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A blood-assisted optical biosensor for automatic glucose determination

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ABSTRACT

A new approach for glucose determination in blood based on the spectroscopic properties of blood hemoglobin (Hb) is presented. The biosensor consists of a glucose oxidase (GOx) entrapped polyacrylamide (PAA) film placed in a flow cell. Blood is simply diluted with bidistilled water (150:1, v:v) and injected into the carrier solution. When reaching the PAA film, the blood glucose reacts with the GOx and the resulting H_2O_2 reacts with the blood Hb. This produces an absorbance change in this compound. The GOx–PAA film can be used at least 100 times. Lateral reactions of H_2O_2 with other blood constituents are easily blocked (by azide addition). The linear response range can be fitted between 20 and 1200 mg dL^{-1} glucose (R.S.D. 4%, 77 mg dL^{-1}). In addition to the use of untreated blood, two important analytical aspects of the method are: (1) the analyte concentration can be obtained by an absolute calibration method; and (2) the signal is not dependent on the oxygen concentration.

A mathematical model relating the Hb absorbance variation during the reaction with the glucose concentration has been developed to provide theoretical support and to predict its application to other compounds after changing the GOx by another enzyme. The method has been applied to direct glucose determination in 10 blood samples, and a correlation coefficient higher than 0.98 was obtained after comparing the results with those determined by an automatic analyzer. As well as sharing some of the advantages of disposable amperometric biosensors, the most significant feature of this approach is its reversibility.

1. Introduction

Since research into blood glucose determination methods continues to be particularly important in bioanalytical chemistry, new alternatives are continuously being proposed and tested.

On the one hand, several body fluids such as aqueous humor from the eye [1], saliva [2] and mainly interstitial fluids (ISFs) [3] are being used as alternatives to blood in order to design a less invasive methodology (very important for glycemic control in diabetic patients). The most interesting is ISF; here, technological alternatives currently being tested include transdermal extraction (iontophoresis [4] or ultrasound [5]), direct spectral dermis measurement [6] or subcutaneous punction [7]. Developments in this field are producing very interesting analytical solutions towards achieving a definitive system. However, the relationship of the glucose levels in these fluids with real blood glucose remains a source of controversy [8,9]. Consequently, improvements in direct glucose determination in blood are still necessary.

On the other hand, the chemical and the transducer parts of the analytical system are simultaneously being developed. Most procedures are based on the use of proteins and enzymes as recognition reagents, given their selectivity. The most commonly used is the classical method based on the glucose oxidase (GOx) reaction:

$$\text{glucose} + O_2 \leftarrow \text{GOx} \rightarrow \text{glucuronicacid} + H_2O_2$$

Several of the previously indicated semi-invasive devices work according to this reaction coupled with H_2O_2 amperometric detection [3–5,7]. Besides the need for frequent recalibration, this method has two main problems [10,11]: the signal dependence with the O_2 concentration and the interference caused by other endogenous substances (uric acid, ascorbic acid) or commonly used pharmaceuticals (acetaminophene). These problems can be partially resolved (but not completely) by using a charge transfer mediator. However, this mediator is usually bio-incompatible and complicates the reversibility of the system, so this methodology is generally used in portable disposable punction sensors.

In recent years optical detection methods based on the use of the intrinsic and extrinsic optical properties (fluorescence and absorption) of proteins are being considered. These methods represent an attempt to avoid the problem caused by electrochemical detection and to make use of their potential reversibility. The conformational-change-based-fluorescence of binding proteins [12–15], the change in fluorescence originated by cellular signaling (in which regulatory proteins are implied) [16,17], and the optical intrinsic properties

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of proteins such as tryptophan [18–20]) or heme groups [21] have proved to be the most interesting bases for these methods. Such methodologies are designed to be selective, reversible, compatible with biological fluids and used as an alternative to synthetic or semi-synthetic organic receptors [22,23]. However, most of these methods require previous steps in order to eliminate interference, and this limits their applicability in future clinical designs. Such previous steps usually involve precipitation or ultra-filtration for the elimination of various blood constituents that can cause interference.

