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Biophysical Chemistry 120 (2006) 81 – 86

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Use of fluorescence to monitor the incorporation of horseradish peroxidase into a sol–gel derived medium

Graham Hungerford *, Ana Rei, M. Isabel C. Ferreira

Departamento de Física, Universidade do Minho, 4710-057 Braga, Portugal

Received 10 August 2005; received in revised form 20 October 2005; accepted 21 October 2005
Available online 16 November 2005

Abstract

The solvatochromic dye nile red has been employed to monitor the incorporation of an enzyme (horseradish peroxidase) into a sol–gel derived medium. The fluorescence spectrum of the dye, when incorporated into the enzyme, was analysed as the sum of Gaussian component spectra and relative changes between these component spectra were monitored upon encapsulation of the dye–enzyme system within the host matrix. Activity of the confined enzyme was verified and the effect of temperature was also investigated, through the examination of nile red fluorescence in the sol–gel derived matrix, where a stabilising effect was noted.

Keywords: Nile red; Energy transfer; Molecular dynamics; Catalytic activity

1. Introduction

The properties of sol–gel derived media [1,2], such as robustness, low processing temperature, high porosity and large internal surface area have made them an attractive choice for the encapsulation and immobilisation of biologically active molecules [3–5]. Their glass-like nature gives them good optical properties and allows for light addressable entities to be incorporated and accessed using spectroscopic techniques. This form of matrix coupled with biologically active species can be used as a protective immobilisation medium [6,7] and for biosensor applications [3,8]. However, although silica derived sol–gel media have been touted as inert hosts, it has been reported that the matrix may not only exert an influence on the catalytic activity of the enzyme by perturbation of its conformation, but that the silica itself may produce some catalytic effect [9]. Also some by-products, such as ethanol, from the sol–gel reaction are detrimental to proteins and this has led to various strategies for biomolecule incorporation [10–13]. Hence it is important to find ways to ascertain the matrix influence on the incorporated bio-molecule.

Although it is possible to make use of the intrinsic protein fluorescence, from tryptophan and tyrosine residues for example, scattering from the matrix in the uv region, where typically fluorescent amino acids absorb and emit, can preclude their use. Thus, application of an extrinsic probe, which absorbs and emits at longer wavelengths, is advantageous. In the present work, the dye nile red (NR) was selected to monitor the incorporation of horseradish peroxidase (HRP) into a sol–gel derived silica matrix (SG), because of its solvatochromic properties [14,15]. Moreover, previous work by the authors has shown the occurrence of a specific interaction between NR and HRP in homogeneous media [16].

In this work we report on the changes in the NR–HRP system, as reported by nile red fluorescence, upon incorporation into the sol–gel derived matrix. The behaviour of NR–HRP in SG upon heating, was also examined. Finally, HRP activity was checked to ensure that NR probing was performed under experimental conditions of interest in the potential application of HRP as a biosensor.

2. Experimental

2.1. Dye–enzyme incubation

As previously reported [16], the incubation of NR into horseradish peroxidase ([EC 1.11.1.7], referred to as HRP, was performed in a pH 7 phosphate buffer, at a concentration of 2 mg of enzyme per 3 ml buffer. A sample of nile red (Aldrich)

* Corresponding author. Fax: +351 253678981.
E-mail address: graham@fisica.uminho.pt (G. Hungerford).

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doi:10.1016/j.bpc.2005.10.009

dissolved in spectroscopic grade dimethylsulfoxide (Aldrich) was added to the previous solution in such amount as to obtain a dye to enzyme ratio of approximately 1:5, thus minimising the possibility of dye–dye interactions. The incubation time was ascertained from following the change in the nile red emission spectrum and set to a time when negligible changes were observed, i.e. 2 h, [16]. Throughout, this system will be referred to as NR–HRP. All chemicals were used as received.

