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A reagentless enzymatic fluorescent biosensor for glucose based on upconverting glasses, as excitation source, and chemically modified glucose oxidase

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ABSTRACT

Upon near-infrared excitation $Tm^{3+}+Yb^{3+}$ doped fluorohafnate glasses present upconversion properties and emit visible light. This property permits to use these glasses (UCG) as excitation sources for fluorescent optical biosensors. Taking this into account, in this work a fluorescent biosensor for glucose determination is designed and evaluated. The biosensor combines the UCG and the fluorescence of the enzyme glucose oxidase chemically modified with a fluorescein derivative (GOx-FS), whose intensity is modified during the enzymatic reaction with glucose. Optical parameters have been optimized and a mathematical model describing the behavior of the analytical signal is suggested. Working in FIA mode, the biosensor responds to glucose concentrations up to, at least, 15 mM with a limit of detection of 1.9 mM. The biosensor has a minimum lifetime of 9 days and has been applied to glucose determination in drinks. The applicability of the sensor was tested by glucose determination in two fruit juices.

1. Introduction

Materials with upconversion properties (UCM) are able to convert NIR into visible radiation, minimizing in this way problems derived from the autofluorescence and made them especially interesting for clinical and biological applications. Other interesting properties of this material such as low penetration length in the skin, negligible photodamage, tunable fluorescence wavelength (by chosen the appropriate UCM composition) and low power laser requirements for excitation, are also highly appreciated in these fields. This is because the use of UCM is continuously increasing for developing analytical methodologies. Fundamentals and applications of these materials have been recently reviewed by Wolfbeis [1], Zhao [2] or Krull [3]. For (bio) sensors design, UCM are generally synthesized as nanoparticles (UCLNP) from doped fluorohafnate [4,5] or other materials (carbon dots [6]), which permits using them as nanolamps. However, in many applications, UCM glass could be more interesting; for example, UCM glass can be used for mechanizing luminometer cells or well-plate, which can act as excitation source of the substances contained inside (solution, sensors films,...).

The continuous monitorization of glucose has an essential role in diabetes management and the development of optical biosensors for this analyte continuous to be of capital importance in scientific research [7]. Despite different biomolecule as Concanavalin A, periplasmic binding proteins or Glut transporters have been frequently used as receptors [8,9], It is very well known that glucose oxidase (GOx) meets the selectivity and reversibility requirements to be successfully used as a recognition reagent for glucose, so different approaches have been studied in order to give it adequate transducer abilities [10].

GOx is a flavoenzyme (contains FAD as prosthetic group) which reversibly reacts with glucose according to a complex mechanism

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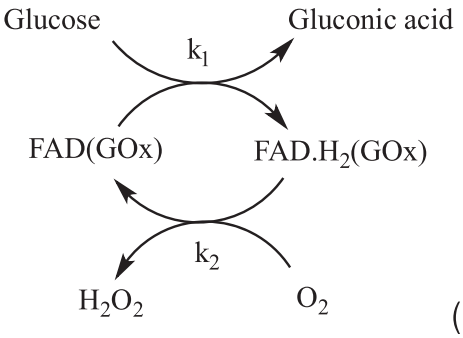
which can be simplified to:



(1)

Glucose reduces FAD(GOx) to FAD.H_2(GOx) and dissolved O_2 regenerates FAD(GOx) (the O_2 of the surrounding media is transferred by diffusion and compensates the consumed O_2). Usually, the use of GOx in glucose optical sensors is based on the measurement of the oxygen consumed during the enzymatic reaction [1,11] monitored by the changes in the fluorescence properties of Ruthenium (or Ir, Pt, Pd,...) fluorophores, which are highly quenchable by O_2. In addition, in some designs it has also been occasionally used the measurement of the changes of the pH in the medium during the reaction (1) due to the gluconic acid formation [12] or monitoring of the generated H_2O_2.

