnections of the probe due to the animals movement. No local anaesthetics were used, either during the fiber implantation or during the measurement phase. The dogs were conscious and immobilised in a dedicated cage only at the beginning of the experiment. Once the fiber reached the correct position within the Teflon guide and relative to the connector, the nylon tubes were connected to the measuring instruments and the physiological solution was pumped through the system. After controlling that the solution flowed without leaks or obstructions, the Teflon guide and the connectors were carefully removed leaving the microfibers in place. Finally, the microdialysis probe was fixed by using surgical tape. This procedure took between 5 and 10 min. The instrument was then fully activated, pumping the perfusion solution into the microdialysis fiber and recording the dog's subcutaneous glucose value every 3 min. After 60 min, which are normally sufficient for stabilisation of glucose concentration in the perfusion solution, in vivo calibration of the instrument was performed. This was done by testing the venous blood glucose level (using the Modular Laboratory Instrument, Hitachi) and matching the signal (current value) obtained from the instruments by the linear formula of Eq. (1). In this case $[i]_c$ is the current at the time of calibration, $[G]_c$ is the blood glucose concentration sampled at the time of calibration, B is the background current, $[i]_n$ is the measured current, $[G]_n$ is the calculated glucose concentration.

In the course of the experiment, the values of subcutaneous glucose concentrations, sampled by the instrument, were compared to those determined from blood samples drawn from the dog's ear.

3. Results and discussion

3.1. Operational stability

As stated in the introduction, the objective of this work was to fully characterise a novel glucose biosensor as to its suitability for the continuous monitoring of glucose in diabetic patients. A previous paper has already reported the encouraging preliminary results obtained with new glucose biosensors based on the use of an electrochemical mediator deposited on screen printed electrodes (Ricci et al., 2005). One of the major issues in the development of glucose biosensors to be used in continuous mode is their operative stability which should be maintained as long as possible to avoid misleading results.

However, at this moment it is unrealistic to expect a glucose sensor that would give a perfectly constant response during the auspicated period of monitoring (which is usually intended to be at least for 48 h). This is especially true when the sensor is based on the use of an enzyme. In this context a valid alternative to the non ideal operative stability of the biosensors is represented by the occasional calibration with finger-prick glucose testing performed at fixed times during the monitoring and by the successive adjustment of the glucose calibration using the real values measured. This makes it possible to obtain reliable results even if the biosensors has a systematic decrease of activity during the monitoring. The result would be a very valuable tool for retrospective analysis.

The objective is then to obtain the greatest stability possible so as to allow the use of as few calibrations as possible during the monitoring. As an example of this approach, the Minimed GCM instrument requires four calibrations a day during the eventual 3 days of monitoring. Glucoday, made by Menarini, allows determination of glucose continuously for 48 h and requires only two calibrations, one at the beginning and one at the end of the monitoring.

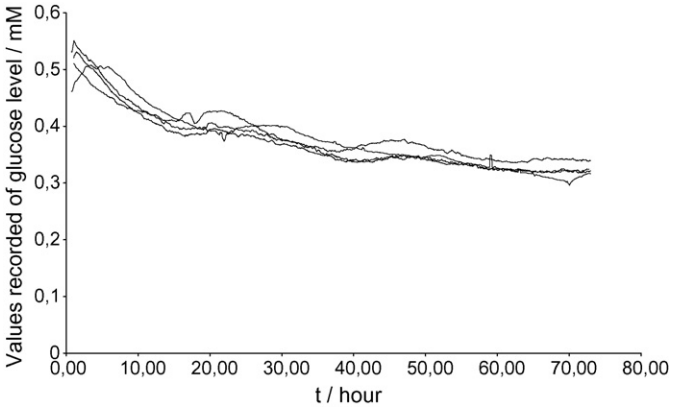

Fig. 1. Glucose continuous monitoring with four different biosensors for a total period of ca. 72 h. Glucose concentration 0.5 mM in buffer solution. Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl. Values at the *y* axes are those recorded by the instrument. One point calibration at the beginning of the monitoring was taken.

The novel glucose biosensors optimised by our group have a very good operative stability which allows the measurement of glucose (0.5 mM) for a period of 72 h with an overall loss of sensitivity of about 30%. The test concentration of glucose utilised with these experiments is usually 0.5 mM and has been chosen taking in account the dilution factor determined by the microdialysis fiber in vivo conditions, which is in the order of 10 times. A normal level of glucose in blood (90 mg/dL, 5 mM) will then result in a final concentration of 0.5 mM for the dialysed solution reaching the electrode surface.