In order to avoid these problems, we have recently developed analytical methods based on the use of the optical properties of hemeproteins [24,25]. These contain heme as the cofactor that shows a molecular absorption spectrum in the visible region which depends on its oxidation state, so that the enzymatic reaction can be followed by means of the absorbance variations during the process. Based on this, we have developed an optical biosensor for the direct glucose determination in blood which uses the horseradish peroxidase (HRP) absorbance variation during the glucose/glucose oxidase/HRP enzymatic reaction [26]. As is known, Hb is a hemeprotein present in the erythrocytes which is made up of two alpha and two beta chains, each containing a heme group with ferrous iron reversibly joined to molecular oxygen ($HbII{\cdot}O_2$). In blood analysis using molecular spectrochemical methods, this protein represents a strong spectral and chemical interference. However, since Hb can react with hydrogen peroxide and this reaction can be followed by the changes in its molecular absorption spectrum, this interference can be converted into a reagent.

Considering the optical transducer qualities of blood Hb [27] and our previous analytical knowledge of the combined glucose oxidase/glucose with Hb/H_2O_2 reaction mechanism as starting points [28], in this paper we demonstrate that these properties can be used as the basis for reversible optical sensors for glucose determination in blood and that they could also be used for automatic analyzers. Unlike the previously developed sensor [26], this sensor does not require an indicating protein (such as HRP) because it is provided by the sample itself. The linear response range can be properly modified and the precision is similar.

In our opinion, this type of sensor can compete with the current commercially available disposable amperometric glucose sensor or with kit assays used in automatic analyzers [29,30]. With slight modifications, this methodology can be used for the design of optical sensors for other blood components.

2. Experimental

2.1. Apparatus

The molecular absorption measurements were carried out in a Hewlett-Packard 8452A diode-array spectrometer and in a PerkinElmer Lambda 5 (spectral bandwidth 2 nm) spectrometer. The measurements were performed with a previously described flow cell (300 μL capacity).

2.2. Reagents

Buffer solution: 0.1 M citrate buffer of pH 5–6 (from solid sodium citrate, adjusted with NaOH 1 M) and 0.1 M phosphate solution of pH 6 (from solid KH_2PO_4 and solid Na_2HPO_4). Sodium azide from Sigma (S-8032). Glucose solutions prepared from solid β-D-glucose (Sigma G-5250) in buffer solution (the solution is left for 2 h to achieve the equilibrium between β-D-glucose and α-D-glucose). Glucose oxidase was taken from *Aspergillus niger*, EC 1.1.3.4 (Sigma G-7141) was of 245,900 IU/g of lyophilised solid. A_0 Human Hemoglobin (A_0) (Sigma H-0267). Blood was taken from 10 volunteers by finger punction.

2.3. GOx film preparation

The preparation of a polyacrylamide (PAA) glucose oxidase film was based on a general enzyme immobilisation procedure described elsewhere [11]. 0.2 mL of a phosphate buffer solution at pH 6.0 containing 0.03 g of acrylamide, 0.002 g of bis-acrylamide and 0.001 g of ammonium persulphate (as a reaction precursor) were mixed with the appropriate amount of the GOx lyophilised solid (about 0.004 g). Dissolved oxygen was eliminated by bubbling nitrogen through the solution. The cocktail was spread in a 0.5 mm hollow, made in a glass film (20 mm × 9 mm × 0.1 mm), covered with a second glass film and irradiated with a UV-lamp (254 nm) for 60 min. The film was then stored in the phosphate buffer solution at 4 °C. The film was located in a flow cell previously described for absorbance measurement [24].

2.4. Blood sample preparation

The only sample treatment was dilution in bidistilled water 1/150 (v:v). The mixture was stirred during 30 s until the hemolysis (erythrocyte lyses and Hb liberation) was completed, detected by the disappearance of the turbidity initially observed.

2.5. Procedure

The phosphate buffer solution with added azide flowed through the flow cell at 0.84 mL min^{-1} and the absorbance at the working wavelength (represented by sub-index λ_w, 412 nm in most cases) and the reference wavelength (represented by sub-index λ_{ref}, 444 nm when 412 nm working wavelength is used) began to be monitored. The initial absorbance of the film was represented by the superscript "film" ($Abs^{film}_{\lambda_w}$ and $Abs^{film}_{\lambda_{ref}}$, respectively). Then 0.8 mL of the blood sample was injected and the flow stopped when the flow cell was completely filled with the sample (in the designed system at 45 s). At this moment the absorbance measured at both wavelengths is represented by the sub-index "0" ($Abs^{meas}_{\lambda_w,0}$ and $Abs^{meas}_{\lambda_{ref},0}$, respectively). The analytical parameters obtained at a given reaction time t are:

$$\Delta Abs_{\lambda_w,t} = Abs^{meas}_{\lambda_w,t} - Abs^{meas}_{\lambda_w,0}$$

$$\Delta Abs_{\lambda_w/\lambda_{ref},t} = \frac{Abs^{meas}_{\lambda_w,t} - Abs^{meas}_{\lambda_w,0}}{Abs^{meas}_{\lambda_{ref},0} - Abs^{film}_{\lambda_{ref}}}$$

$Abs^{meas}_{\lambda_w,t}$ and $Abs^{meas}_{\lambda_{ref},t}$ being the absorbances at a time t at the working and reference wavelengths, respectively.

