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ORIGINAL PAPER

Autoindicating optical properties of laccase as the base of an optical biosensor film for phenol determination

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Abstract In the context of sustainable analytical chemistry, phenol has been determined through its enzymatic reaction with laccase. The method has been studied and optimized through the autoindicating optical properties of laccase both by intrinsic molecular absorption and fluorescence. The method shows a linear range from $9.79 \cdot 10^{-6}$ to $7.50 \cdot 10^{-4}$ M with a relative standard deviation of 1.07 %. The molecular absorption methodology has been implemented in a polyacrylamide film for the design of an autoindicating optical sensor. In order to increase the lifetime of the sensor, the reversibility study of the enzymatic reaction has proposed, as a novelty, the regeneration of laccase with an oxidase-type enzyme (glucose oxidase). The lifetime of the sensor film has improved from 15 to 30 measurements. The reaction mechanism has also been studied and confirmed by fluorescence and molecular absorption. The method leads to the determination of phenol in environmental samples.

Keywords Laccase · Phenol · Autoindicating · Protein · Sensor film · Enzymatic

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Introduction

Sustainability is nowadays one of the main aims of scientific research, particularly in the field of green chemistry. In this context, many analytical methods have focused on developing simple and reversible procedures with biocompatible compounds [1, 2]. In recent years, analytical procedures based on the use of the autoindicating optical properties of proteins have been developed [3, 4] enabling the determination of analytes of clinical [5–8] or environmental interest [9].

Of these autoindicating optical properties, the most frequently used are the intrinsic fluorescence of enzymes owing to the tryptophan groups in the UV region or that of the flavin groups (flavin adenine dinucleotide (FAD) or FMN) in the visible region. Nevertheless, proteins have also been genetically or chemically modified with green fluorescence protein or fluorophores. Several research groups have studied these fluorescence properties in different kinds of proteins:

Periplasmatic-binding proteins These proteins are linked to their ligands (or to DNA fragments) with high selectivity. As a result of the union, the protein suffers a folding that induces changes in the intrinsic fluorescence of the tryptophan or fluorophores previously linked to the protein. Cass [10, 11] began this line of research, which is currently also being developed by Hellinga et al. [12, 13].

Flavo-enzymes As the FAD and its reduced form, $FADH_2$, have different fluorescence properties, it is possible to

follow the enzymatic reaction through these changes in fluorescence. The main problem of this methodology is the weak fluorescence of the flavin groups which has led to (a) the use of the changes in the fluorescence of the tryptophan due to an energy transfer from the flavin group [14, 15] or (b) the chemical modification of the enzyme with a fluorophore [16].

Apo-enzymes Lakowicz [17] affirms that, in flavoenzymes, the union of the substrate to the enzyme produces a conformal change amending the intrinsic fluorescence of the tryptophan and thus avoiding the redox reaction.

Whole cells Daunert's group [18–20] uses the fluorescence of the proteins in live cells. The main disadvantages of this methodology are the paucity of preservation methods for long-term storage and the physiological signal background.

Other important group of autoindicating optical properties which have been studied is the intrinsic molecular absorption of proteins due to hemeproteins. Most of the studies with hemeproteins such as HRP, catalase or haemoglobin, have focused on the reaction mechanisms from the biochemistry point of view. However, in recent years our research group has used hemeproteins for the quantitative determination of analytes [21, 22]. The most interesting application has been the use of blood haemoglobin as a sustainable reagent [8].

Apart from all the characteristics mentioned above, proteins having autoindicating optical properties have several analytical advantages such as the fact that the analytical methodology is substantially simplified, there is analytical and kinetic control (the analytical signal is provided by the protein itself) and, finally, the advantage of reversibility. One example is laccase (Lac) [23, 24], a copper-containing protein which could be used as an autoindicating protein.

Lac (E.C. 1.10.3.2, 4-benzenediol oxygen oxidoreductase) is an oxidoreductase which catalyzes the oxidation of aromatic compounds by the reduction of O_2 to H_2O; the active centre contains four atoms of copper [25, 26] with intrinsic spectroscopic properties. During the enzymatic reaction of Lac with phenol different intermediates with different spectroscopic properties appear [27]. As far as we know, these intrinsic optical properties have never been used to follow the enzymatic determination of phenol.