However, the intrinsic spectroscopic properties of GOx lead to alternative methodologies for optical glucose sensors. By the kinetic study of the enzymatic reaction mechanism it is deduced that the concentrations of FAD(GOx)/$FADH_2$(GOx) change during the reaction with glucose concentration. After glucose addition, $FADH_2$(GOx) concentration initially remains constant in a very low value (which also depends on the glucose concentration) and at a certain time (appearance time, t_m) it increases gradually until the whole FAD becomes reduced to FAD.H_2; then, it remains constant for a time (which also depends on the glucose concentration) and decreases gradually again to the initial value (FAD is regenerated). As glucose concentration increases, the t_m decreases and the total time of the reaction increases. A mathematical model relating the t_m with glucose ([G]) concentration was proposed for the quantitative determination of glucose [13] whose simplified equation is given by:

$$t_m = \frac{[O_2]_0}{k_1[G]_0[GOx]_0} \quad (2)$$

The intrinsic spectroscopic properties of GOx depend on the flavine oxidation state. The fluorescence of FAD (at 520 nm) almost disappears in $FADH_2$(GOx), so batch methods and optical sensors [14] have been designed based on them. Despite this fluorescence could be used for sensing purposes and avoids the necessity of monitoring the O_2 consumed, the signal are too small for some applications, so different strategies have been proposed for improving them: 1) Taking advantage of the different inner filter effect properties that FAD and FAD.H_2 produce on a fluorophore; 2) By chemical modification of GOx with an appropriate fluorophore, as hydroxicoumarine, pyrene and specially fluorescein (GOx-FS) [10,15], the excitation and detection wavelengths are moved to the visible region, so the problem is apartially solved; 3) Linking GOx-FS to UCM nanoparticles have allowed to move the excitation wavelength to the NIR region [16].

In this work, we present an optical biosensor based on combining a sensor film (consisting of GOx-FS immobilized in a polyacrylamide film) with a UCM glass [17]. The glass is used as the excitation source for GOx-FS, avoiding the use of a visible excitation, preferable for glucose determination in biological samples.

2. Materials and methods

2.1. Apparatus

All upconversion fluorescence measurements were performed using a Photon Technology International modular spectrofluorometer equipped with a RLDH980-200-3 CW diode laser, 980 nm, 200 mW from Roithner.

The FIA system consisted of a peristaltic pump Minipuls 3 from Gilson and 0.5 mm inner-diameter PTFE tubes.

The chemically modified enzyme GOx-FS was separated from the excess of FS using a low-pressure chromatography system. It consisted of a glass column (13 × 1 cm) filled with Sephadex G-50. The eluent was supplied by a peristaltic pump Minipuls 3 (Gilson). The end of the column was connected to a UV–vis spectrophotometer 8453 A from Agilent. Optical absorption measurements were performed on a Diode-Array HP8453 spectrophotometer.

2.2. Chemicals

The following starting materials were used for the upconverting glasses (doped fluorohafnate glasses) (UPG) synthesis: HfF_4, BaF_2, LaF_3, AlF_3, CsBr, TmF_3 and YBF_3.

pH 8.5, 0.1 M carbonate solutions were prepared from solid $NaHCO_3$ and Na_2CO_3; pH 6.0, 0.1 M MES buffer solutions were prepared from solid MES and NaOH. Glucose oxidase (GOx) type X-S from *Aspergillus niger* (EC 1.1.3.4) with an activity of 147.9 U mg^{-1} of lyophilized solid was purchased from Sigma-Aldrich. The FS solution was prepared by dissolving 2 mg of 6-[fluorescein-5(6)-carboxamido]hexanoic acid N -hydroxysuccinimide ester (Sigma-Aldrich) in 1 mL of dimethyl sulfoxide. Glucose solutions were prepared by dissolving the appropriate amount of β-D(+)-glucose (Sigma-Aldrich) in the MES buffer solution. Polyacrylamide films were synthesized using acrylamide, bis-acrylamide, ammonium persulfate (Sigma-Aldrich) and N,N,N, N-tetramethylenediamine (TEMED, from BioRad).

