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Short communication

# Immobilization of a trienzymatic system in a sol–gel matrix: A new fluorescent biosensor for xanthine

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ABSTRACT

In this work we report the development of a highly sensitive fluorescent multienzymatic biosensor for quantitative xanthine detection. This biosensor is built by the simultaneous encapsulation of three enzymes, xanthine oxidase, superoxide dismutase and peroxidase, in a single sol–gel matrix coupled to the Amplex Red probe. The sol–gel chemistry yields a porous, optically transparent matrix that retains the natural conformation and the reactivity of the three co-immobilized proteins. Xanthine determination is based on a sequence of reactions, namely catalytic oxidation of xanthine to uric acid and superoxide radical, and subsequent catalytic dismutation of the radical, resulting in the formation of hydrogen peroxide, which reacts stoichiometrically with non-fluorescent Amplex Red to produce highly fluorescent resorufin. The optimal operational conditions for the biosensor were investigated. Linearity was observed for xanthine concentrations up to 3.5 μM, with a detection limit of 20 nM, which largely improved the sensitivity of the current xanthine biosensors. The developed biosensor is reusable and remains stable for 2 weeks under adequate storage conditions.



## 1. Introduction

The development of new biosensor devices is currently the subject of extensive research in areas such as clinical diagnostic, food technology, biomedical and environmental analysis (Borisov and Wolfbeis, 2008; Rich and Myszka, 2007; Xu et al., 2005; Velasco-García and Mottram, 2003; Wolfbeis et al., 2000). The importance of enzyme-based biosensors has increased considerably in the last years thanks to their exceptional properties which include high specificity, rapid response and low cost (Choi, 2004). The configurations of enzymatic biosensors range from simple one-enzyme devices to multienzyme systems. Generally, additional enzymes are necessary because the products obtained in the first enzymatic reaction are not detected directly by a transducer and/or because the sensitivity and selectivity of the reaction of product recognition by the transducer is improved. Consequently, the use of more than one enzyme species considerably extends the range of possible applications of biosensors for detection of numerous biological substrates, but complicates the manufacture of these devices as well as their basic design (Wollenberger et al., 1993).

One of the most important steps in the development of these kind of biosensors is the immobilization of the multienzymatic system in an appropriate solid support which integrates the biomolecules maintaining their functionality, allowing to perform reactions in a sequential order while providing accessibility towards the target analyte and subsequent substrates (Xu et al., 2006). In recent years, the renaissance of sol–gel chemistry has provided a versatile method for immobilizing and stabilizing a wide variety of enzymes and other biological molecules in transparent inorganic matrices. Compared to other immobilization methods, such as adsorption to solid supports, covalent attachment and polymer entrapment, the sol–gel glasses show numerous advantages, including entrapment of large amount of enzymes, thermal and chemical stability of the matrix, enhanced stability of the encapsulated biomolecules, excellent optical transparency and flexibility in controlling pore size and geometry. Furthermore, thanks to the porous nature of the matrix, the immobilized proteins remain accessible to interact with external specific analytes with negligible protein leaching, making possible conduction of multienzymatic reactions (Pierre, 2004; Kandimalla et al., 2006; Jerónimo et al., 2007; Gupta and Chaudhury, 2007).

Multienzymatic entrapment in the same sol–gel matrix allows sequential reactions to occur in a confined space, leading to high conversion efficiencies due to the proximity of analytes and intermediates to the enzymes active sites (Jin and Brennan, 2002). Several types of enzymatic biosensors have been developed based on two enzymes sol–gel immobilization (Martinez-Pérez et al., 2003; Pastor et al., 2004; Cui et al., 2007; Singh et al., 2007).

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However, co-immobilization of three or more enzyme species in a single sol–gel glass has only been scarcely reported (Gallarta et al., 2007), due to the difficulty to get the optimal operational conditions for different enzymes in the same matrix. In addition, in spite of the sensitivity and reliability being higher for these biosensors than for the mono and bienzymatic devices, the response time should be also higher due to the increasing number of reactions that take place within the sol–gel matrix.