Phenolic compounds are widespread in nature. They can be found in fruits and vegetables, and they are responsible for the organoleptic properties of wine and olive oil. Their antioxidant properties [28] help to prevent cancer and cardiovascular diseases. However, some phenols have been recognised as toxic, carcinogenic or allergenic [29, 30] and a new methodology for their determination and elimination is being studied. The maximum concentration of phenols allowed in waste water by the European Union is $1.06 \cdot 10^{-5}$ M [31]. Most of the methodology for determining phenolic compounds is based on HPLC or CG-MS with a previous derivatisation or preconcentration step [32, 33].

In this paper, an autoindicating analytical procedure for the determination of phenol based on the intrinsic optical spectroscopic properties of Lac is presented, the final purpose being the design of an optical autoindicating biosensor.

Experimental

Apparatus

The molecular absorption measurements were carried out in a Hewlett-Packard 8453 diode-array spectrometer. The fluorescence was measured in a Perkin-Elmer LS50B luminometer and in a Time Master Photon Technology International (PTI) model TM-2/2003-PTI.

Oxygen was measured with a Fibre Optic Oxygen R-Sensor Probe (Ocean Optics).

Reagents

Lac from *Trametes Versicolor* (21.8 U/mg solid) E.C. 1.10.3.2 and glucose oxidase (GOx) from *Aspergillus niger* (155 U/mg solid) EC 1.1.3.4 were obtained from SIGMA. Tyrosinase from *Mushroom* (1,444 U/mg solid) E.C. 1.14.18.1 was obtained from THERMO SCIENTIFIC. Diluted solutions of each enzyme were daily prepared by dissolving the respective quantity in phosphate buffer solution of pH 6.

Phenol (Sigma P 3653) and hydrogen peroxide (Scharlab, 30 %) solutions were daily prepared in the phosphate buffer solution. Acrylamide, *N*,*N*′-methylenebis(acrylamide) and peroxodisulphate (SIGMA) were used for the sensor film preparation.

The phosphate buffer solution (0.1 M and pH 6) was prepared from solid KH_2PO_4 and solid Na_2HPO_4.

Procedure

Absorbance measurements

Two millilitres of the corresponding Lac solution in phosphate buffer was added to a 1-cm path length quartz cell, and the absorbance at the working wavelength (represented with sub-index λ_{max}, 400 nm in most cases) began to be monitored. Then 0.1 mL of the corresponding phenol solution was added. The analytical parameters are given by the following expressions:

$$\Delta\mathrm{Abs} = \mathrm{Abs}_{\lambda_{\max,t}} - \mathrm{Abs}_{\lambda_{\max,0}}$$

$$V_0 = \frac{\Delta\mathrm{Abs}_{\lambda_{\max}}}{\Delta t} = \frac{\mathrm{Abs}_{\lambda_{\max,t}} - \mathrm{Abs}_{\lambda_{\max,0}}}{t}$$

$\mathrm{Abs}_{\lambda_{\max,0}}$ and $\mathrm{Abs}_{\lambda_{\max,t}}$ being the absorbance at a time t or 0, at the working wavelength.

Fluorescence measurements

Two millilitres of the corresponding Lac solution in phosphate buffer was added to a 1-cm path length quartz cell. The fluorescence intensity at the working wavelengths (λ_{ex}=340 nm and λ_{em}=450 nm) began to be monitored, after which 0.1 mL of the corresponding phenol solution was added. The analytical parameter is given by the following expression:

$$V_0 = \frac{\Delta I_f}{\Delta t} = \frac{I_t - I_0}{t}$$

I_t and I_0 being the absorbance at a time t or 0, at the working wavelength.

Results and discussion

The enzymatic reaction of phenol with Lac was studied and optimised based on the autoindicating optical properties of Lac both by the intrinsic molecular absorption and fluorescence.

Molecular absorption in solution

Figure 1 shows the variation of the absorption spectra of Lac before and after the addition of phenol. It is noticed that as the reaction takes place a new maximum appears at 400 nm. According to the enzymatic reaction mechanism (Fig. 2), the new peak is assumed to be due to the reduced form of Lac ($\mathrm{Lac_{red}}$). The observed decrease in absorbance at this wavelength, after a maximum of the signal is reached, indicates the regeneration of the oxidised form of Lac (this was confirmed by a new addition of phenol, observing a new increase in the absorbance signal at 400 nm). From the absorption spectra, it can also be seen that after a certain time, a new maximum appears at 425 nm.

Optimisation

The optimal conditions for the enzymatic reaction were studied working with two different analytical parameters, the increase in absorbance at 400 nm (ΔAbs) and the initial slope of the enzymatic reaction (V_0) at this wavelength.