3. Results and discussion

3.1. Blood hemoglobin as a transducer reagent

To use Hb as an analytical reagent, lyses induction in erythrocytes without coagulation is required. To achieve this, simple blood dilution in a hypotonic media such as bi-distilled water in an appropriate ratio is sufficient [24,27,28]. In this study, a dilution ratio of 150:1 (v:v) was selected considering the concentrations of glucose in blood, natural hemoglobin concentration and the elimination of possible interferences with the Hb/H_2O_2 reaction. These diluted solutions are stable during more than 24 h in a closed vessel, away from the light and at room temperature.

Apart from Hb, other proteins (mainly enzymes) present in blood can consume H_2O_2. Considering the Hb and glucose concentrations, and the level at which the sample is diluted, only blood catalase

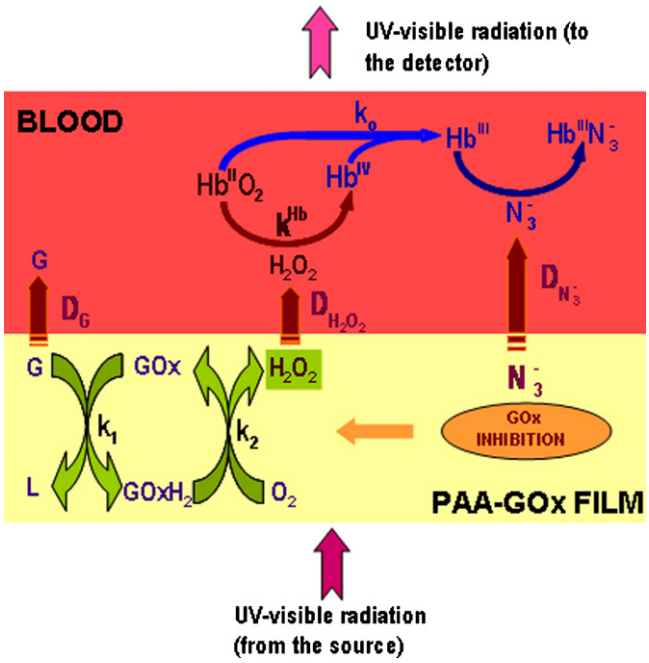

Fig. 1. Overall reaction mechanisms occurring in the sensor flow cell. G: glucose; L: glucolactone; $HbIIO_2$ (ferrous blood hemoglobin linked to O_2); HbIII (ferric hemoglobine); $HbIII{\cdot}N_3^-$ (azide linked ferric hemoglobine); GOx (glucose oxidase, oxidized form); $GOx{\cdot}H_2$ (reduced form); D_G, $D_{H_2O_2}$ and $D_{N_3^-}$: glucose, H_2O_2 and azide diffusion coefficients.

can act as a H_2O_2 scavenger so the H_2O_2/catalase reaction must be inhibited. This inhibition can be efficiently performed by azide addition to the carrier [31], which coordinates iron(III) of the catalase heme group blocking its reaction with H_2O_2. Obviously, azide also binds ferric hemoglobin (HbIII) to form the $HbIII{\cdot}N_3^-$ complex which is unable to react with hydrogen peroxide, but as the Hb/H_2O_2 reaction mechanism becomes simplified, far from being a problem this fact is an advantage. In Fig. 1 (upper part) a simplified reaction mechanism of Hb with H_2O_2 in the presence of azide is shown (see Fig. S1 in the Supplementary Material for a full explanation). HbII and HbIII are the hemoglobin in ferrous (Fe^{2+}) and ferric (Fe^{3+}) states, respectively. $HbII{\cdot}O_2$ reacts with hydrogen peroxide giving HbIV, an oxyferryl intermediate with the heme group in an oxidation state of +4, which quickly comproporcionates with $HbIIO_2$ giving HbIII which in the presence of azide forms $HbIII{\cdot}N_3^-$. The intramolecular and intermolecular reduction of HbIV and the reduction of HbIV by reducer substrate are negligible, so a direct $HbII{\cdot}O_2/HbIII{\cdot}N_3^-$ transition is observed. As both species have different optical absorption spectra (Fig. S2 in the Supplementary Material) it is possible to follow the reaction through the absorbance variations observed.