Some typical glucose monitoring profiles obtained using a fixed concentration of glucose of 0.5 mM are shown in Fig. 1. The loss of sensitivity during 72 h was found to be around 30% (R.S.D.% = 7%, $n = 4$ sensors) and was almost the same for all the electrodes tested.

A study of the stability of the biosensors in the absence of any analyte (only buffer) and with H_2O_2 allowed us to understand the cause of the loss in sensitivity. The overall decrease of the signal (ca. 30%) is in fact due to different concomitant events. Fifty percent of this decrease can be attributed to the inactivation of glucose oxidase which results in a decrease of the signal. Thirty percent has instead to be ascribed to the loss of mediator leaching from the electrode surface. Twenty percent of the overall decrease of the signal is provoked by the ineluctable decrease of the background current level which then results in an apparent decrease of the signal. This is due to the fact that during the monitoring it is not possible to check the background current. This last contribution should be not properly ascribed to the loss of sensitivity of the sensor being an apparent loss of sensitivity caused by decrease of the background component.

The stability that was observed for these glucose biosensors makes them in any case applicable for retrospective analysis. For this kind of application, the sensors require the calibration of the results with more than one single point. Fig. 2(a) shows a monitoring profile which was calibrated with two points, one at the beginning and one at the end of the monitoring. In this example, all the values between these two points are calculated using a mathematical algorithm which takes into account the

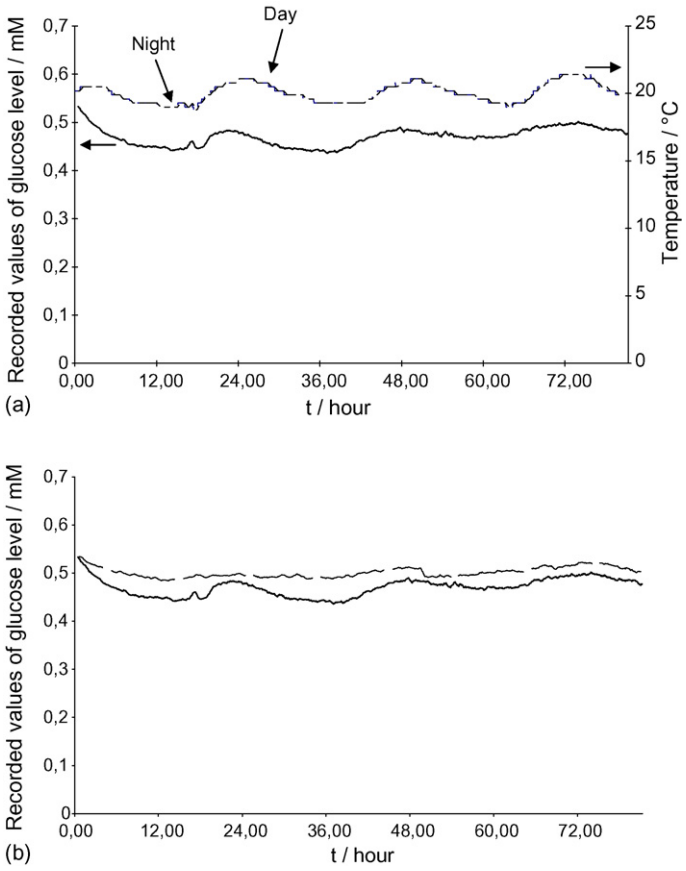

Fig. 2. Glucose continuous monitoring for a total period of ca. 72 h. Glucose concentration 0.5 mM in buffer solution. Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl. Values at the *y* axes are those recorded by the instrument. In (a) and (b) solid line represents the monitoring with two calibration points one at the beginning and one at the end of the monitoring. Dashed line in (a) temperature monitoring recorded in parallel and in (b) monitoring calibrated with the temperature values. In (a) the night/day transition of the first 24 h is also shown.

distance of each point in time from the two calibration points (see experimental part). As can be seen, the use of two calibration points provided for more reliable behaviour of the glucose biosensors. However, a strong oscillation of the glucose signal is still observed during the monitoring period. This corresponded to the night–day transition, as is indicated in Fig. 2(a), due to the fact that the experiments were carried out in a non thermostated ambient. Studies of temperature dependence were then required as a means to compensate for such effects.