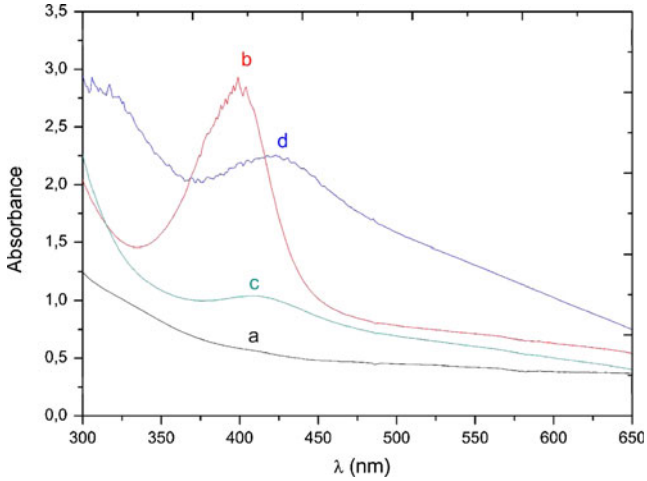

Fig. 1 Variation of the absorption molecular spectra of Lac ($1.56{\cdot}10^{-5}$ M) with phenol ($5.00{\cdot}10^{-3}$ M) in buffer phosphate of pH 6. (*a*) Oxidised form of Lac at t=0 min, (*b*) reduced form of Lac at t=5 min, (*c*) regenerated oxidised form of Lac at t=30 min, and (*d*) polymer formed for enzymatic reaction at t=150 min

Buffer solution and pH From the bibliography [34–37], it was obtained that Lac from Trametes Versicolor works effectively, both with phosphate or acetate buffers. After a preliminary study, we decided to use the phosphate buffer for the optimisation, given that higher ΔAbs and V_o values were obtained. Figure 3 shows the variation in absorbance at 400 nm with the reaction time at different pH values. As can be seen, the higher absorbance variations are obtained at pH 5. However, the regeneration of the enzyme is better at pH 6. For this reason, the latter value was used during the optimisation.

Lac concentration Table 1 shows how the V_0 and the ΔAbs increase by increasing the enzyme concentration until $1.56{\cdot}10^{-5}$ M. From this concentration, no significant differences were observed either in both parameters; as result it was used as the optimum enzyme concentration.

Analytical characteristics

The analytical characteristics (Table 2) were obtained working at pH 6 in a 0.1 M phosphate buffer solution and with a (Lac) of $1.56{\cdot}10^{-5}$ M. In any case, it was observed that at phenol concentrations higher than $7.50\ 10^{-4}$ M the new maximum at 425 nm starts to increase (Fig. 1d). As stated above, the analytical parameters were calculated as ΔAbs and as V_0. As can be seen, working with ΔAbs the linear range is very narrow, mainly due to the irreproducibility of the initial absorbance (each enzyme solution was prepared immediately before use). However, two linear ranges can be deduced, one for low phenol ($9.79{\cdot}10^{-6}$ to $7.50{\cdot}10^{-4}$ M) concentrations and the other for higher concentrations

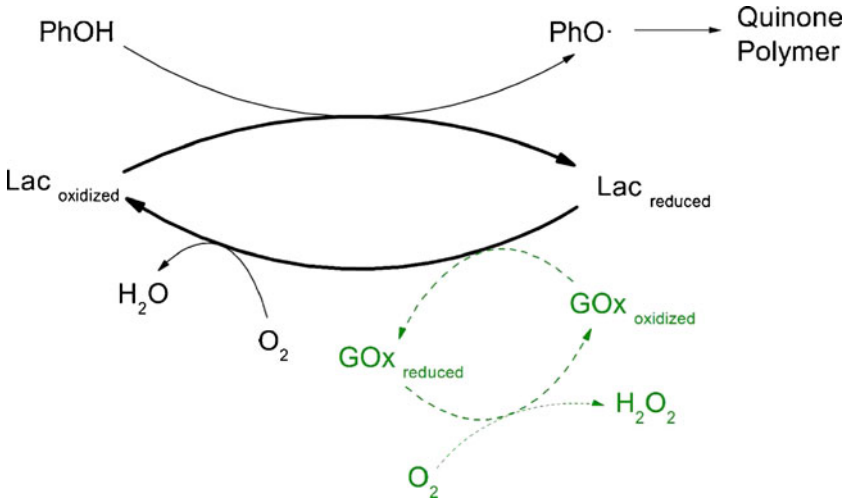

Fig. 2 Schematic representation of the enzymatic reaction of phenol with Lac (*solid line*) and enzymatic regeneration proposed (*dashed line*)

($7.50 \cdot 10^{-4}$ to $5.00 \cdot 10^{-3}$ M). Moreover, working with this parameter the analytical characteristics such as the sensitivity, relative standard deviation (RSD) and limit of quantification (LOQ) were better. These results verify that it is possible to determine phenol in the conditions described and using ΔAbs as the analytical parameter.

