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Glucose concentration determination by means of fluorescence emission spectra of soluble and insoluble glucose oxidase: some useful indications for optical fibre-based sensors

M. Lepore[a], M. Portaccio[a], E. De Tommasi[a], P. De Luca[a], U. Bencivenga[b], P. Maiuri[a], D.G. Mita[a,b,*]

[a] *Department of Experimental Medicine, Faculty of Medicine and Surgery, Second University of Naples, Via S.M. di Costantinopoli 16, 80136 Naples, Italy*
[b] *Institute of Genetics and Biophysics "Buzzati Traverso" of CNR, Via Guglielmo Marconi 12, 80125 Naples, Italy*

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Abstract

By using soluble and insoluble glucose oxidase, the changes in intrinsic emission fluorescence in the visible spectral region were studied as a function of glucose concentration. Insoluble glucose oxidase (GOD) was obtained by entrapment in a gelatine membrane or by covalent attachment on an agarose membrane grafted with hexamethylendiamine. The intensity of the fluorescence emission peak at 520 nm or the value of the integral fluorescence area from 480 to 580 nm were taken as physical parameters representative of the glucose concentration during the enzyme reaction. By using these parameters, linear calibration curves for glucose concentration were obtained. The extension of the calibration curve and the sensitivity of the adopted systems were found to be dependent on the enzyme state (free or immobilized) and on the immobilization method. In particular, it was found that the extent of the linear range of the calibration curves is increased of one order of magnitude when the glucose oxidase is immobilized, while the sensitivity of the measure is decreased of one order of magnitude by the immobilization process. Measures carried out by using the integral fluorescence area resulted more sensitive than those obtained with the peak size. Useful indications for the construction of optical fibre-based sensors were drawn from the reported results.
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1. Introduction

Determination of glucose concentration takes a relevant place in controlling various food and biotechnological processes [1,2] as well as in diagnosing many metabolic disorders, mainly in diagnosis and therapy of diabetes [3]. Among the various methods employed to this aim, those utilising the enzyme glucose oxidase (GOD) are the most widespread. GOD is an oxidoreductase, which catalyses the oxidation of glucose to gluconic acid.

Raba and Mottola [4] published an interesting review on the analytical use of GOD. Amperometric, potentiometric, chemolumetric, spectrophotometric or fluorometric methods are used for the enzymatic measurements of glucose concentration. Amperometric systems are used for the determination of H_2O_2 or O_2 [5–9], while potentiometric methods [10,11] are employed for the determination of the production of gluconic acid following the pH change. Trettnak and Wolfbeis [12] developed a sensor based on the changes in the emission fluorescence properties of GOD in the visible region during the enzyme reaction. Enzymes, indeed, exhibit intrinsic fluorescence [12–14]. An intense emission fluorescence is observed in the UV spectral region, due to the presence of tyrosine and thryptophan residues; while a weak fluorescence due to flavine residues occurs in the visible zone. For this reason, glucose oxidase has been used as glucose sensing mainly in the UV spectral region, while the effectiveness of

* Corresponding author. Tel.: +39 081 2395887; fax: +39 081 2395887.
E-mail address: mita@iigb.na.cnr.it (D.G. Mita).

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the fluorescence of the GOD–FAD complex was the subject of a serious debate. Some authors [14–16] affirmed that the flavinic coenzyme embedded in the protein does not generate any detectable emission, while other authors [17,18] argued the contrary. Indeed oxidised free flavines exhibit a relatively strong fluorescence with a quantum yield of 0.3 [19] and an emission maximum around 530 nm. It was also found that the energy intensity of this emission depends on solvent polarity and temperature [19–21], as well as on the formation of complexes with a variety of molecules [22,23].

In this paper, we will discuss the changes in the emission fluorescence spectra in the visible region when GOD, free or immobilized, interacts with different glucose concentrations. In the case of insoluble GOD, the enzyme was entrapped in a gelatine membrane or covalently attached to an agarose membrane, grafted with hexamethylendiamine. The study of the intrinsic fluorescence of the immobilized GOD was undertaken in view of the employment of these catalytic membranes as biological element constituting the interface with an optical fibre-based sensor. Within this frame of reference the extension of the linear calibration curves as well as the sensitivity of the soluble and insoluble GOD will be discussed, together with the dependence of the kinetic and optokinetic parameters from the state, free or immobilized, of GOD. This comparison allows us to know how the immobilization process [24–30] affects the kinetic of the enzyme reaction.