3.2. Temperature dependence

The temperature dependence of the glucose biosensors could be due to two possible factors. One is related to the enzymatic activity of glucose oxidase and the other to the H_2O_2 response of the mediator (Prussian Blue). However, continuous monitoring of H_2O_2 during 72 h with temperatures ranging from 22 to 37 °C demonstrated that the mediator was not influenced by temperature changes. Thus, the temperature dependence of the biosensor can be ascribed completely to the decrease in enzyme activity. This dependence is in turn due to two

different factors: (a) variation of the actual enzymatic activity and (b) variation of oxygen concentration as a function of temperature.

The first point is related to the classic dependence of enzymatic activity on temperature. The second point is instead strictly related to the reaction catalysed by GOx:

$$\text{Glucose} + O_2 \rightarrow \text{Gluconic} \quad \text{Ac.} + H_2O_2$$

It is clear that the reaction rate will be directly dependent on the concentration of dissolved oxygen in the working buffer, which in turn depends on the ambient temperature. These two effects are both important and are in competition in the range of 22–37 °C. In the case of the enzymatic activity, it tends to increase with increasing temperature values (up to 37 °C). This effect is offset, especially at high glucose concentration, by the lower solubility of oxygen at higher temperatures. By continuously measuring the temperature during the glucose monitoring, it was possible to observe the effective temperature dependence of the glucose biosensors (Fig. 2(a)) and to calculate that there was a 4% variation of the current signal for each centigrade degree change. By recording temperature values during the course of the monitoring, it is then possible to re-calibrate the values determined by the instrument by using a simple mathematical formula:

$$\text{Calibrated current values} = i_t + (i_t \times 0.04)(T_0 - T_t)$$

where i_t is the current recorded at the time t, 0.04 the 4% correction for each centigrade degree, T_0 the temperature recorded in correspondence of the first calibration point and T_t is the temperature at the time t.

Applying this formula, an attenuation of the night–day variation could be observed (Fig. 2(b), 0.5 mM). As it is clear from the figure, the temperature based calibration reduced the fluctuations thus resulting in a steady response of the biosensors. Although it has been demonstrated here that the glucose biosensor is temperature-sensitive, in vivo conditions will normally prevent the presence of strong temperature variations. This is due to the fact that fluid temperature tends to be constant and then the dialysed sample exiting the microdialysis probe and entering the wall-jet cell are expected to be almost at a fixed temperature. Still, it could be possible to observe a 2–3 °C variations in the subcutaneous fluid temperature and also low ambient temperature could cause a change in the dialysed sample temperature before reaching the sensor surface. The monitoring of the fluid temperature thus becomes extremely important in the practical application of this biosensor.

To confirm these results, and in addition to mimic future in vivo application of the glucose biosensor, an experiment was performed in which a 0.5 mM glucose solution at a fixed temperature of 37 °C (to simulate the subcutaneous fluid) was monitored using our calibration regimes. The profiles are shown in Fig. 3 for both the one and two calibration methods. It could be observed that the overall loss of sensitivity was the same as observed at room temperature (ca. 30%) thus demonstrating that, at least in the tested temperature range, the activity decrease is not dependent on temperature. Also it can be observed that at a given temperature, the night–day variations disappeared. It could be seen that with two calibration points, a constant level of glucose results for the analysis.

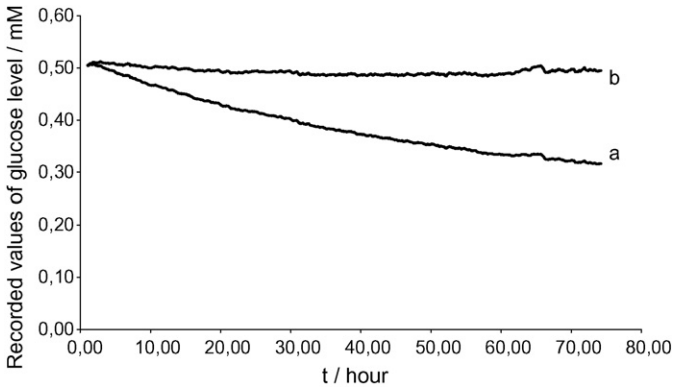

Fig. 3. Glucose continuous monitoring for a total period of ca. 72 h at 37 °C fixed temperature. Glucose concentration 0.5 mM in buffer solution. Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl. Values at the *y* axes are those recorded by the instrument. In the case of curve 'a' one point calibration at the beginning of the monitoring was taken. For the curve 'b' two calibration points one at the beginning and one at the end of the monitoring were taken.