In addition, working with V_0 and by plotting $1/V_0$ vs. 1/(phenol), the Michaelis–Menten constant (km=$3.12 \cdot 10^{-3}$ M) and the V_{max} ($5.00 \cdot 10^{-2}$ Abs·s^{-1}) were calculated (see Fig. S2 in the Electronic supplementary material (ESM)).

Molecular fluorescence in solution

A 3D spectrum of Lac (see Fig. S1a in the ESM) confirmed the presence of different fluorescence maxima, one at λ_{ex}=280 nm and λ_{em}=340 nm due to the presence of tryptophan groups, and two more at λ_{ex}=340 nm or 360 nm and λ_{em}=450 nm due to the Cu centres, which is consistent with data given in the literature [38]. A 3D spectrum of the solution after the addition of phenol gave rise to the disappearance of the fluorescence peak at 450 nm. This decrease was confirmed to be due to both an inner filter effect of the product of the reaction and to the lower fluorescence signal of the reduced Lac, at the same time that a new maximum appears at λ_{ex}=300 nm and λ_{em}=460 nm. Figure S1 in the ESM shows the 3D spectrum of the Lac–phenol reaction before and after the elimination of the polymer by centrifugation.

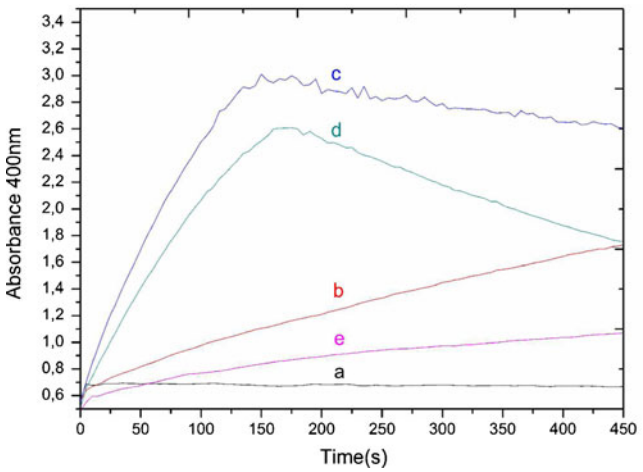

Fig. 3 Variation of Abs_{400nm} with reaction time at different pH values. Reaction conditions: (Lac)=$1.56 \cdot 10^{-5}$ M, (Phenol)=$2.50 \cdot 10^{-3}$ M in buffer phosphate of (*a*) pH=3, (*b*) pH=4, (*c*) pH=5, (*d*) pH=6, and (*e*) pH=7

Optimisation

The optimal conditions were studied working at the Cu centre wavelengths (λ_{ex}=340 nm and λ_{em}=450 nm). The pH and the buffer solution were those optimised within the molecular absorption methodology.

Lac concentration Through the representation of the variation of the fluorescence intensity with the concentration of Lac, it can be seen that by increasing the Lac concentration the fluorescence intensity shows a linear dependence until $4.78 \cdot 10^{-6}$ M. It can also be seen that due to the Lac autoabsorption, a quench on the fluorescence intensity value is observed from $7.16 \cdot 10^{-6}$ M and above. However, working at this Lac concentration the enzymatic reaction is very slow and very long reaction times would be necessary to obtain significant changes in the fluorescence intensity with the phenol concentration. Taking these considerations into account, (Lac)=$1.56 \cdot 10^{-5}$ M was chosen as the optimum value.

Analytical characteristics

As stated in the previous paragraph, due to the Lac autoabsorption, the Lac concentration used would greatly increase the time required to stabilise the reaction. Thus, only the V_0 was chosen as the analytical parameter. In these conditions,

Table 1 Optimisation of Lac concentration

(Lac) M	$5.21\cdot10^{-6}$	$8.66\cdot10^{-6}$	$1.21\cdot10^{-5}$	$1.56\cdot10^{-5}$	$2.26\cdot10^{-5}$	$2.60\cdot10^{-5}$	$3.02\cdot10^{-5}$
ΔAbs	1.94	1.91	1.90	2.01	1.91	1.87	1.75
V_0	$1.25\cdot10^{-2}$	$1.77\cdot10^{-2}$	$2.53\cdot10^{-2}$	$3.64\cdot10^{-2}$	$3.62\cdot10^{-2}$	$3.58\cdot10^{-2}$	$3.52\cdot10^{-2}$

All measurements in 0.1 M phosphate buffer of pH 6 and $5.00\cdot10^{-3}$ M of phenol

the linear range of the method is very narrow and the analytical characteristics very poor (RSD around 27 %).