2.3. Upconverting glasses (UCG) preparation

Two UCG were prepared as reported elsewhere [17]. The composition of these glasses is given in Table 1. The density of the glasses was measured and a mean value of 5.50 ± 0.05 g cm^{-3} was obtained. Since the integrated absorption coefficients for Yb^{3+} and Tm^{3+} optical transitions follow a linear relationship with the nominal rare earth content, rare earth concentrations have been calculated from the glasses densities and nominal compositions. The emission spectra of these crystals under excitation at 980 nm are given in Fig. 1.

2.4. Chemically modified enzyme (GOx-FS) preparation

The chemically modified GOx was prepared by mixing 300 μL of carbonate solution (pH 8.5, 0.1 M) containing 20 mg of GOx with 200 μL of FS. The mixture was allowed to react in the dark at room temperature for 90 min. The excess of FS was then separated using the chromatographic system described in the instrumentation section. The MES buffer solution (pH 6.0, 0.1 M) was used as

Table 1
Composition (mol%) of the fluorohafnate glasses studied.

	HfF_4	BaF_2	LaF_3	AlF_3	BrCs	TmF_3	YbF_3
UCG-A	50.0	16.5	2.45	1.55	24.25	0.75	4.5
UCG-B	50.1	16.5	2.59	1.68	24.38	0.25	4.5

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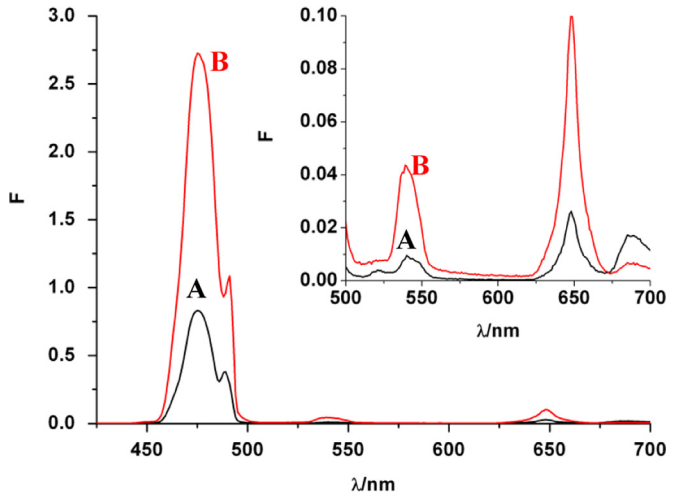

Fig. 1. Emission spectra of doped fluorohafnate upconverting glasses (see composition in Table 1) at λ_{ex}=980 nm : (A) – UCG-A; (B) – UCG-B.

eluent at a flow rate of 1.5 mL min^{-1}. The first fraction, which absorbed at 280 nm and 470 nm, contained the chemically modified enzyme GOx-FS and was recovered. The second fraction contained the excess of FS and was discarded.

2.5. GOx-FS polyacrylamide films preparation

Polyacrylamide films were prepared by dissolving 75 mg of acrylamide and 5 mg of *N,N′*-methylenebisacrylamide in 500 µL of a solution containing 455 U mL^{-1} of GOx-FS. Subsequently, 4 µL of ammonium persulfate solution (10% w/v) were added and the dissolved oxygen was removed by bubbling nitrogen through the solution. Finally, 0.5 µL of TEMED were added and the mixture was immediately spread on a glass cast ($0.7 \times 0.5 \times 0.8$ mm). The mixture was left to react for one hour, after being covered with a glass slide. The GOx-FS film was then kept in MES buffer solution (pH 6.0, 0.1 M).