2. Materials and methods

2.1. Materials

Glucose oxidase (GOD, EC 1.1.3.4) from *Aspergillus niger* (154 U mg^{-1}) was used in this study. GOD catalyses the oxidation of glucose to gluconic acid through the reaction:

$$\text{D-glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-gluconolactone} + H_2O_2$$

$$\text{D-gluconolactone} + H_2O \rightarrow \text{D-gluconic acid}$$

The reaction mechanism is the following: glucose reduces FAD of glucose oxidase to $FADH_2$ with formation of Gluconolactone, which is rapidly hydrolysed to gluconic acid. At this point the dissolved oxygen reoxidises $FADH_2$ to GOD and produces H_2O_2. Horseradish peroxidase (POD, EC 1.11.1.7) (1,119 U mg^{-1}) was used to measure H_2O_2 concentration produced by GOD. POD catalyzes the oxidation of organic substrates with hydrogen peroxide as the ultimate electron acceptor. In particular, in the presence of 2,2′-azino-bis(3-ethyl-benzothyazoline-6-sulfonic acid) (ABTS) the following reaction occurs:

$$H_2O_2 + 2\text{ABTS} \xrightarrow{\text{POD}} 2H_2O + 2\text{ABTS}^*$$

The presence of the radical $ABTS^*$ gives a green color to the reaction mixture. The intensity of the solution colour, proportional to $ABTS^*$ concentration, was spectrophotometrically measured at 405 nm. The absorbance is also proportional to H_2O_2 concentration. POD was used to characterise, from the biochemical point of view, the catalytic activity of our soluble and insoluble GOD.

Bovine gelatine (average molecular weight 100,000 Da) or agarose were used to prepare the membranes on which GOD was immobilized by entrapment or by covalent attachment, respectively. In the latter case, hexamethylenediamine (HMDA) and glutaraldehyde (GA) were used as spacer and coupling agent, respectively.

All chemicals, including the enzymes, were purchased form Sigma (Sigma–Aldrich, Milano, Italy) and used without further purification. Gelatine was a gift of Deutsche Gelatine Fabric Stoess, Eberbach, Germany.

2.2. Methods

2.2.1. Preparation of the catalytic membranes

Two catalytic membranes were prepared. The first one was obtained by immobilizing the glucose oxidase by entrapment in a gelatine membrane; the second one was obtained by covalently attaching the enzyme to an agarose membrane grafted with HMDA.

2.2.1.1. Enzyme immobilization by entrapment. A 10% gelatine (w/v) aqueous solution was heated in a water-bath at 90 °C for 15 min, then the solution was cooled at 40 °C before adding a 50% (v/v) ethanol/formaldehyde solution to give a 1% final HCOH concentration. After 20 min of treatment at this temperature, GOD (final concentration 1 mg mL^{-1}) was added under vigorous stirring and then the mixture was poured on a Plexiglas flat surface into a Plexiglas square frame (0.5 mm in thickness, and 12 cm in size). The preparation was quickly put into a freezer at −24 °C and after 16 h brought back to room temperature, thus obtaining a flexible gelatine membrane, which was extensively washed with distilled water. At this point rectangular pieces of the same size (6 mm × 15 mm) were cut and used for the experimentation. In this way, catalytic membrane comparable for dimension and amount of entrapped GOD were obtained. The crosslinked gelatine membranes had a lattice structure, which efficiently held the biocatalyst and allowed diffusion of substrate and reaction products across the membrane. When not used, the membranes were stored at 4 °C in buffer solution pH 5.0.

2.2.1.2. Enzyme immobilization by covalent attachment. To obtain this membrane type a 5% (w/v) agarose aqueous solution was prepared by dissolving, under vigorous stirring, agarose in distilled water kept at 100 °C for 10 min. At the end of this treatment the agarose solution was poured into the same Plexiglas device used to prepare the gelatine mem-

brane and stored overnight at 4 °C. The subsequent step consisted in grafting the agarose membrane with HMDA. To this aim, the membrane was allowed to interact 90 min at room temperature with a 3% (v/v) HMDA aqueous solution. After repeated washings with distilled water to remove the unbound amines, the graft agarose membrane was treated for 120 min at room temperature with a 2.5% (v/v) glutaraldehyde aqueous solution. After further washing with distilled water, the activated membrane was treated overnight at 4 °C with a 0.1 M acetate buffer solution pH 5.0, containing GOD at the concentration of 1 mg mL^{-1}. At the end of this treatment the catalytic membrane was cut in rectangular pieces as for the gelatine membrane. Also in this case, catalytic membranes comparable for dimension and amount of immobilized enzyme were obtained. When not used, the catalytic membranes were stored at 4 °C in 0.1 M buffer acetate pH 5.0.

2.2.2. Biochemical measurements of GOD activity

GOD activity was measured by enzymatically determining the concentration of the produced hydrogen peroxide by means of POD, according to the following procedure: 0.1 mL of the sample to be analyzed and 50 μL of POD (5.55 U mL^{-1}) were added to 2 mL of 0.1 M acetate buffer, pH 5.0, containing ABTS at a concentration of 10 mM. The concentration of ABTS was in excess in respect to that of H_2O_2 so that the reaction was limited by the concentration of the latter product. The reaction was allowed for 10 min at 25 °C, till the amount of H_2O_2 was consumed. A calibration curve of the H_2O_2 concentration as a function of the absorbance gave an angular coefficient equal to 2 mM^{-1}.