Reversibility studies

The final purpose of the methodology based on the use of the autoindicating properties of enzymes is the design of an optical biosensor. This is the reason why an in-depth study of the reversibility of the enzymatic reaction was proposed.

The first studies focused on the absorption properties of Lac. According to the proposed mechanism (Fig. 2), the re-oxidation of Lac starts once the phenol has totally reacted and is re-oxidised by the oxygen dissolved in solution. However, as can be seen in Fig. 1c, this reversibility is barely reached, mainly due to the concentration of oxygen in the solution ($(O_2)=2.20\cdot10^{-4}$ M). Several oxidant solutions were studied and, although the reversibility improved, in no case was 100 % regeneration of Lac achieved.

Enzymatic regeneration

It is known that tyrosinase (Tyr) catalyses the hydroxylation of phenols to catechols and then the further oxidation of catechols to ortho-quinones with the presence of molecular oxygen [39]. The optical properties of Tyr for phenol determination were tested, and it was concluded that although it presents an absorption maximum at around 400 nm, the changes with the addition of phenol lack sufficient sensitivity to be able to follow through the determination with this methodology. Nevertheless, it was supposed that the addition of two phenol sensitive enzymes would improve the kinetics of the enzymatic reaction and lead to its total reversibility. However, as both Lac and Tyr present absorbance maxima at 400 nm which change during the enzymatic reaction, it is difficult to distinguish which changes in the absorbance are due to the regeneration of Lac only.

At this point the possibility was studied of implementing an oxidase type enzyme which was thought to regenerate the Lac according to the scheme proposed in Fig. 2. The best results were obtained with GOx for which, as can be seen in Fig. 4a, the 100 % regeneration of Lac was easily achieved. After studying the effect of the GOx concentration on the regeneration process, a molar ratio of 1:1 (Lac/GOx) was chosen as the working concentration.

To confirm the results, the regeneration of Lac was studied in the presence and in the absence of GOx following the changes in the fluorescence signal of Lac (λ_{ex}=340 nm and λ_{em}=450 nm) (Fig. 4b). These results were consistent with those obtained when studying the enzymatic regeneration by molecular absorption.

The regeneration mechanism was also followed by the consumption of the dissolved oxygen in solution, using a Fibre Optic Oxygen R-Sensor Probe (OCEAN OPTICS). As expected, higher oxygen consumption and a higher consumption rate was obtained in the presence of GOx. The oxygen depletes due to both the Lac and the GOx regeneration. Table 3 shows these results.

It is important to point out that in both cases (AM and FM) the presence of GOx helps the regeneration of Lac, but although the V_0 of the reaction is the same, the changes in the signal (ΔAbs or Δ*I*) are lower, mainly due to the regeneration mechanism. As the solution contains both Lac and GOx, the regeneration of the Lac_{red} starts simultaneously with its enzymatic reaction with phenol.

Table 2 Analytical characteristics of the autoindicating Lac–phenol determination by molecular absorption both in solution and in the sensor film

	Linear range (M)	Slope	% RSD	LOD (M)	LOQ (M)
ΔAbs[a]	$9.79\cdot10^{-6}$ – $7.50\cdot10^{-4}$	$4.20\cdot10^{3}$	1.07	$2.94\cdot10^{-6}$	$9.79\cdot10^{-6}$
V_0[a]	$1.36\cdot10^{-4}$ – $5.00\cdot10^{-3}$	$1.70\cdot10^{1}$	2.61	$4.07\cdot10^{-5}$	$1.36\cdot10^{-4}$
ΔAbs[b]	$1.09\cdot10^{-4}$ – $2.50\cdot10^{-3}$	$5.40\cdot10^{1}$	3.11	$3.27\cdot10^{-5}$	$1.09\cdot10^{-4}$

[a] In solution with $(Lac)_{solution}=1.56\cdot10^{-5}$ M

[b] In the sensor film with $(Lac)_{sensor\ film}$=87.2 IU

Sensor film

The next step of the study is to develop a system for continuously measuring the phenol concentration in samples without pre-treatment of the sample and thus without a regeneration step.