2.6. Measurement procedures

2.6.1. GOx-FS films measurement system

A home-made flow cell (Fig. 2) was used. The GOx-FS film was placed in the hole of the stainless steel piece and covered with two windows (0.17 mm thickness), for providing optical transparency, and the UCG was fixed next to the frontal window. Both windows together with the UCG were held with Hoffmann clamps. The flow cell was fixed in the sample compartment of the spectrofluorometer (additional details are given in Supplementary material, Fig. S1). The diode laser previously described was used as excitation source (at 0° regarding to the detection), instead of the xenon arc lamp of the spectrofluorometer, which was previously blocked. During the measurements, the MES buffer solution (pH 6, 0.1 M) flowed across the cell at 0.5 mL min^{-1} and 1 mL of different concentrations of glucose was injected. The fluorescence intensity was simultaneously monitored at 520 nm and at a reference wavelength of 673 nm. The Area (A) and the height (H) of the quotient signal were used as the analytical parameters.

2.6.2. Fluorescence measurements in solution

The solutions were placed in a conventional quartz cuvette. The UCG was fixed next to the outside face of the cuvette. The diode laser previously described was situated in-line (at 0°) with the detector in the sample compartment of the spectrofluorometer (the original xenon arc lamp was blocked during the measurements). FS solutions were prepared in MES buffer (pH 6, 0.1 M)

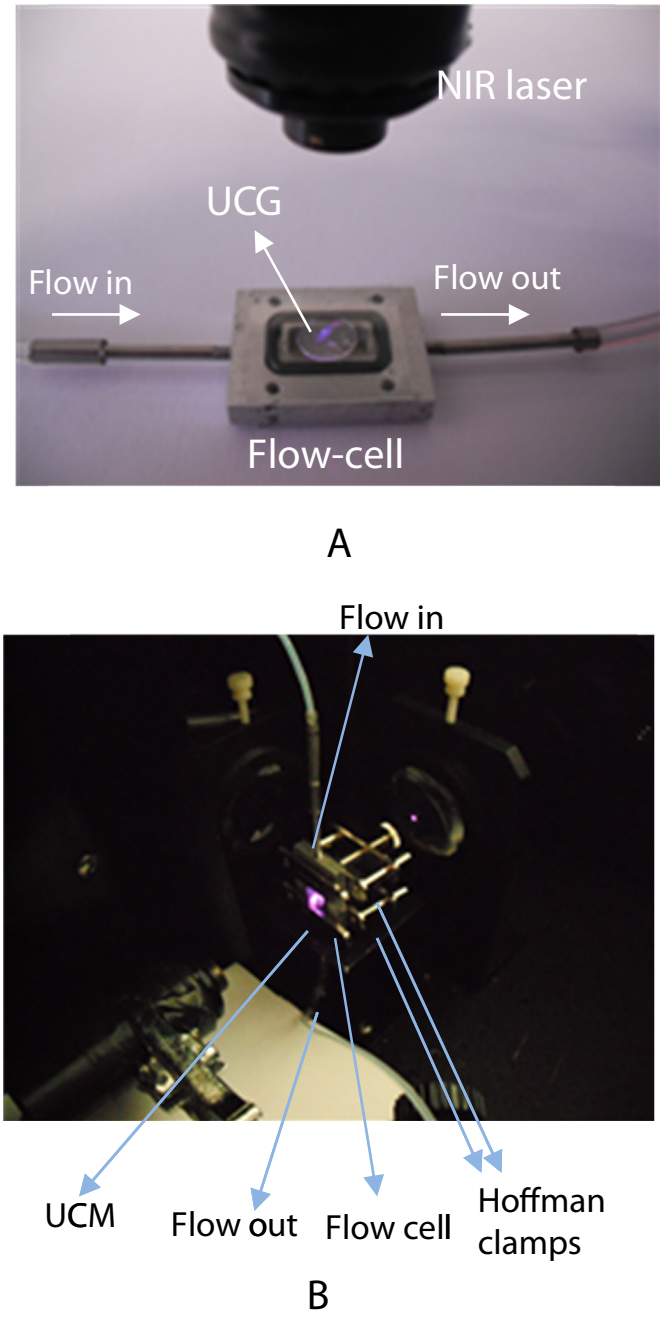

Fig. 2. Fluorescence arrangement. **(A)** Biosensor flow cell (**B**) Luminometer sample holder with the biosensor flow cell inside.

and the emission was acquired at 520 and corrected by the fluorescence intensity at 650 nm, which was used as the reference wavelength.