Xanthine is a product generated during chemical degradation of purine nucleotides found in DNA and RNA. It penetrates cell membrane and accumulates in extracellular fluids. Quantitative determination of xanthine in biological samples is of great interest for the diagnostic of several pathologies such as perinatal asphyxia, adult respiratory distress syndrome, cerebral ischemia, tumor hyperthermia and pre-eclampsia (Jimenez et al., 2007). This analyte is routinely measured in plasma and urine samples by analytical techniques such as HPLC, chemiluminiscence or UV-spectroscopy, whose usual drawback is the laborious procedure for sample preparation (Horozova et al., 2008). Consequently, the development of alternative xanthine detection methods, which minimize this problem, continues to be a challenging analytical task (Rehak et al., 1994; Hlavay et al., 1994; Zhao et al., 1996; Arslam et al., 2006; Rahman et al., 2007). A biosensor, combining the properties of using sequential enzymatic reactions coupled to a fluorescent transducer with those of sol–gel immobilization, may offer the advantages of a sensitive, selective, low cost, and ready to use method for quantitative analyte determination. In this work we have developed a fluorescent trienzymatic biosensor for xanthine determination based on the immobilization of xanthine oxidase, superoxide dismutase and peroxidase, in a single sol–gel matrix coupled to the Amplex Red probe. Activity of the three immobilized enzymes was analysed and compared to that obtained in solution. Sensitivity, stability and reusability of the developed biosensor were also investigated.

## 2. Materials and methods

### 2.1. Reagents

Peroxidase (HRP) (E.C.1.11.1.7; from horseradish; 1310 U/mg), superoxide dismutase (SOD) (E.C.1.15.1.1; from bovine erythrocytes, 2610 U/mg), xanthine oxidase (XO) (E.C.1.1.3.22; from buttermilk, 0.5 U/mg), xanthine sodium salt, tetraethyl orthosilicate (TEOS), from Sigma–Aldrich Chemical (Milwauke, WI, USA). *N*-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red), was obtained from Molecular Probes (Eugene, OR, USA). Amplex Red stock solutions were prepared in dimethyl sulfoxide (20 mM) and stored at −20 °C. All other chemicals were of analytical or spectroscopic reagent grade. Sodium phosphate buffers (10 mM, pH 7.4) were prepared with deionized doubly distilled water.

### 2.2. Spectroscopic measurements

Absorption measurements were carried out using a Shimadzu spectrophotometer (UV-1603, Tokyo, Japan). Fluorescence measurements were performed at 37 °C using a SLM-8000C spectrofluorimeter (SLM Instruments Inc., Urbana, IL, USA).

### 2.3. Immobilization of enzymes in sol–gel monoliths

Enzymes were immobilized using the alcohol free sol–gel route described by Ferrer et al. (2002). Protein concentrations were selected to be the same that produced optimal results for the previously developed superoxide biosensor (Pastor et al., 2004). Briefly, 4.46 mL of TEOS, 1.44 mL of $H_2O$ and 0.04 mL HCl (0.62 M) were mixed at 22 °C in a closed vessel. After 1 h, 1 mL of the resulting sol was mixed with 1 mL of deionised water, and rotaevaporated for a weight loss of 0.62 g. Then 500 μL of aqueous sol was mixed with 500 μL of buffered XO ($4 \times 10^{-4}$ U/mL), HRP (0.4 U/mL) and SOD (80 U/mL) solution in a cuvette at room temperature. Gelation occurred readily after mixing. The freshly formed monoliths, having a size of ∼4 mm × 9 mm × 17 mm, were aged during 48 h, washed with buffer and stored in dark at 4 °C. For absorption measurements, glass monoliths (9 mm × 9 mm × 17 mm) were prepared using a similar procedure but with higher concentrations of enzymes (0.08 U/mL for XO; 622.9 U/mL for SOD; 818.8 U/mL for HRP).

### 2.4. Xanthine assays

The xanthine fluorimetric assays were conducted on a quartz cuvette by immersion of the sol–gel monolith in a solution containing Amplex Red (10 μM) and different xanthine concentrations. The system was directly incubated in the spectrofluorimeter, under dark at 37 °C. For each xanthine concentration, three assays were performed to asses the reproducibility and reliability of the method. The excitation and emission wavelengths were placed at 530 and 590 nm, respectively. Appropriate blanks (*i.e.*, identical samples without xanthine) were subtracted from the sample emission to eliminate the background fluorescence coming from some residual resorufin formation occurring during the time of assay.