From the batch process, it was established that the absorption method shows better analytical characteristics and was selected as the method to implement the enzyme in a sensor film.

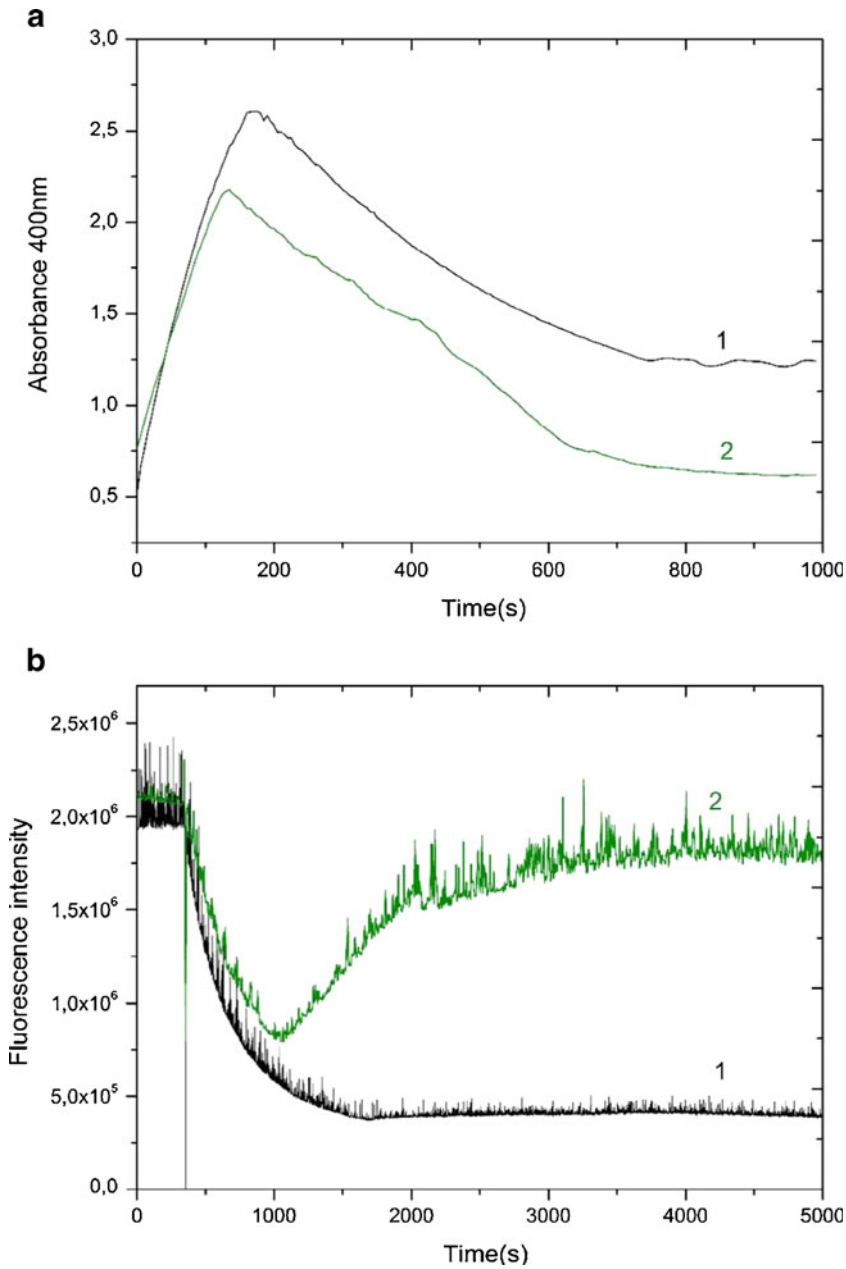

Fig. 4 Effect of GOx ($1.56 \cdot 10^{-5}$ M) on the regeneration of Lac ($1.56 \cdot 10^{-5}$ M). (*1*) In absence of GOx and (*2*) in presence of GOx. (*a*) Variation of the Abs_{400nm} of Lac for enzymatic reaction with phenol ($2.50 \cdot 10^{-3}$ M) in buffer phosphate of pH 6. (*b*) Variation of the fluorescence intensity (λ_{ex}=340 nm and λ_{em}= 450 nm) of Lac for enzymatic reaction with phenol ($7.50 \cdot 10^{-4}$ M) in buffer phosphate of pH 6

Table 3 Oxygen consumption of the Lac–phenol reaction in presence and in the absence of GOx