3.2. Some considerations on sensor design

The sensor film must be as transparent as possible at the working wavelength in order to minimize the background scattering signal. Regarding the flow cell, several aspects have to be considered. The inner volume has to be as small as possible to limit the sample volume consumption (around 300 μL). Furthermore, the thickness of the flow cell has to be as thin as possible (0.12 cm, GOx film thickness 0.03 cm) in order to both concentrate the hydrogen peroxide formed and fit the optical pathway of the system. Regarding the kind of flow, when a non-stop-flow mode is used the H_2O_2 diffusing to the sample is cleaned out of the flow cell and the sensitivity is low. In this work the flow is stopped when the cell is completely filled with the sample, reaching the maximum sensitivity given that hemoglobin and glucose concentrations are at a maximum in this condition.

Fig. 2 shows the changes in the absorption spectrum of the GOx film-blood system during the reaction (A: $\lambda < 500$ nm; B: $\lambda > 500$ nm). Comparing these spectra with those shown in Fig. 2, the following conclusions can be derived: (1) the absorption and scattering in the film due to the GOx remains as a constant during the reaction; (2) absorbance at 412, 538 and 576 decreases during the reaction; these wavelengths correspond to the $HbII{\cdot}O_2$ absorption; (3) the isosbestic points at 444, 526 and 586 nm owing to $HbII{\cdot}O_2/HbIII{\cdot}N_3^-$ remain. This all demonstrates that a $HbII{\cdot}O_2/HbIII{\cdot}N_3^-$ transition is produced during the reaction, which is very important in order to formulate the correct mathematical model of the system. Maximum sensitivity is obtained at 412 nm.

3.3. Mathematical model

According to the overall model given in Fig. 1, the measured absorbance in the flow cell at any wavelength and any time ($Abs_{\lambda,t}^{meas}$) is the given by

$$Abs_{\lambda,t}^{meas} = Abs_{\lambda,t}^{film} + Abs_{\lambda,t}^{sol} \quad (1)$$

$Abs_{\lambda,t}^{film}$ and $Abs_{\lambda,t}^{sol}$ being the absorbance of the film and the solution (blood sample), respectively. The $Abs_{\lambda,t}^{film}$ solely depends on the entrapped GOx. The molecular absorption spectrum of GOx depends on whether it is in the initial oxidized form (GOx) or in the reduced form ($GOx{\cdot}H_2$). As has been indicated before [24,27], the oxidized to reduced ratio depends on the O_2 concentration. In our system, since the O_2 concentration is much higher than the glucose concentration, the $GOx{\cdot}H_2$ is quickly oxidized, so that the enzyme is mainly in this form (which is consistent with the absorbance variations shown in Fig. 2) and then:

$$[GOx]_t = [GOx]_0 \quad (2)$$

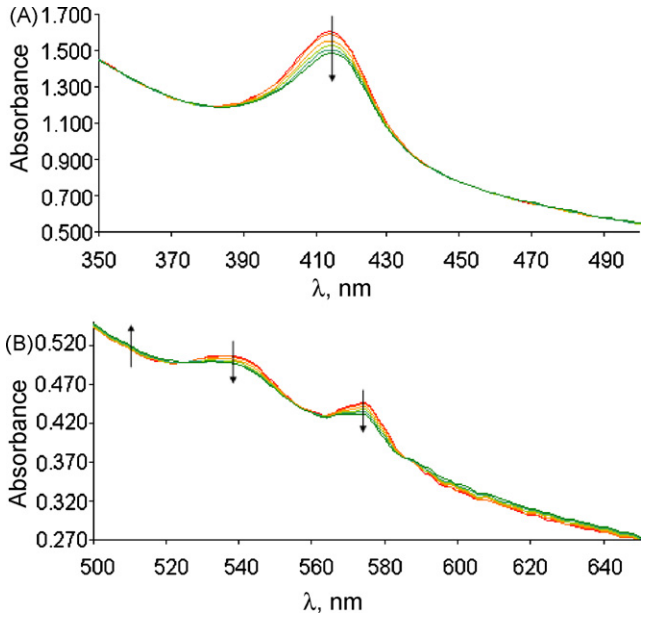

Fig. 2. Changes in the molecular absorption spectra of the blood sample–GOx film system. The spectra variations correspond to a $HbII{\cdot}O_2/HbIII{\cdot}N_3^-$ transition (A, from 350 to 500 nm; B, from 500 to 650 nm). Arrows show increasing time (40 mg mL^{-1} GOx concentration in the PAA polymerization mixture; 150:1 (v:v) blood dilution; 10^{-3} M azide concentration and 1.38×10^{-4} M blood glucose concentration).