## 3. Results and discussion

### 3.1. Immobilization of the XO–SOD–HRP system

In order to develop a stable and reusable biosensor for quantitative xanthine determination, HRP, SOD and XO were simultaneously encapsulated in sol–gel matrix. These three enzymes had already been independently immobilized in sol–gel glasses (Ellerby et al., 1992; Niu and Lee, 2000; Ferrer et al., 2003; Yang et al., 2006) and HRP and SOD were successfully co-immobilized in a single sol–gel matrix to develop a biosensor for superoxide radical (Pastor et al., 2004). However, the simultaneous encapsulation of the coupled XO–SOD–HRP system was carried out for the first time in this work. A preliminary analysis was done to prove the suitability of the co-immobilization method. Fig. 1 shows the absorption spectra of the three enzymes in solution, and co-immobilized in the sol–gel matrix. The peak observed at 403 nm corresponds to the HRP

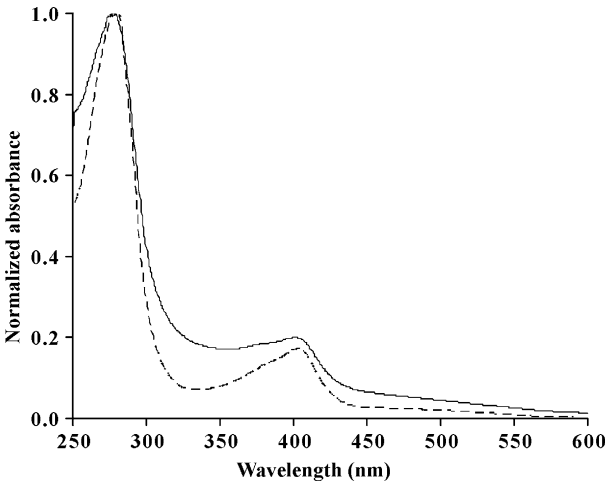


**Fig. 1.** Normalized absorption spectra of XO, SOD and HRP in solution (- - -) and co-immobilized in sol–gel monoliths (–).

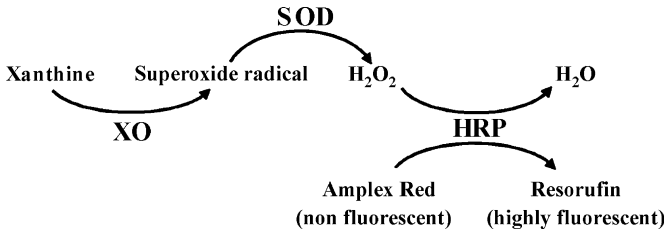


**Fig. 2.** Scheme of the resorufin formation catalysed by the coupled XO–SOD–HRP enzymatic system.

heme group absorption, while that one observed approximately at 280 nm corresponds to the total of the tryptophan absorption of HRP and XO and tyrosine absorption of SOD (although the latter practically negligible). The shape of the absorption spectrum of the enzymes in solution and in sol–gel was very similar, suggesting that these enzymes were successfully immobilized and that the immobilization process did not affect the enzymatic properties. The small differences observed in these spectra can be attributed to the higher turbidity of the sol–gel matrix as compared with that one in the aqueous solution (turbidity of the monolith without protein used as blank was always slightly lower than that of the monolith containing proteins).