	Lac[a]	Lac/GOx (1:1)[b]
ΔI[c]	133	164
Oxygen consumption ($\Delta I/\Delta t$)	0.189	0.234

(Lac)=$1.56 \cdot 10^{-5}$ M, (phenol)=$7.00 \cdot 10^{-4}$ M, 0.1 M phosphate buffer of pH 6

[a] In absence of GOx

[b] In presence of (GOx)=$1.56 \cdot 10^{-5}$ M

[c] Changes on the fluorescence intensity of the sensor probe (λ_{ex}= 450 nm and λ_{em}=600 nm)

Sensor film preparation and measure

Lac was immobilised in a polyacrylamide film following the procedure described elsewhere [40] but modifying the concentration of the enzyme. In this case, 5 mg (87.2 IU) of Lac were dissolved in 100 mL of the MES buffer solution. The film was placed in a homemade flow cell [40] and settled in the cuvette compartment of the spectrophotometer. The phosphate buffer solution was used as the carrier flow and 2.5 mL of phenol of different concentrations were injected each time.

The variation of the absorbance at 400 nm was recorded and, as can be seen in Fig. 5, the changes were lower than

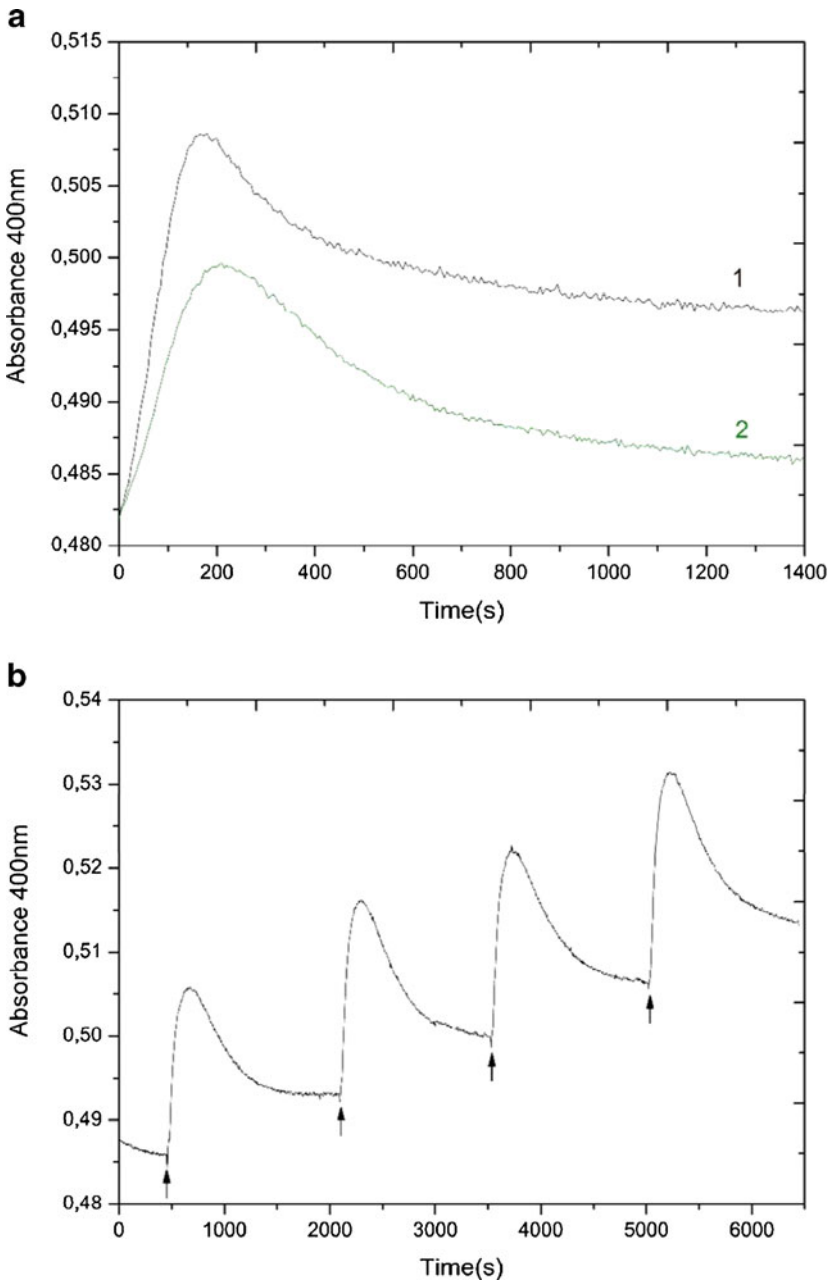

Fig. 5 (**a**) Effect of GOx (about 1100 UI) on the regeneration of Lac in sensor film (about 87.2 UI). Variation of the Abs_{400nm} of Lac for enzymatic reaction with phenol ($1.00 \cdot 10^{-3}$ M, injection 2.50 mL) in buffer phosphate of pH 6. (*1*) In absence of GOx and (*2*) in presence of GOx. (**b**) Variation of the absorbance of Lac in sensor film for successive injection of phenol ($3.00 \cdot 10^{-3}$ M, injection 2.5 mL) in buffer phosphate of pH 6. The repeatability of successive injections is RSD=3.11 %