Thus the $Abs^{film}_{\lambda,t}$ always remains constant ($Abs^{film}_{\lambda,t} = Abs^{film}_{\lambda}$) and then (1) is given by

$$Abs^{meas}_{\lambda,t} = Abs^{film}_{\lambda} + Abs^{sol}_{\lambda,t} \quad (3)$$

According to (3), when blood fills the flow cell and before the reaction starts, the measured absorbance at the working wavelength (λ_w) is given by

$$Abs^{meas}_{\lambda_w,0} = Abs^{film}_{\lambda_w} + \varepsilon^{HbII\cdot O_2}_{\lambda_w} L[Hb]_0 \quad (4)$$

L being the optical pathlength of the sensor cell flow. Since $\varepsilon^{HbII\cdot O_2}_{\lambda_w}$ can be accurately measured, the hemoglobin concentration in blood can be determined. When the reaction starts, the hemoglobin begins to change but, as has been stated before, the only species which are in significant concentrations are $HbII\cdot O_2$ and $HbIII\cdot N_3^-$ and the measured absorbance at any time at the working wavelength will be given by

$$Abs^{meas}_{\lambda_w,t} = Abs^{film}_{\lambda_w} + \varepsilon^{HbII\cdot O_2}_{\lambda_w} L[HbII\cdot O_2]_t + \varepsilon^{HbIII\cdot N_3^-}_{\lambda_w} L[HbIII\cdot N_3^-]_t \quad (5)$$

and considering that the mass balance for hemoglobin is

$$[Hb]_0 = [HbIIO_2]_t + [HbIIIN_3^-]_t \quad (6)$$

Eq. (5) gives

$$Abs^{meas}_{\lambda_w,t} = Abs^{film}_{\lambda_w} + \varepsilon^{HbII\cdot O_2}_{\lambda_w} L[Hb]_0 + (\varepsilon^{HbIII\cdot N_3^-}_{\lambda_E} - \varepsilon^{HbII\cdot O_2}_{\lambda_w}) L[HbIII\cdot N_3^-]_t \quad (7)$$

It is very usual to detect slight drifts in the absorbance signals in optical sensors. This can be avoided by measuring the absorbance during the reaction simultaneously at a reference (λ_{ref}) wavelength for which the absorbance does not change (in this case an isosbestic wavelength for the $HbII\cdot O_2$ and $HbIII\cdot N_3^-$ species) and will be given by

$$Abs^{meas}_{\lambda_{ref},t} = Abs^{meas}_{\lambda_{ref},0} = Abs^{film}_{\lambda_{ref}} + \varepsilon^{HbII\cdot O_2}_{\lambda_{ref}} L[Hb]_0 \quad (8)$$

Eqs. ((4), (7) and (8)) can be combined in different forms. In many cases we will use one of the two following analytical parameters:

$$\Delta Abs_{\lambda_w,t} = Abs^{meas}_{\lambda_w,t} - Abs^{meas}_{\lambda_w,0} = (\varepsilon^{HbIII\cdot N_3^-}_{\lambda_w} - \varepsilon^{HbII\cdot O_2}_{\lambda_w})[HbIII\cdot N_3^-]_t = \Delta\varepsilon_{\lambda_w}[HbIII\cdot N_3^-]_t \quad (9a)$$

$$\Delta Abs_{\lambda_w/\lambda_{ref},t} = \frac{Abs^{meas}_{\lambda_w,t} - Abs^{meas}_{\lambda_w,0}}{Abs^{meas}_{\lambda_{ref},0} - Abs^{film}_{\lambda_{ref}}} = \left(\frac{\varepsilon^{HbIII\cdot N_3^-}_{\lambda_w} - \varepsilon^{HbII\cdot O_2}_{\lambda_w}}{\varepsilon^{HbII\cdot O_2}_{\lambda_{ref}}}\right)\frac{[HbIII\cdot N_3^-]_t}{[Hb]_0} = \Delta\varepsilon_{\lambda_w/\lambda_{ref}}\frac{[HbIII\cdot N_3^-]_t}{[Hb]_0} \quad (9b)$$