3. Results

3.1. Effect of the GOx-FS on UCG fluorescence

3.1.1. Choice of materials

Most of the optical biosensors for the determination of glucose in biological samples require the use of visible excitation sources (laser or LEDs), which, in some cases, cause damaging problems in the sample. As stated in the introduction section, UCM glasses can be used as a visible excitation source avoiding the use of the more

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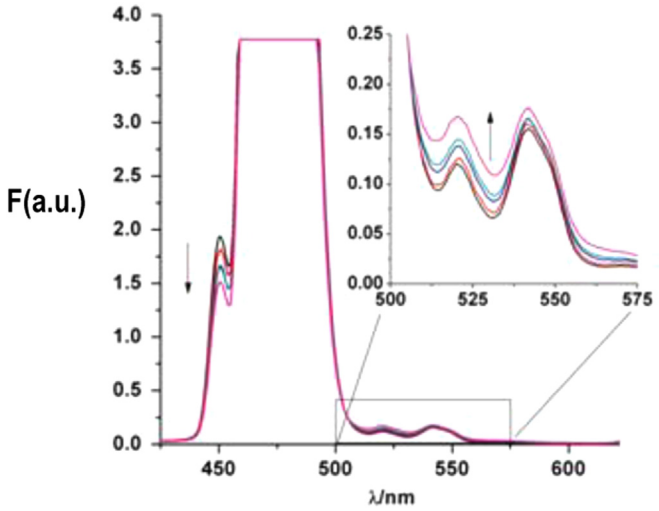

Fig. 3. Emission spectra of UCG-A in a solution containing different concentrations of GOx-FS (in U mL^{-1}) in MES buffer: – 0, – 2.7, – 5.4, – 8.0, – 13.3. (λ_{excit}=980 nm). Insert: spectra for 500–575 nm.

energetic laser or LEDs sources. These because the effect of the GOx-FS in the fluorescence of UCGs was studied.

3.1.2. Detection scheme

The capabilities of the UCG as excitation source of the GOx-FS was studied both in solution (according to procedure described in the *Measurements in solution* section) and in a biosensor film of GOx-FS immobilized in polyacrylamide (according to the *GOx-FS films measurement system* section).

Fig. 1 shows that under excitation at 980 nm, the upconverting glasses fluorescence spectra possess a very intense emission maximum at 475 nm, and secondary maxima at 450, 520, 540 and 650 nm (with relative intensities of 1, 0.065, 0.085 and 0.65 respectively). In the presence of different GOx-FS concentrations (Fig. 3), the three different behaviors expected were observed: 1) intensity decrease in the crystal at those wavelengths fitting with the excitation spectrum of the modified enzyme was observed (450 nm and 475 nm, the last one is not shown in the figure because of the signal appears saturated); 2) intensity increase at those wavelengths overlapped with the fluorescence spectra of the GOx-FS (520 nm and 540 nm); and 3) no intensity changes at those wavelengths which do not match neither the excitation nor the fluorescence spectra of the fluorophore (650 nm). The decrease in UCG fluorescence at 450 and 475 nm can be explained in terms of the inner filter effect (IFE) produced by GOx-FS. By the application of the classical IFE equation [18] to the fluorescence intensity of the UCM at any wavelength affected by IFE ($F_{IFE,\lambda}$) (see Supplementary Material for detailed deduction), the decrease in the fluorescence intensity at 450 nm given in Fig. 3 follows the equation:

$$-\log\left(\frac{F^c_{IFE,\lambda}}{F^c_{0,\lambda}}\right) = 2,18{*}10^5\left[GOx-FS\right] - 3,96{*}10^{-2} \quad r = 0,995 \tag{3}$$