2.2.2.1. Activity measurements of soluble enzyme. Soluble GOD activity was measured according to the following procedure: 0.1 mL of GOD solution (154 U mL^{-1}) were added to 1.9 mL of the β-D-glucose solution at the appropriate concentration, temperature and pH, so that the enzyme concentration was 7.7 U mL^{-1}. Every 2 min, 0.1 mL of the reaction solution was taken out and added to 0.4 mL of a 0.1 M HCl aqueous solution to stop the enzyme reaction. The H_2O_2 concentration was then measured according to the procedure described above. In this way, the H_2O_2 production as a function of time was obtained. The angular coefficient of the straight line fitting the initial H_2O_2 production gives the enzyme activity measured as μmoles min^{-1}.

2.2.2.2. Activity measurements of insoluble enzyme. To measure the activity of the catalytic membranes, they were put in a reaction vessel filled with 20 mL of glucose solution at the required concentration, temperature and pH. Samples of 0.1 mL were extracted at regular time intervals and processed in the same way than for the free enzyme. The angular coefficient of the straight line fitting H_2O_2 production as a function of time gives the activity of the catalytic membranes, expressed as μmoles min^{-1}.

2.2.3. Intrinsic fluorescence emission measurements

GOD is a typical flavoprotein. GOD from *A. niger* is a dimer having two very tightly bound FAD molecules per dimer. Each subunit of holo GOD (that is the enzyme associated to its flavinic coenzyme) has two distinct domains: one binding the flavin moiety and the other having the catalytic site binding the substrate. As all flavoproteins, GOD shows absorption maxima at about 380 and 450 nm and intrinsic fluorescence with an emission maximum at about 530 nm, at pH 7.0. Changes in the fluorescence of soluble GOD have been found during its interaction with glucose, since the oxidized and reduced flavines have been found to exhibit different fluorescences [12–14].

The emission fluorescence spectra have been collected by means of a spectrofluorimeter, model LS 55, purchased from Perkin-Elmer (Perkin-Elmer Italia, Milano, Italy), by using as light source a Xenon discharge lamp with an emission spectrum ranging from 200 to 800 nm. Sample excitation was performed at 450 nm, while the emission spectrum was recorded in the range 480–580 nm. Just to give one example, in Fig. 1 the fluorescence emission spectra of soluble GOD in the presence (5 mM) or in the absence of glucose are reported. From Fig. 1 it is evident a fluorescence increase (about 7% for both peak and integral area values) when glucose is present into the aqueous solution. For analytical purposes the emission fluorescence spectrum can be treated as size of the peak value or as size of the integral area under the spectrum. The spectra have been acquired with entrance and exit slit fixed at 5 nm and with a scan speed of 100 nm s^{-1}. Attention must be done in subtracting the fluorescence background. Our apparatus automatically carries out this operation.

In the following, the experimental data will be discussed as peak size at 530 nm or as integral area in the 480–580 nm region.

2.2.4. Treatment of experimental data

Each experimental point in the figures reported in the following is the average of six different experiments, performed

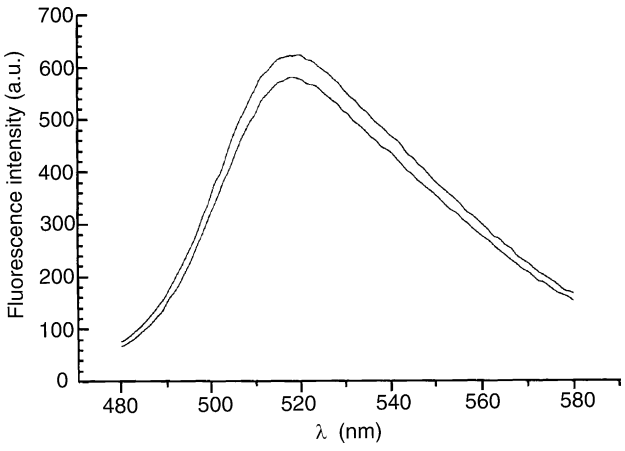

Fig. 1. GOD fluorescence emission spectrum in the presence (5 mM—upper spectrum) and in the absence of glucose.