As can be seen from Fig. 1, the overall reaction includes the diffusion of the glucose from solution to film, the diffusion/reaction of glucose inside the film, diffusion of the H_2O_2 formed from the film to the solution and the reaction of this compound with hemoglobin. Mass transport and reaction kinetic considerations for glucose and H_2O_2 enable us to relate the $[NbIII\cdot N_3^-]_t$ with glucose and hemoglobin concentrations and thermodynamic constants (see

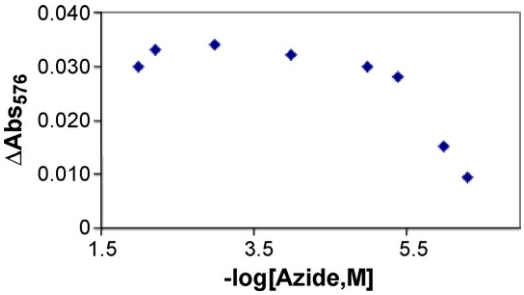

Fig. 3. Effect of azide concentrations (as −log[]) on the absorbance variation (40 mg mL^{-1} GOx concentration in the PAA polymerization mixture; 150:1 (v:v) blood dilution).

"Model derivation" section in Supplementary Material for an explanation of the deduction and terms) and the following equation is obtained:

$$[HbIIIN_3^-]_t = K[Hb]_0 t^2 [G]_0 \quad (10a)$$

K being a constant grouping several thermodynamic parameters, G being the glucose concentration and t being the reaction time. Eq. (10a) is merely a simplification; in fact the $[HbIIIN_3^-]_t$ is exponentially related to G according to:

$$[HbIII\cdot N_3^-] = [Hb]_0(1 - e^{-2k^{Hb}(D^f_G/LX_f)[G]_0((t^2/2)-(1/6)(D^S_G/LX_S)t^3))} \quad (10b)$$

(see Eq. (S26) in the Supplementary Material for an explanation), which can be simplified to a straight line for low exponent values. After substitution of (10a) in (9), the global model, working with or without the reference wavelength, is finally found:

$$\Delta Abs_{\lambda_w/\lambda_{ref},t} = \Delta\varepsilon_{\lambda_w/\lambda_{ref}} K t^2 [G]_0 \quad (11)$$

$$\Delta Abs_{\lambda_w,t} = \Delta\varepsilon_{\lambda_w,t} K [G]_0 [Hb]_0 t^2 L \quad (12)$$

Eq. (10) demonstrates that the analytical signal is not dependent on the O_2 concentration.

3.4. Mathematical model validation: analytical figures of merit

As stated previously, azide has three effects: (a) it inhibits blood catalase, an enzyme that consumes H_2O_2 which would make the sensitivity lower; (b) it combines with HbIII to form $HbIII\cdot N_3^-$ which inhibits the $HbIII/H_2O_2$ reaction and simplifies the reaction mechanism; (c) it partially inhibits GOx. Effects (a) and (b) play in favour of the method sensitivity while effect (c) works against it, therefore there will be an optimal azide concentration at which the sensitivity is at a maximum (Fig. 3). Taking into account the hemoglobin concentration in the diluted blood sample (minimum dilution for complete hemolysis 50:1 (v:v) and normal hemoglobin levels in blood in the range of 12–18 g dL^{-1}) and the experimental data, 10^{-3} M was finally chosen.

The GOx concentration in the polyacrylamide film was also studied. Table 1 shows the slopes for four calibration lines $\Delta Abs_{\lambda_w/\lambda_{ref},t}$ *versus* the glucose concentration (lineal zone) at different GOx concentrations. As can be seen, and according to the model, the GOx concentration does not affect the method sensitivity (the R.S.D. of the slopes is lower than 2%) in this concentration range and using 10^{-3} M azide concentration. This is very important from the point of view of the robustness of the system and permits the biosensor to be easily made. However, for high azide concentrations (about 0.01 M), the signal depends on the GOx concentration as the percentage of GOx inhibition increases.