$F^c_{0,\lambda}$ being the corrected fluorescence intensity in the absence of IFE and $F^c_{IFE,\lambda}$ the corrected fluorescence affected by IFE. The fluorescence intensity (F_λ) at any wavelength in the GOx-FS fluorescence range (see Supplementary Material for deduction), and particularly to the fluorescence intensity at 520 nm given in Fig. 3, fit to the following equation:

$$\frac{F^c_{F,520}-F^c_{0,520}}{F^c_{0,520}} = 8,30{*}10^5\left[GOx-FS\right] + 0,98 \quad r = 0,995 \tag{4}$$

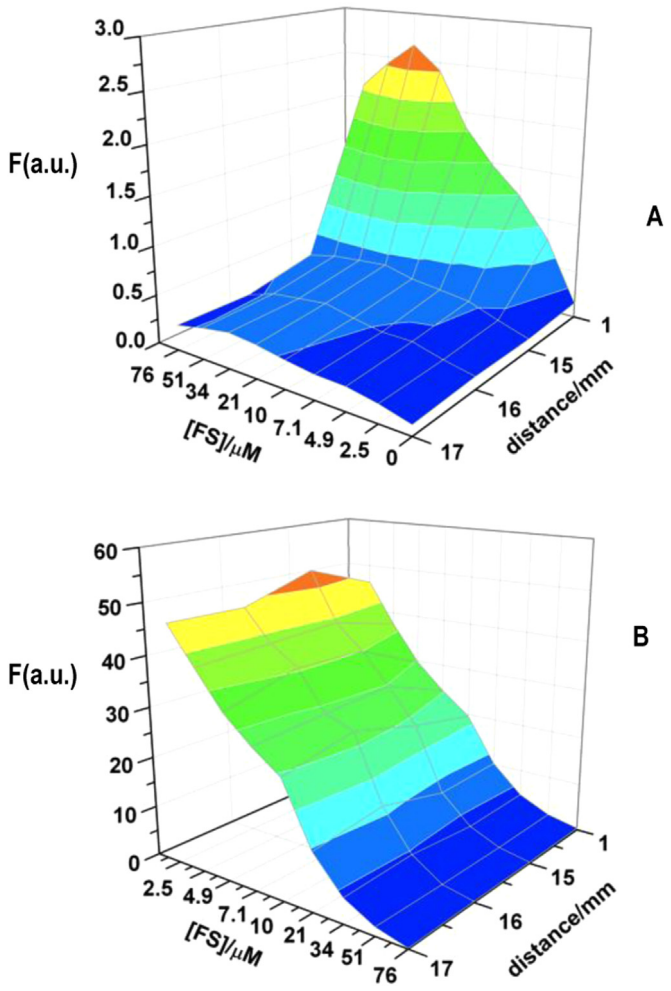

Fig. 4. Effect of the distance between the UCG and the FS solution in the fluorescence intensity values (F, arbitrary units). A set of four distances (1, 15, 16, 17 mm) and 9 FS concentrations (0, 2.5, 4.9, 7.1, 10 ,21, 34, 51 and 76 μM) were tested. **(A)** Measured at 475 nm (inner filter effect); **(B)** Measured at 520 nm (fluorescence). Working conditions indicated in "Section 2.6".

These results indicate that the fluorescence intensity coming from the UCG linearly depends on the GOx-FS concentration.