Table 1
Catalytic membrane properties

Membrane type	Amount of immobilized enzyme (mg cm^{-3})	Coupling yield (%)	Absolute activity $\times 10^2$ (μmoles min^{-1} cm^{-3})	Specific activity $\times 10^2$ (U mg^{-1})	Activity retention (%)
GOD into gelatine membrane	1.00	100.0	1.17	1.17	23.4
GOD on agarose membrane	1.75	30.0	1.14	0.65	13.0

The specific activity of free GOD was 5×10^{-2} U mg^{-1}.

under the same conditions. In the measurements of GOD activity the experimental error never exceeded 3%, while in the experiments relative to the emission spectra the error never exceeded 3.2%.

3. Results and discussion

Before discussing the catalytic behaviour of soluble and insoluble GOD, as well as the sensing power of free and immobilized glucose oxidase, we report in Table 1 a summary of some biochemical properties of the two enzyme derivatives employed in this research. Absolute activity is referred to the activity of 1 cm^3 of catalytic membrane, while specific activity is referred to 1 mg of immobilized GOD. Activities reported in Table 1 were measured under the experimental conditions: $T = 25\,^{\circ}$C and 20 mM glucose in 0.1 M acetate buffer, pH 5.0.

3.1. Biochemical characterization of free and immobilized GOD

To biochemically characterise the biocatalytic membranes used for the determination of glucose concentration by means of intrinsic fluorescence emission spectra, the kinetic parameters of the immobilized GOD were determined. Being known that the immobilization process affects these parameters, the dependence of the soluble GOD activity on the glucose concentration was also studied under the same experimental conditions. In Fig. 2, the results of these studies are reported. Fig. 2a refers to free enzyme, while Fig. 2b and c refer to GOD immobilized on gelatine or agarose membrane, respectively. The experimental conditions were: 0.1 M acetate buffer, pH 5.0, and $T = 25\,^{\circ}$C. Results in Fig. 2 exhibit a Michaelis–Menten behaviour either for the soluble GOD or for both forms of immobilized enzyme. When data of Fig. 2 are reported in form of Hanes plots, the apparent K_m and V_{max} values relative to the soluble and insoluble form of GOD are obtained. These values, reported in Table 2, clearly indicate that the immobilization procedures affect the enzyme affinity towards the substrate, the affinity being expressed by the value of the apparent K_m. Interesting enough, the affinity value of covalently immobilized GOD is smaller than that of entrapped GOD, indicating in this way that the diffusion limitations across the gelatine membrane are greater than in the

Table 2
Kinetic parameters

GOD form	K_m (mM)	$V_{max} \times 10^2$ (U mg^{-1})
Free	2.14	25
Immobilized into gelatine membrane	1.73	14
Immobilized on agarose membrane	0.84	10

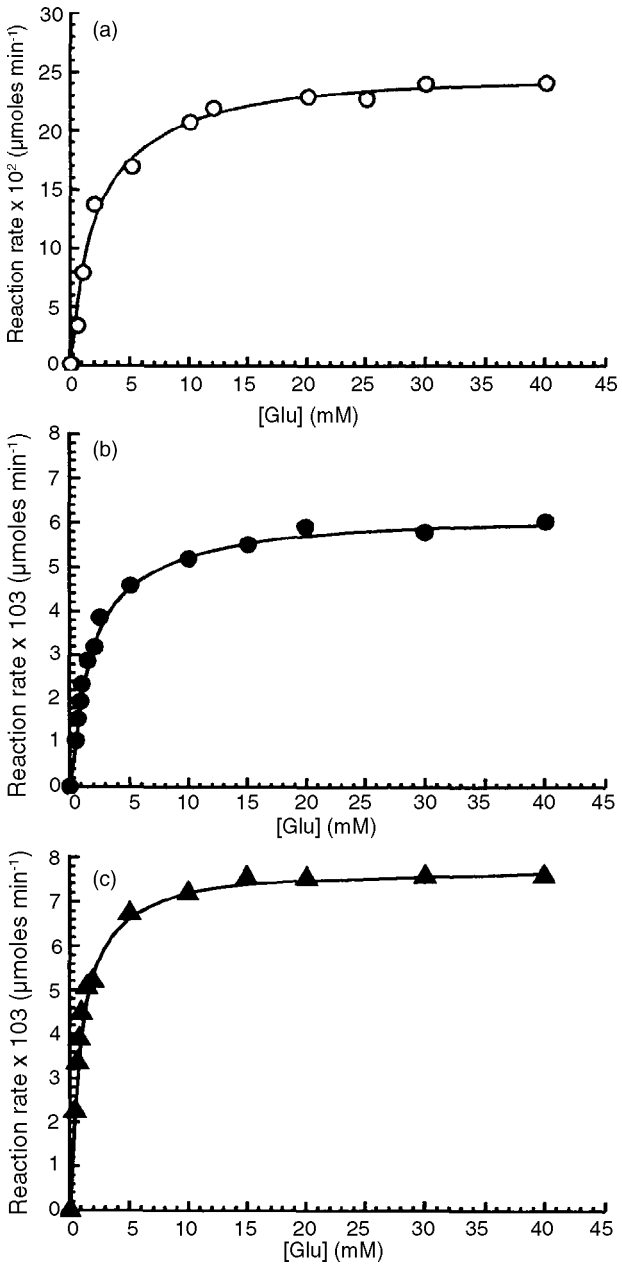

Fig. 2. GOD reaction rate as a function of glucose concentration, (a) soluble GOD, (b) GOD–gelatine membrane, and (c) GOD–agarose membrane.