3.3. Biosensor coupled with microdialysis probe

The glucose biosensors optimised and utilised in this work are intended for use in conjunction with a microdialysis probe. The effect on the operative stability and temperature variations should then be carefully studied with this configuration. A preliminary study to this effect has already been reported for a limited time with a dialysed sample continuously pumped over the electrode surface (Ricci et al., 2005). Here, the biosensor was instead directly connected to a microdialysis probe. The probe was inserted in a serum sample in constant agitation to mimic the subcutaneous liquid. Serum glucose concentration was fixed at 5 mM and kept constant in the presence of a preservative agent. The operative stability of the glucose biosensor under these conditions was very similar to that observed with standard glucose solution; a loss of ca. 30% was observed after 72 h. This indicates that the matrix components of the serum do not negatively affect the overall stability of the biosensor and also that the microdialysis fiber recovery is constant in this period. The dilution and protective barrier effect provided by the microdialysis probe are in part responsible for this stability. Using two points calibrating the monitoring a constant profile could be obtained (Fig. 4). Despite this, the application of the temperature correction with a 4% factor failed to avoid temperature fluctuations. This is due to the fact that the microdialysis probe recovery is also temperature dependent. With high temperature in fact, the permeability of the fibre increases thus allowing a higher recovery of glucose. To stabilise these fluctuations it is necessary to apply a 12% factor for each centigrade degree so that steady behaviour could be observed (Fig. 4). A slight oscillation of the recorded calibrated values are probably to be ascribed to the microdialysis fiber recovery which is not perfectly constant during the entire monitoring period. Even if the temperature dependence of the microdialysis probe is significant, it should be considered that

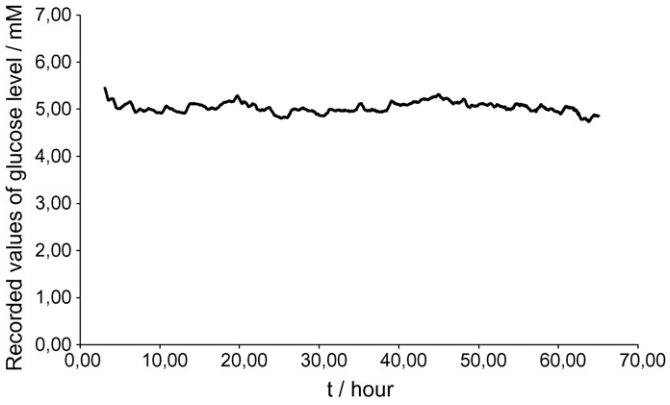

Fig. 4. Glucose continuous monitoring for a total period of ca. 70 h with glucose biosensor coupled with a microdialysis probe. Microdialysis fiber inserted in a stirred human serum sample with a glucose concentration of 5.0 mM. Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl. Values at the *y* axes are those recorded by the instrument. Two points one at the beginning and one at the end of the monitoring were taken. Calibration with the temperature values recorded simultaneously was also performed (see text for details).

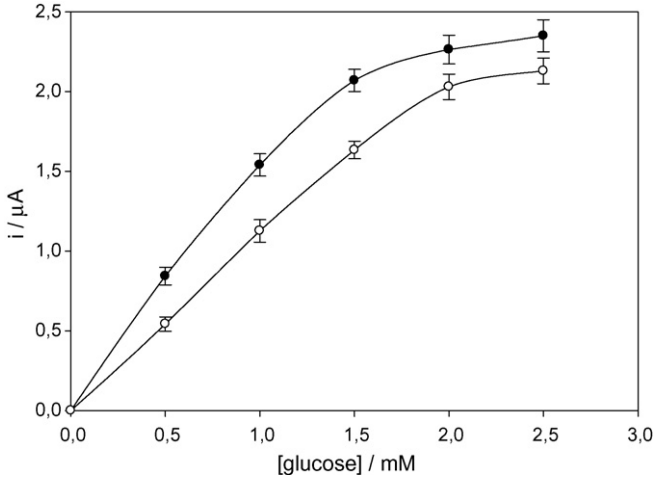

Fig. 5. Glucose calibration plot at 22 °C (◯) and 37 °C (●). Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl.

being inserted in the subcutaneous tissue it will be protected by large temperature variations.