Hemoglobin is a reagent necessary for the method which is supplied by the sample itself, so the hemoglobin concentration can not be changed by the analyst. Nevertheless, the model predicts the effect of the hemoglobin concentration and checking this

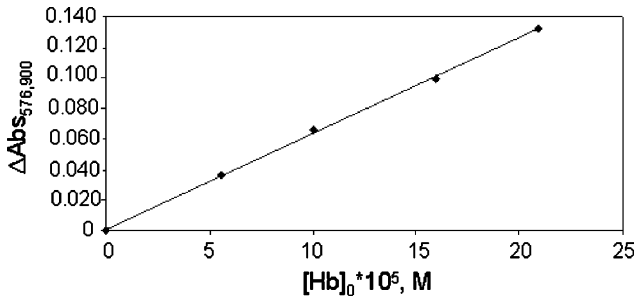

Fig. 4. Hb concentration effect of the analytical signal (60 mg mL^{-1} GOx concentration in the PAA polymerization mixture; 10^{-3} M azide concentration; 3.7×10^{-5} M blood glucose concentration and 900 s reaction time).

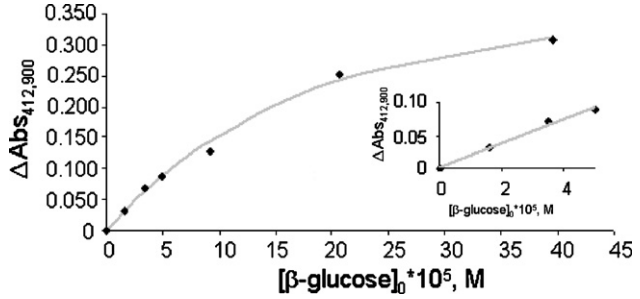

Fig. 5. $\Delta Abs_{412,900}$ *versus* glucose concentration using a fixed reaction time (900 s). The grey curve corresponds to the theoretical equation: $\Delta Abs = 0.3478(1 - e^{-5833.18[G]_{0\beta}})$ $r = 0.994$. *Inset*: $\Delta Abs_{412,900}$ *versus* glucose concentration in the linear response range (40 mg mL^{-1} GOx concentration in the PAA polymerization mixture; 150:1 (v:v) blood dilution; and 10^{-3} M azide concentration).

effect is important in order to make corrections. To study this effect, several mixtures of commercial hemoglobin (A_0, the majority form in blood (>99%)) with a constant glucose concentration were prepared and the absorbance variation in optimal conditions measured. The results obtained (Fig. 4) indicated a linear relationship between $\Delta Abs_{\lambda_w,t}$ and the hemoglobin concentration as the model predicts (Eq. (12)). Furthermore, this linear relationship is very interesting because it allows us to omit the dependence of the hemoglobin concentration on the signal as described above (Eq. (11)). We have determined that the percentage of $HbII{\cdot}O_2$ consumed during a 1250 s reaction time and with a glucose concentration of 3.73×10^{-5} M is $60 \pm 2\%$. This value is independent of the hemoglobin concentration, given the linear relationship between $\Delta Abs_{\lambda_w,t}$ and $[Hb]_0$.

Fig. 5 shows the representation of the analytical parameter *versus* glucose concentration. This equation corresponds mathematically to Eq. (10b). For low glucose concentrations, the model (Eqs. (11) or (12)) predicts a linear relationship between the absorbance variation and the analyte concentration. Nevertheless, the method sensitivity expressed as the slope of the calibration line depending of the measuring time (Table 2). The lower the reaction time, the higher the measuring time where the exponential approximation is fulfilled and thus the higher is the top limit of the linear range. For example, working with 150/1 dilution, the most usual concentrations can be analysed in less than 8 min. It is important to consider that the response range for a given time can be extended using the exponential calibration or modifying the sample dilution. The relative standard deviation for the analytical parameter of a 77 mg dL^{-1} glucose was 4% ($n = 5$).

Table 1
GOx effect on the slope of the calibration line.

[GOx] (mg mL^{-1})[a]	Slope (M^{-1})[b]
20	202
40	201
60	205
80	198

Conditions: Blood dilution 150:1 (v:v) in water and azide 10^{-3} M, time reaction 900 s.

[a] GOx concentration in the polymerization mixture for polyacrylamide film preparation.

[b] Slope of the calibration line of $\Delta Abs_{576,1150}$ *versus* glucose concentration in the linear range.

Table 2
Sensitivity and linear range (lower and upper limit) as a function of the reaction time.

Reaction time (s)	Sensitivity (M^{-1})	Lower limit (mg dL^{-1})	Upper limit (mg dL^{-1})
100	5.9	620	1200
300	28.1	135	950
450	72.1	54	810
700	105	40	675
900	140	25	540
1100	163	20	540

Conditions: GOx concentration in the polymerization mixture for polyacrylamide film preparation 40 mg mL^{-1}, blood dilution 150:1 (v:v) in water and azide 10^{-3} M. Sensitivity is expressed as the slope for the calibration line; lower limits for the linear range correspond to the detection limit (data for 576 nm).