### 3.2. Enzyme activity and characterization of sensor performance

The above results confirmed the success of the enzyme immobilization but did not guarantee that the enzymes remain active after encapsulation. Activity of both enzymes was tested by the xanthine assays described in Section 2. This is based on the sequential action of XO, SOD, HRP and Amplex Red to yield one molecule of hydrogen peroxide per xanthine molecule (Fig. 2). The newly formed $H_2O_2$ is used by HRP to oxidize Amplex Red into one molecule of the fluorescent dye resorufin, therefore linking xanthine levels with a measurable fluorescence signal. The time required to complete the oxidation reaction was determined by monitoring the fluorescence increase as a function of the incubation time at a constant concentration of xanthine (0.5 μM) and compared to that obtained in solution. An increase in the fluorescence signal was recorded at 530 nm to reach a constant value at ~60 min (data not shown). This time was slower than that obtained when the assay was performed for the three enzymes in solution (*i.e.* 45 min) but similar to that observed for free XO and HRP/SOD entrapped in the sol–gel matrix (Pastor et al., 2004). This result suggests that oxidation of xanthine within the sol–gel matrix does not modify the response time of the biosensor and that it is the slow diffusion of superoxide radical through the matrix the limiting factor of the biosensor response.

The comparative catalytic activities of the free and immobilized forms of the three enzymes were then examined by calibration curves in which sensitivity of the biosensor to xanthine was measured. Samples containing XO, SOD and HRP (free and immobilized in the sol–gel matrix), and Amplex Red were incubated at 37 °C with increasing concentrations of xanthine, up to 1.06 μM, for 45 min with free enzymes and 60 min in sol–gel. Fig. 3a shows the calibration curve for the sol–gel biosensor and for the free-coupled XO–SOD–HRP system. The increase in the fluorescence signal, recorded at 590 nm, was proportional to the concentration of xanthine. Both calibration curves were quite similar. Their linear ranges spanned very low concentrations of xanthine, up to 1.06 μM, with good correlation coefficients ($r^2$ = 0.994 and 0.986, respectively) and similar slopes, indicating that no activity loss occurs upon enzyme immobilization. The same linear response was found up to 3.5 μM xanthine (see insert in Fig. 3a), although under these conditions, xanthine concentrations >1.2 μM would need a different instrumental setup and higher amounts of Amplex Red. The lower detection limit, calculated following IUPAC recommendations (Thomsem et al., 2003), was also the same for both systems (20 nM), and similar to that determined for other Amplex Red-based enzymatic assays (Chapman and Zhou, 1999; Martinez-Pérez et al., 2003; Pastor et al., 2004). This value largely improves the sensitivity of the currently xanthine biosensors, as is shown in Table 1, and allows to handle diluted samples to further reduce the presence of interference substances (Martinez-Pérez et al., 2003).

### 3.3. Storage stability of enzyme system

The stability of the trienzymatic biosensor was checked over a period of 24 days with the Amplex Red assay. Ten monoliths containing the three enzymes were fabricated and stored in buffer solution at 4 °C after measuring 0.5 μM of xanthine. We found that the biosensor exhibited a 100% of the initial signal during the first 12 days. A reduction to ~75% of the sensor response was observed at day 16 and response continued to fall to 60% at day 20 and to 5% at day 24 (data not shown). Since in a previous work we found that the enzymatic activity of the sol–gel encapsulated HRP–SOD coupled system was maintained for at least 1 month (Pastor et al., 2004), it is suggested that the half-life of the immobilized XO is lower than that of HRP and SOD, although it is clearly higher than that determined in buffer (~67 h) (Radi et al., 1997). This result is in agreement with that reported by other authors who found that, even immobilized, XO looses its catalytic activity faster than other enzymes (Dimcheva et al., 2002; Zhao and Luong, 1994). Stabilization of the enzyme solution before