3.4. Linearity of the glucose biosensor

One of the major issues associated with the use of glucose biosensors for clinical application is their linearity range. Due to the high concentration of glucose in the blood and to limitations of the oxygen concentration, the problem of obtaining a wide linear range in hyperglycaemic region, while still maintaining the necessary sensitivity, has always affected GOD-based biosensors. In this work, as already mentioned, the conjunction with the microdialysis probe allows a dilution of the subcutaneous liquid of about 10 times. This means that the biosensor only has to respond linearly to concentrations of glucose up to 1.5–2.0 mM; corresponding to glucose levels in the subcutaneous liquid of about 15–20 mM (270–360 mg/dL). Glucose levels in case of severe hypoglycaemic periods will be around 30–40 mg/dL (ca. 2.0 mM); useful biosensors then have to accurately measure concentrations of glucose as low as 0.1 mM. The optimised glucose biosensor used in this work allows the detection of glucose in a range between 0.025 mM (s/n = 3) and 2.0 mM with a sensitivity of 35 mA/M cm^2 (Fig. 5) and is thus appropriate for an in vivo application. Despite this, the study performed at high temperature (37 °C) has highlighted an important issue which should not be underestimated. As already stated, the temperature dependence of the sensor can be totally ascribed to the enzyme activity and related to the factors of enzymatic activity and oxygen concentration. In the study of linearity, the aspect of oxygen concentration was the most important. The enzymatic reaction directly depends in fact on the concentration of the dissolved oxygen in the solution, which in turn is dependent on the external temperature by the Henry's law. A shift from 23 to 37 °C will result in a decrease in dissolved oxygen concentration in solution from 8.1 ppm (ca. 0.5 mM) to 6.8 ppm (ca. 0.44 mM). This variation (i.e. 14%), although apparently low, could lead to a different behaviour of the biosensor at high glycaemic levels where oxygen concentration is limiting. This is demonstrated in Fig. 5 where the calibration plots obtained at 22 and 37 °C are shown. The current values obtained at 37 °C are higher than those observed at 22 °C thanks to the already discussed temperature dependence of the enzymatic activity. However, the linearity of the glucose biosensor at 37 °C is diminished relative to that observed at 22 °C. To overcome this problem for practical application at 37 °C, a simple diminution of the enzyme amount to be immobilised on the electrode surface has been performed. This leads to a lower consumption of oxygen in the measuring solution, thus allowing extension of the linear range to sufficiently high concentrations. Oxygen limitation is in fact responsible for the low linearity and its consumption must be controlled in order for it not to be limiting for the enzymatic reaction. However, diminishing the enzyme amount on the electrode surface leads obviously to a lower sensitivity of the sensors (25 mA/M cm^2). This disadvantage is mitigated by the fact that with high temperature the enzyme activity is increased thus giving a higher current response. The overall sensitivity of the biosensor in these conditions (37 °C and lower enzyme amount) allows the measurement of glucose from 0.04 mM (s/n = 3) to 2.0 mM, that is a range similar to what is obtained at 22 °C.

The stability of the glucose biosensors thus has to be confirmed with this new configuration and also with higher glucose concentrations, also considering the possibility of long-term hyperglycaemia. For this reason, further stability experiments were performed with a glucose concentration of 2.0 mM which, taking into account the dilution performed by the microdialysis probe, corresponds to a glycaemic level of 20 mM (360 mg/dL). The same loss of sensitivity (ca. 30%) was observed with this concentration of glucose after 72 h, thus demonstrating that high glucose levels do not negatively affect the overall stability of the biosensors. Also, as a confirmation, a complete monitoring of 100 h was performed with four different glucose concentrations (0.5, 1.0, 1.5, and 2.0 mM) and demonstrated a steady response of the biosensors (Fig. 6).

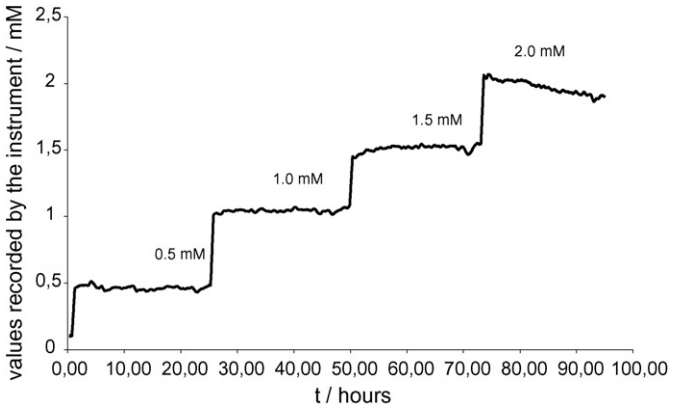

Fig. 6. Glucose continuous monitoring for a total period of ca. 100 h. Glucose concentration 0.5, 1.0, 1.5 and 2.0 mM in buffer solution. Flow rate = 10 μL/min; applied potential = −50 mV vs. Ag/AgCl. Values at the *y* axes are those recorded by the instrument. Two points calibration at the beginning and at the end of the monitoring were taken. Calibration with temperature data was also performed.