case of agarose membrane. Both insoluble forms, anyway, have a K_m value smaller than that of soluble enzyme. Similar results were obtained by us with laccase [30], where we also have found an affinity decrease as a consequence of the immobilization process. It is interesting to note that laccase and glucose oxidase are enzymes involving electric charge transfer during the reaction.

3.2. Calibration curves of glucose concentration through emission spectra

3.2.1. Soluble GOD

In Fig. 3a the peak intensities, $P_{C,\text{free}}$, of the emission spectra of soluble glucose oxidase are reported as a function of glucose concentration. Subscripts "C" and "free" indicate the substrate concentration and the enzyme form, respectively. $P_{C,\text{free}}$ values in Fig. 3a have been decreased by $P_{0,\text{free}} = 586$ arbitrary units, i.e. the peak value of soluble glucose oxidase in the absence of glucose ($C = 0$). The experimental conditions were: temperature 25 °C and glucose solution in 0.1 M acetate buffer, pH 5.0, and GOD concentration. Data in figure exhibit a Michaelis–Menten behaviour, i.e. a range in which the size of the fluorescence peak is linear with the glucose concentration, and a range where the emission is quite constant with the glucose concentration. Moreover, data in Fig. 3a are well fitted by an equation of the type:

$$P_{C,\text{free}} = \frac{P_{\text{sat,free}} C}{K_{P,\text{free}} + C} \quad (1)$$

where now the subscript "sat" indicates the peak value at saturation and K_P is a pseudo Michaelis–Menten constant from which it is possible to derive the $K_{P,\text{free}}$ and $P_{\text{sat,free}}$ values reported in Table 3. In analogy with the free biochemical kinetics parameters we have called K_P and P_{sat} optokinetic parameters.

Fig. 3b shows the range where it is possible to appreciate a linear relationship between the size of the emission peak and the glucose concentration, the correlation being of the type:

$$P_{C,\text{free}} = S_{P,\text{free}} C \quad (2)$$

The $S_{P,\text{free}}$ coefficient measures the sensitivity of the method. The $S_{P,\text{free}}$ value calculated from the data of Fig. 3b is reported in Table 3.

In Fig. 3c the integral area values, $A_{C,\text{free}}$, of the intrinsic fluorescence emission spectra in the range 480–580 nm are reported as a function of the glucose concentration. Also in this case data of Fig. 3c have been corrected for the value of the integral area of the GOD emission spectrum in the absence of glucose, i.e., $A_{0,\text{free}} = 35195$ arbitrary units. The experimental conditions were the same that in Fig. 3a. In Fig. 3d the linear range between the integral area values and the glucose concentration is reported.

Also in the case of the integral area the considerations done for the peak intensity are good. This means that data in Fig. 3c can be treated with an equation similar to Eq (1):

$$A_{C,\text{free}} = \frac{A_{\text{sat,free}} C}{K_{A,\text{free}} + C} \quad (3)$$