3.2. Optimization of the optical system

As has been indicated in the experimental section, the laser, the flow and the detector are placed in the same optical line. To obtain the strongest excitation, the laser beam should as close as possible to the glass. The fluorescence radiation leaving the UCM diverges, so the longer the distance at which the cuvette is located, the higher the aperture of the excitation beam; this increases the volume of the cuvette excited but decreases the intensity received per area unit. This is the reason why we studied the effect of the distance between the UCG and the cuvette. For this study only FS was measured.

A set of 4 different distances and 9 different FS concentrations were tested. The results were measured at 475 nm (IFE) and 520 nm (fluorescence) and they are indicated in Fig. 4 as a 3D plot. The obtained results clearly indicated that the lower the distance the higher the effect of the FS concentration in both fluorescence and IFE, so the UCG should be located in contact with the cell (d=0, or less than 1 mm if possible).

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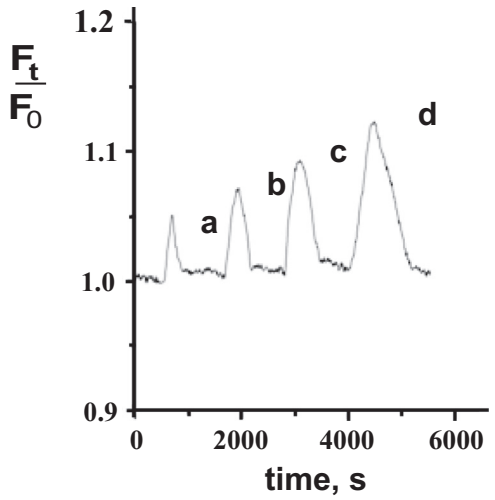

Fig. 5. Variation of the relative fluorescence intensity (F_t/F_0) at 520 nm of a UCG-A and PAA-GOx.FS sensor film during the reaction; this parameter was obtained by dividing the fluorescence intensity at each moment (F_t) by the fluorescence intensity baseline (F_0). Glucose concentrations used (mM) were: (a) 4.6, (b) 5.7, (c) 6.8, (d) 11. Working conditions indicated in "Section 2.6".

3.3. Sensor based on UCG/GOx-FS for glucose determination

3.3.1. Theoretical model

As has been indicated, GOx-FS in the oxidized form (FAD) presents a higher absorption and a lower fluorescence than the reduced form (FAD.H_2), so when FAD is reduced to FAD.H_2 the IFE on the UCG (450 and 475 nm) decreases and the fluorescence coming from the UCG (520 and 540 nm) increases. During the enzymatic reaction FAD becomes partially reduced to FAD.H_2, proportionally to the glucose concentration ($[G]_0$). Working in FIA mode, transient positive signals are observed (Fig. 5).

A theoretical treatment permits to relate the peak height (H) and the peak area (S) with the glucose concentration ($[G]_0$) according to the following equations (see Supplementary material for deduction):

$$H = M[G]_0 \tag{5}$$

$$S = \frac{M\,[O_2]_0}{4k_1[GOx - FS]_0}\left(\frac{[G]_0^2}{8[G]_{min}^2} + \frac{[G]_0}{2[G]_{min}} - 1\right) \tag{6}$$

M being a parameter depending on the mass transport and the instrument properties of the instrumental system and $[G]_{min}$ being the quantification limit.

3.3.2. Dopants concentration in fluorohafnate glasses

Despite both signals can be used (IFE or fluorescence), the fluorescence intensity at 520 was finally chosen for the biosensor performance because it gives more sensitivity. Most of the parameters related to the chemical reaction (working pH and GOx-FS concentration), membrane composition (monomers concentration, GOx chemical modification with FS and entrapment) and flow conditions (sample volume injected, carrier flow,...) were optimized elsewhere [16]. The optical arrangement was previously optimized so only the UCG will be deal with here.