3.5. *In vivo experiments*

Fig. 7a shows the glucose profiles recorded during a preliminary in vivo experiment with a dog for about 20 h. The figure also reports the values of the control points taken at various intervals. These results demonstrate how the fiber system connected to the biosensor reliably follows the blood glucose concentration. During the night period (from 23:00 p.m. till 7:00 a.m.), no control points where taken. Given the fact that the experiment were performed in a temperature controlled room, no temperature control was necessary. The sensor response time is fast enough and the stability of the sensitivity observed quite acceptable. The profile in Fig. 7b shows a part of a different monitoring of ca. 2 h. During this period an oral infusion of glucose was performed. It can be seen how the biosensor adequately responds to high glucose levels during this period. Also the biosensor showed good stability and high sensitivity during the entire monitoring period. A medical examination of the animal's skin after the monitoring has confirmed the absence of any problems due to the fiber's presence.

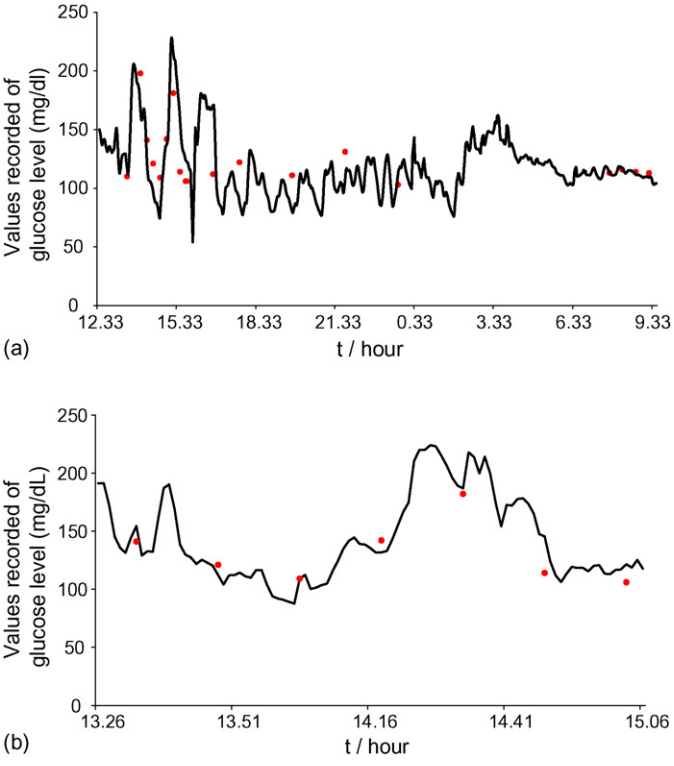

Fig. 7. Glucose continuous monitoring in dog. (a) Entire monitoring of about 20 h; (b) ca. 2 h monitoring where hyperglycaemic levels are visible. Red dots identify blood glucose control points. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

To provide an overall estimation of the accuracy, the dog's plasma venous glucose determinations were plotted versus the glucose values determined by the instrument in an error grid analysis form. One hundred percent of the 18 points falls within the A + B region. The mean bias was 4 mg/dL and the correlation coefficient was equal to 0.9489.

4. Conclusions

A detailed study of the operative stability of glucose biosensors has been performed, showing good behaviour for up to 72 h continuous monitoring at different glucose concentrations (0.5, 1.0, 1.5, and 2.0 mM) and with temperature as high as 37 °C. A strong temperature dependence has been observed, resulting in a 4% variation in the current value for each centigrade degree change. The need of a parallel monitoring of the temperature has been then highlighted in order to avoid misleading results due to possible temperature changes. Monitoring performed with serum samples in conjunction with microdialysis fiber demonstrated efficient behaviour for 72 h continuous monitoring with little matrix effect on operative stability. Linearity of the sensors was also studied taking in consideration the dissolved oxygen concentration in the working solution and its dependence upon temperature changes. Also in this case, care is required in order to take this issue into account for future in vivo applications. Under the optimised conditions, sensors were capable of detecting glucose at concentration as low as 0.04 mM and with a good linearity up to 2.0 mM (at 37 °C). In vivo experiments with dogs for ca. 20 h have demonstrated the feasibility of the system proposed in this study suggesting the utility of successive clinical studies on diabetic patients.

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