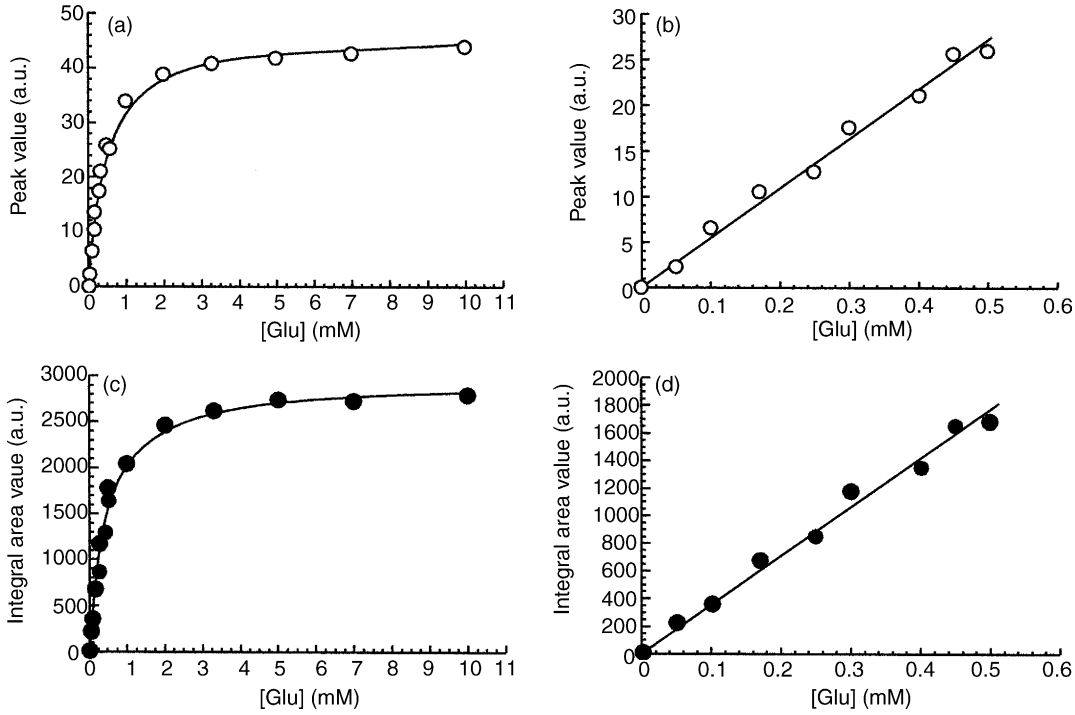

Fig. 3. Soluble GOD. (a) Fluorescence emission peak as a function of glucose concentration. (b) Glucose calibration curve with reference to the value of the size of the emission peak. (c) Fluorescence emission integral area as a function of glucose concentration. (d) Glucose calibration curve with reference to the value of the size of the emission integral area.

Table 3
Optokinetic parameters

GOD form	K_P (mM)	K_A (mM)	P (a.u.)	A (a.u.)	Linear range (mM)	S_P (a.u. mM^{-1})	S_A (a.u. mM^{-1})
Free	0.47	0.47	46.8	2962	Up to 0.5	54	3530
Immobilized into gelatine membrane	8.68	8.68	90.9	5406	Up to 8.0	5.6	355
Immobilized on agarose membrane	5.34	5.34	54.6	3277	Up to 5.0	6.0	350

Similarly data in Fig. 3d can be represented by the equation

$$A_{C,\text{free}} = S_{A,\text{free}} C \quad (4)$$

where now $A_{C,\text{free}}$ is the integral area of the intrinsic emission spectrum, and $S_{A,\text{free}}$ the sensitivity of the method. The values of $K_{A,\text{free}}$, $A_{\text{sat,free}}$ and $S_{A,\text{free}}$ are reported in Table 3.

3.2.2. GOD–gelatine membrane

In Fig. 4a the values of the peak intensity of the intrinsic fluorescence emission spectrum of GOD entrapped in the gelatine membrane are reported as a function of glucose concentration, having taken in account for the fluorescence peak intensity of the GOD–gelatine membrane in the absence of glucose, i.e. $P_{0,\text{gel}} = 30$ arbitrary units. The experimental conditions were the same that in Fig. 3. Also in this case a Michaelis–Menten behaviour is observed. It follows that all the considerations done for the soluble GOD are valid for GOD–gelatine membrane, together with Eq. (1). In Fig. 4b the linear range between the peak intensity of the emission spectrum and the glucose concentration is reported. The values of $K_{P,\text{gel}}$, $P_{\text{sat,gel}}$ and $S_{P,\text{gel}}$ are reported in Table 3. These values have been calculated by means of equations identical to Eqs. (1) and (2), by substituting the subscript free with the subscript gel.

When reference is done to the integral area of the fluorescence emission spectrum of GOD–gelatine membrane one obtains the results reported in Fig. 4c. Also in the case of the results of Fig. 4c the reported experimental data have been obtained taking in account for the integral area of the emission spectrum of the GOD–gelatine membrane in the absence of glucose, i.e. $A_{0,\text{gel}} = 1785$ arbitrary units. In Fig. 4d the linear range between the integral area of the emission spectrum and the glucose concentration is reported. Also in the case of Fig. 4c and d the considerations done for the soluble enzyme are still good, so that through equations similar to (3) and (4) one obtains the values of $K_{A,\text{gel}}$, $A_{\text{sat,gel}}$ and $S_{A,\text{gel}}$, reported in Table 3.