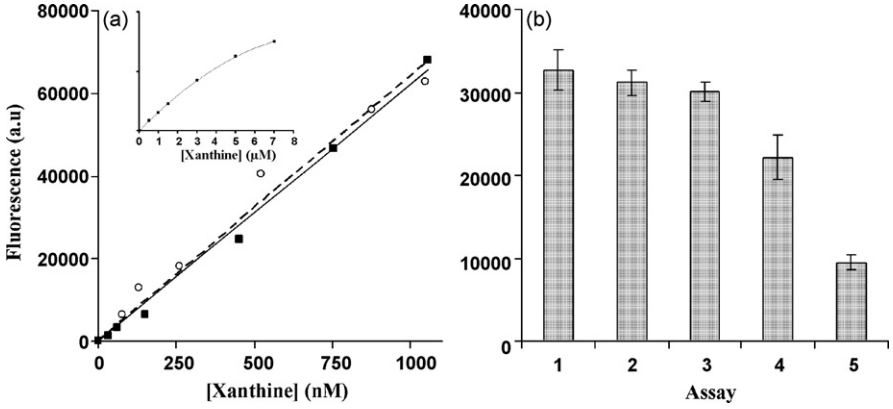


**Fig. 3.** (a) Xanthine calibration curves obtained by the Amplex Red assay for free (○) and sol–gel encapsulated XO–SOD–HRP system (●). Inset: calibration curve for the encapsulated system with the *x*-axis expanded in the range of 0–7 μM xanthine. (b) Reusability of the biosensor for five consecutive assays. [Xanthine] = 0.5 μM.

**Table 1**
Analytical characteristics of some xanthine biosensors

| Technique | Analytical range (μM) | Detection limit (μM) | Useful time (days) | Reference |
|---|---|---|---|---|
| Amperometric | 10–400 | 1 | 10 | Arslam et al. (2006) |
| Amperometric | 0.5–40 | 0.1 | – | Kirgöz et al. (2004) |
| Amperometric | Up to 40 | 4.5 | 1 | Dimcheva et al. (2002) |
| Amperometric | 310–6800 | 150 | 21 | Villalonga et al. (2007) |
| Amperometric | Up to 30 | 0.2 | 7 | Zhao et al. (1996) |
| Amperometric | Up to 1000 | 20 | 5 | Rehak et al. (1994) |
| Amperometric | 0.5–100 | 0.09 | 30 | Rahman et al. (2007) |
| Chemiluminescence | 3–216 | 0.55 | – | Hlavay et al. (1994) |
| Fluorescence | 0–3.5 | 0.02 | 14 | This work |

immobilization probably will improve the long-term stability of the biosensor.

### 3.4. Enzyme system reusability

Reusability is one of the major advantages of the sol–gel immobilized enzymes. After investigating the stability, suitability and sensitivity of the encapsulated XO–SOD–HRP system, we checked the capability of the biosensor to be reused after washing thoroughly both the analyte and the probe. Fig. 3b displays the fluorescence response of the encapsulated system at 0.5 μM of xanthine for seven consecutive assays (washing was performed for 3 days every 8 h). Results show that the same monolith can be used three times with no loss of activity. In the 4th cycle the biosensor response was reduced to 63%, and response continued to fall to 24% at cycle 5. The activity loss was higher than that reported when only HRP and SOD were immobilized in a single sol–gel matrix (Pastor et al., 2004). In this last case, the co-immobilized system could be used four times with no loss of activity, and subsequent activity loss was attributed to the degradation of the HRP heme group (Ferrer et al., 2003). Taken together, these results show that XO is less robust than HRP and SOD when incorporated in a sol–gel glass, as we observed from the stability experiments above.

## 4. Conclusions

We have simultaneously encapsulated for the first time a coupled XO–SOD–HRP system in a single sol–gel matrix to prepare a fluorescent biosensor for the accurate determination of xanthine. The co-immobilized system maintained 100% of its activity as compared to free enzymes in solution counterpart, allowing its reusability, and was stable for two weeks under adequate storage conditions. The fluorescent biosensor was able to detect concentrations at submicromolar level with a minimum detection limit of 20 nM, which largely improves the sensitivity of the currently xanthine biosensors. Given its high sensitivity, the biosensor would be useful to determine xanthine in biological fluids by sample dilution, reducing the interfering species to negligible levels. Preliminary results obtained in diluted urine and plasma samples resemble the ones obtained with an acid uric biosensor developed in our laboratory and based on a similar scheme (a multienzymatic system in a single sol–gel matrix coupled to the Amplex Red probe). For the latter, accurate values of the uric acid concentration were obtained in highly diluted body fluids, e.g. serum, plasma and blood (Martinez-Pérez et al., 2003). In addition, the biosensor could be also applied in the food industry, since the level of xanthine is generally used as an index for evaluating meat or fish freshness. All these possible applications, together with the search for new strategies to improve reusability and long-term stability of the biosensor, are now under investigation and will be the scope of a future work.

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