those observed working in solution. The lower sensitivity of the continuous methodology was expected because of the measurement procedure used and since the rate of the reaction kinetics is not very high.

Analytical characteristics of the sensor film

It has been shown that the enzymatic reaction of Lac–phenol is not fully reversible but, as can be seen in Fig. 5, the reversibility greatly increases working in the continuous method, possibly due to the low rate of the reaction. In these conditions, the RSD of the flow system was calculated to be in the order of 3.11 % and the sensor film can be used for at least 15 measurements. Which was also observed, it was that the lifetime of the sensor film does not depend on the stored time (it can be stored for more than 6 months without decrease on the enzymatic activity of Lac) but only on the number of measurements.

The linear range was calculated measuring the increase in absorbance at 400 nm and was from $1.09 \cdot 10^{-4}$ to $2.50 \cdot 10^{-3}$ M of phenol.

The response of the Lac sensor films was also studied to catechol and *o*-nitrophenol, however, no changes on the absorption signal of Lac were observed. This fact was related to the lower sensitivity of immobilised Lac to these analytes.

Enzymatic regeneration of the sensor film

The regeneration of Lac through the addition of GOx was tested by co-immobilisation of the two enzymes in the polyacrylamide film. In this case, the sensor film was prepared by dissolving 8 mg of GOx (1,123 IU) and 5 mg Lac (87.2 IU) in 100 mL of the MES buffer solution and polymerised as described in the previous section. Figure 5 shows the improvement in the Lac regeneration. In these conditions, the analytical characteristics of the methodology were similar, but the lifetime of the sensor film improved considerably (more than 30 measurements).

Application

The absorption molecular method has been used to measure phenol in waste water and in all cases the phenol concentration was under the LOD of the method. In the case of doped waste water samples, the recuperation was in the order of 95 % (RSD of 7 %).

Enzymatic reaction mechanism

Apart from the regeneration study, the complete enzymatic reaction mechanism was studied. As stated above, as the enzymatic reaction takes place, an increase in the absorption signal at 425 nm was observed (Fig. 1d). According to the literature [41, 42], this new maximum is due to polymerisation through the formation of phenoxy radicals. When studying the enzymatic reaction through the intrinsic fluorescence of Lac, it was observed that the generated polymer leads to an inner filter effect on that fluorescence.

After studying the products of the reaction by exclusion chromatography, an increase in the absorption signal at 425 nm was confirmed at a retention time longer than that of the reduced form of Lac, which suggested the formation of a new compound different to that of the initial product of the enzymatic reaction.

This mechanism was also confirmed working with very low concentrations of phenol. In this case the maximum at 425 nm was not observed (and therefore no polymerisation was observed).

It was also seen that in the presence of GOx there were a decrease in the polymer formation. This effect is probably related to the formation of H_2O_2 which was shown to inhibit the polymerisation reaction (see Fig. S3 in the ESM).

From these results, the enzymatic reaction mechanism could be established as represented in Fig. 2.

Conclusions

The determination of phenol has been optimised following the enzymatic reaction with Lac, both by fluorescence and molecular absorption, using the intrinsic optical properties of the Lac. The final purpose is to develop a methodology to be applied in the design of an autoindicating optical sensor, and thus Lac has been immobilised in a polyacrylamide sensor film. The analytical characteristics have led to a linear range for phenol determination from $9.79 \cdot 10^{-6}$ to $7.50 \cdot 10^{-4}$ M (the LOQ is below the legal limits in waste waters). On the basis of the lifetime of the sensor film, an in-depth study of the regeneration mechanism has been carried out. As a novelty, the regeneration of Lac with an oxidase type enzyme (GOx) has been proposed. The lifetime of the sensor film was improved from 15 to 30 measurements. The reaction mechanism was also studied and confirmed by fluorescence and molecular absorption. The method allows the effective determination of phenol in environmental samples.

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