UCG of different composition were synthetized [17] but finally, due to their optical properties, only two were considered as candidates (UCG-A and UCG-B) (Fig. 1). As can be seen, the fluorohafnate glass UCG-A containing 0.25 mol% Tm^{3+}, 4.5 mol% Yb^{3+} exhibit a higher upconversion fluorescence at 475 nm than glasses UCG-B with 0.75 mol% Tm^{3+}, 4.5 mol% Yb^{3+} and hence, in the presence of a GOx-FS film, a higher fluorescence at FS emission wavelengths was observed. In the biosensor system, the use of the highest efficient glass could imply a reduction of the noise in fluorescence intensity and an improvement of the analytical signal. In order to confirm this, the noise values of the fluorescence intensity (F_b/F_a) over time, signal to noise ratios (SNR) as well as area and height values of the peaks obtained after glucose injection (1 mL of 9.0 mM glucose) were calculated for both glasses (Table 2). Best results were obtained using the UCG-A.

Table 2
Noise and SNR of the fluorescence intensity. RSD for Area and Height values (for 1 mL of 9.0 mM glucose solutions, n=5) obtained for the doped fluorohafnate glasses studied.

Upconverting glass	Noise	SNR	A (RSD)	H (RSD)
A	0.0804	12.4	10%	5%
B	0.0470	21.3	12%	6%

3.4. Analytical figures of merit and application

Aliquots containing different glucose concentration were injected at working conditions indicated in Section 2.6.1 and the fluorescence increase at 520 nm was monitored. From the corresponding transient signal obtained, both A and H were measured. After applying least squares regression, the results obtained were fitted to the mathematical model previously deduced and the following equations were obtained:

$$H = 11,5[G]_0 - 0,0015 \qquad r = 0,994 \tag{7}$$

$$S = 3,45.10^5[G]_0^2 + 3,48.10^3[G]_0 - 13,1 \quad r = 0,998 \tag{8}$$

The H changes linearly with the glucose concentration up to 11 mM. The A values fit a second order polynomial equation in the whole concentration range assayed (up to 14 mM). By comparing (Eq. (6)) and (Eq. (8)) an average $[G]_{min}$ value of 1.9 mM was obtained (similar values are obtained from the coefficient of the second degree term and the intercept). From the Table 2 and the Fig. S.5 the uncertainty of the different calibration points can also be obtained. The average RSD (n=5) was about 5% for H and 10% for A.

A very first evaluation of the sensor life was carried out. Measurements were performed during 9 days (one measurement each day). The relative standard deviation obtained were 15% for A and 10% for H and no tendencies in the values were observed.

The sensor was finally applied to glucose determination in two commercial fruit juices from orange and peach respectively; the only treatment applied was the appropriate dilution in the buffer solution. The results obtained, using the H parameter, were 17.7 ± 0.9 g l^{-1} for the orange juice (n=3) and 42.2 ± 1.6 g l^{-1} for the peach juice (n=3). Bot juices were previously analyzed (by triplicate) by the classical spectrophotometric-enzymatic method based in GOx/Horseradish Peroxidase/ABTS; concentrations of 16.6 ± 0.8 g l^{-1} for the orange juice and 45.4 ± 1.2 g l^{-1} for the peach juice. These results did not show statistical differences (*t*-test).

4. Conclusions

The results obtained demonstrate that the combination of the upconversion fluorescence of doped glasses and the spectroscopic properties of GOx-FS allows the determination of glucose using near-infrared laser excitation. This is a great advantage for

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biological applications due to the minimization of interferences and autofluorescence at the working wavelengths. Moreover laser modules required are affordable and the methodology could be easily extended to other biochemical analytes by the use of the corresponding flavoenzymes. Finally, UCG are easy to manufacture and water-resistant, so they can be also incorporated into conventional optical material (cells, well-plates,…) or even in polyacrylamide films (using powdery UCG) for taking advantage of these capabilities.

Novelty statement

The fluorescence of an Upconverting crystal is used as an excitation source of a polyacrylamide film having glucose oxidase. This idea could be implemented in different kinds of conventional optical materials (as well-plates) for taking advantage of this type of fluorescence.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.07.062.

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