3.2.3. GOD–agarose membrane

In Fig. 5a the values of the peak intensity of the fluorescence emission spectrum of GOD covalently attached to the agarose membrane are reported as a function of glucose concentration, having taken in account for the fluorescence peak intensity of GOD–agarose membrane in the absence of glucose, i.e. $P_{0,\text{agar}} = 47$ arbitrary units. The experimen-

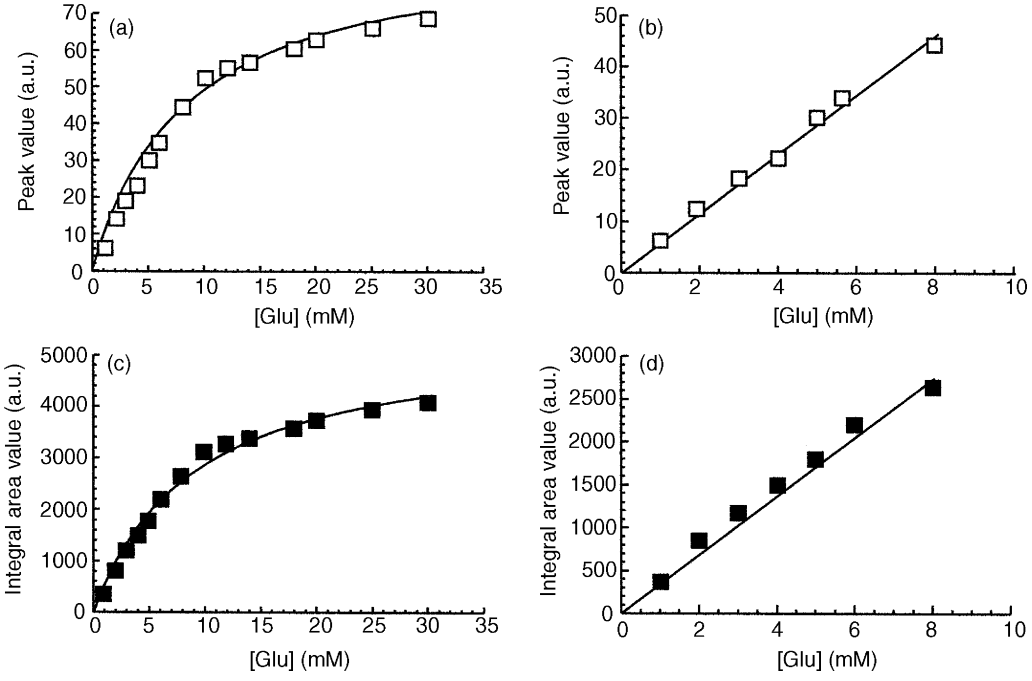

Fig. 4. GOD–gelatine membrane. (a) Fluorescence emission peak as a function of glucose concentration. (b) Glucose calibration curve with reference to the value of the size of the emission peak. (c) Fluorescence emission integral area as a function of glucose concentration. (d) Glucose calibration curve with reference to the value of the size of the emission integral area.

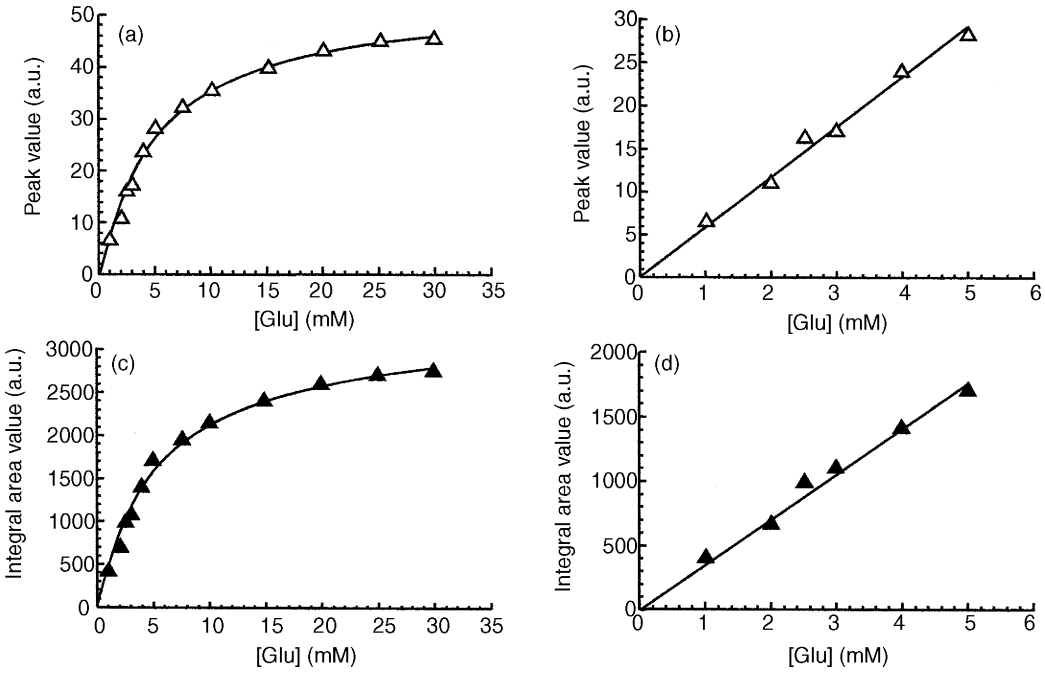

Fig. 5. GOD–agarose membrane. (a) Fluorescence emission peak as a function of glucose concentration. (b) Glucose calibration curve with reference to the value of the size of the emission peak. (c) Fluorescence emission integral area as a function of glucose concentration. (d) Glucose calibration curve with reference to the value of the size of the emission integral.