As stated previously, the wavelengths at which the maxima appear were 412, 538 and 576 nm. The sensitivities experimentally obtained (slopes of the calibration lines) for these three wavelengths were 1219, 103 and 166 M^{-1}, respectively, which are proportional to $\Delta\varepsilon_{\lambda_w/\lambda_{ref}}$ (42,132, 4068 and 5833 M^{-1} cm^{-1}, respectively) as the model predicts (Eq. (12)). The quotient between sensitivity and $\Delta\varepsilon_{\lambda_w/\lambda_{ref}}$ for the different wavelengths was 0.028 ± 0.002 cm ($n = 3$). The slopes of the calibration lines given can be used for testing the global quality of the model. To do this, Eq. (12) will be considered. In this equation, the following considerations were applied: (a) the $\Delta\varepsilon_{\lambda/\lambda_{ref}}$ were obtained from the spectra; (b) a previously obtained [8] value of 14 M^{-1} s^{-1} for k_{Hb} was taken; (c) the hemoglobin concentration in the sample was calculated from Eq. (2) and the ε_{HbIIO_2} value used [27] was 13,257 M^{-1} cm^{-1}; (d) the glucose diffusion coefficients in the sample solution (D_s^G) and in the film (D_f^G) were assumed to be equal. From these considerations the K value was calculated and from this value it is possible to compare the experimental absorbance variation signals with the theoretical profiles (Eq. (S27) in the Supplementary Data). Fig. 6 compares the experimental with the theoretical (expected) values; as can be seen the model fits very well with the experimental results. A GOx in PAA sensor film lifetime is about 6 months for glucose determination in fruit juices (based on the chemically modified GOx fluorescence) [25]. With this method the same sensor film has been used during 2 months (more than 100 measurements) without damage.

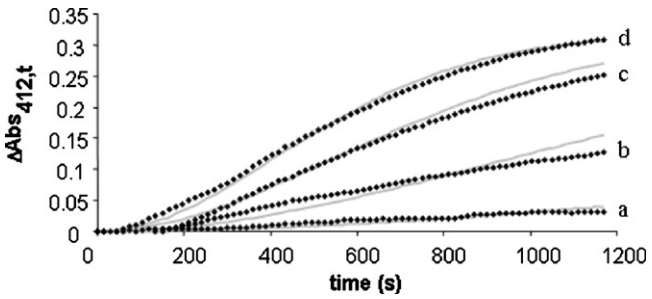

Fig. 6. $\Delta Abs_{412,t}$ *versus* time for different glucose concentrations: (a) 1.60×10^{-5} M, (b) 9.20×10^{-5} M, (c) 2.06×10^{-4} M and (d) 3.96×10^{-4} M (40 mg mL^{-1} GOx concentration in the PAA polymerization mixture; 150:1 (v:v) blood dilution; and 10^{-3} M azide concentration).

3.5. Biosensor validation

From the previously reported values the slope of the calibration line can be stated and a self-calibration method for glucose determination in blood can be applied according to the following equation (900 s measurement time):

$$\Delta \mathrm{Abs}_{412/444,900} = 2722[G]_0 \quad (13)$$

This equation was tested in order to be used for direct glucose determination by an absolute calibration method. Ten blood samples were analysed using this method and quantified with both the equation and a Reflotron® instrument (see Fig. S3 in Supplementary Material). The results obtained were submitted to a correlation study and the line obtained was:

$$[\mathrm{Glucose}]_{\mathrm{reference}} = 0.972[\mathrm{Glucose}]_{\mathrm{this\ method}} + 3.6 \quad r^2 = 0.98 \quad (13)$$

In this equation the slope and the intercept are statistically equal to 1 (0.97 ± 0.14, 95% confidence interval) and 0 (3.6 ± 8.5, 95% confidence interval), respectively, so both methods give similar results.

4. Conclusions

In this paper it has been demonstrated that the molecular absorption properties of blood hemoglobin can be used as an analytical signal for glucose determination in blood, without an additional indicating reaction and without O_2 dependence. The mathematical model developed can be used as a starting point in order to design a methodology for the direct determination of other blood compounds with the appropriate enzyme producing H_2O_2. The method can be applied without calibration and can therefore be easily implemented in automatic blood analyzers. These results open the door to new designs for automatic blood glucose analyzers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2008.12.060.

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