tal conditions were the same that in Fig. 3. Also in this case a Michaelis–Menten behaviour is observed. It follows that all the considerations done for soluble GOD and for GOD–gelatine membrane are still valid for GOD–agarose membrane. Fig. 5b reports the linear range between the fluorescence peak intensity of GOD–agarose membrane and the glucose concentration, as deduced from Fig. 5a. The values of $K_{P,agar}$, $P_{sat,agar}$ and $S_{P,agar}$ are reported in Table 3. These values have been calculated by means of equations identical to (1) and (2) by substituting the subscript free with the subscript agar.

When reference is done to the integral area of the emission fluorescence spectrum of the GOD–agarose membrane one obtains the results reported in Fig. 5c. Also in this case, data in the figure have been obtained by subtracting the integral area value of the emission spectrum of the GOD–agarose membrane in the absence of glucose, i.e. $A_{0,agar} = 2848$ arbitrary units from the measured values of the integral area of the emission spectra in the presence of glucose In Fig. 5d the linear range between the integral area of the emission spectrum and the glucose concentration, is reported. Also in the case of Fig. 5 the considerations done for the soluble enzyme are still good, so that through equations similar to (3) and (4) one obtains the values of $K_{A,agar}$, $A_{sat,agar}$ and $S_{A,agar}$ listed in Table 3.

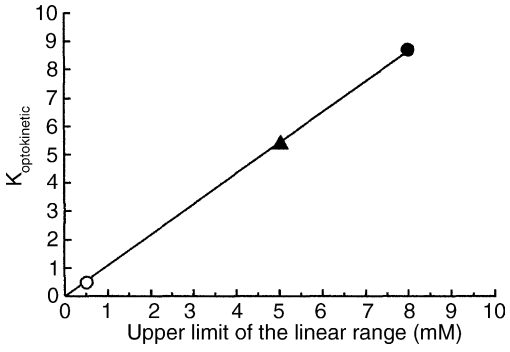

Fig. 6. Optokinetic parameter K as a function of the extent of the linear range.

4. Conclusions

Before concluding, it is advisable to evidenziate some remarks coming out from the results reported in Table 3. The use of immobilized GOD increases one order of magnitude, the extent of the range in which the measures of the emission fluorescence spectra are linearly proportional to glucose concentration. More interesting is the linear relationship between the $K_{optokinetic}$ values of the emission spectra and the corresponding extents of the linear range, as one can see in Fig. 6. Fig. 6 has been obtained by combining the data reported in Table 3. Being the extension of the linear range proportional to the $K_{optokinetic}$ values, and because the latter parameter is related to the immobilization procedure it is easy to conclude that one can obtain linear ranges of different extent by using different immobilization methods or different graft monomer. From our results in Tables 2 and 3 no correlation is observed between the biochemical K_m and the optokinetic K. Further investigations are necessary to this aim.

Looking at the sensitivity of the measure method it is evident that the sensitivity decreases of one order of magnitude when GOD is immobilized. This is an unexpected result since usually one expects to have a sensitivity proportional to the affinity of the enzyme for its substrate, i.e to the value of the biochemical K_m. A further indication can be drawn from the percentage changes in the fluorescence due to the glucose presence. Looking to the percentage changes in fluorescence due to the presence of glucose, our results are greater than those reported by White and Harmon [31]. These authors immobilized reversibly GOD on $CTPP_4$. The $CTPP_4$–GOD derivative gave an absorbance peak at 427 nm. The absorbance intensity decreased during the interaction with glucose. The changes in percentage spanned from 0.2 to 0.6% for glucose concentration varying from 0.1 to 11 mM. In our case the percentage changes are higher, as can be deduced by considering data reported in Figs. 3–5. In particular, for soluble GOD the average percentage changes are about 6% in the linear range both for integral area and for peak value. Higher percentage changes are obtained by us for immobilized GOD (from 5 to 30%). This means that our systems show higher sensitivities than that of White and Harmon. On the contrary, Wolfbeis et al., by using sol–gel-based glucose biosensors, obtained sensitivity higher than ours, but they employed exogenous luminescent probes during the immobilization process, and this trick increased the sensitivity of the method [32]. Measures carried out with reference to the integral area resulted more sensitive than those relative to the peak intensity. This is a clear indication that to obtain calibration curves by using fluorescence emission spectra the measure of the integral area is a physical parameter more sensitive than the measure of the peak intensity.

On the basis of these results, experiments addressed to obtain optical fibre glucose biosensors working in different concentration ranges by using different catalytic membranes are at present carried out in our laboratory.

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