2.2. Sample preparation

Sol–gel derived media were produced in the form of a monolith using a standard 10 mm pathlength cuvette for a mould. The sol was produced by mixing 9 ml of tetraethyl orthosilicate (Aldrich) with 3 ml of water containing 0.2 ml of 0.01 M HCl. This mixture was placed in an ultrasonic bath for 1 h before being placed in a freezer (−18 °C) for about a month. The matrices were formed by taking 2 ml of the sol and mixing it with 2 ml of buffer solution containing the labelled enzyme. Gelling occurred within minutes and the gels were stored in a refrigerator (at 4 °C). After approximately 12 h it was possible to remove the monoliths from their moulds and they were rinsed with distilled water. The resultant monoliths at time of their first measurement were approximately $9 \times 9 \times 30$ mm in size. Monoliths were initially washed daily to remove any leached enzyme and expelled solvent. After a month their size was ca. 1/8 of the initial volume.

2.3. Enzyme activity

Relative horseradish activity was obtained from the oxidation of ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, from Sigma) by hydrogen peroxide (H_2O_2, 37% Aldrich). The absorption spectra were measured at various times, as well as the formation of the oxidised ABTS radical (monitored at 414 nm) [18]. For the studies on NR–HRP system, 100 μl of NR–HRP solution were taken and added to a cuvette containing 3 ml of $\sim 2.5 \times 10^{-5}$ M ABTS and 4 μl H_2O_2 in buffer solution. This gave a ratio of approximately 1:50:500 for HRP:ABTS:H_2O_2. The absorption spectrum was recorded in the range 250–500 nm, both before the addition of the enzyme and after the kinetic study was complete. The kinetic study commenced on the addition of the enzyme and followed the formation of the ABTS radical by monitoring absorption changes at 414 nm [17,18]. Sol–gel (cut into slices) of NR–HRP–SG were analysed by placing them in a buffer solution containing ABTS and H_2O_2 (1:50:500 for HRP:ABTS:H_2O_2), as the sample under study. At various times the block was removed and the spectrum of the solution measured, in order to follow the formation of the ABTS radical by monitoring absorption changes at 414 nm [18].

2.4. Emission data and analysis

Spectra were recorded using a Shimadzu UV-3101PC (absorption) and a SPEX Fluorolog (fluorescence) spectrophotometer. The study of the effect of temperature on the emission of NR was performed using a circulating water bath connected to the sample holder and the temperature monitored via a thermocouple attachment. Unless otherwise stated, measurements were made at room temperature. Spectral analysis of the steady state emission spectra was carried out using Microcal Origin 6 software to perform the decomposition of the measured spectra as a sum of Gaussian components, in agreement with previous studies [16,19]. Best fits were obtained through minimizing the parameter φ defined as:

$$\varphi = \left(\frac{\sum \left(I_{\mathrm{exp}} - I_{\mathrm{cal}} \right)^2}{\sum \left(I_{\mathrm{exp}} \right)^2} \right)^{1/2}. \quad (1)$$

Fluorescence lifetimes were measured by means of a single-photon counting apparatus equipped with a LED excitation source with a peak emission at 495 nm (HORIBA, Jobin Yvon, IBH Ltd. Glasgow, Scotland). The fluorescence emission was wavelength selected using a 550 nm cut-off filter and detected with a Hamamatsu R2949 photomultiplier. Data were analysed as the sum of exponential components (Eq. (2)), each with a fractional amplitude (f) given by Eq. (3), and performed using IBH DAS6 software. The goodness of fit judged in terms of a χ^2 value and weighted residuals.

$$I(t) = \sum_{i=1}^{n} a_i \exp(-t/\tau_i) \quad (2)$$

$$f_i = \frac{\alpha_i \tau_i}{\sum_{i=1}^{n} \alpha_i \tau_i}. \quad (3)$$

3. Results and discussion

3.1. Nile red–HRP interaction

The first encounter between dye and enzyme occurs during the incubation period. As reported elsewhere [16], the spectra obtained from the incubation study were the superposition of component spectra that result from a non homogeneous local environment within the enzyme network. In fact, previous spectral analysis has shown that, when incorporated into HRP in buffer solution, NR displays a complex emission spectra, composed of five bands, as the result of NR fluorescence originating from a low polarity environment (LPM) and NR emission typical of NR probing a polar medium (PM). The LPM was ascribed to the heme moiety of HRP, whereas PM is generally ascribed to the network of HRP, possessing a great number of polar regions. The same work has also revealed that, upon incubation of NR into the network of HRP, a significant drop in NR fluorescence quantum yield occurs as the result of resonance energy transfer (RET) between NR molecules located in the vicinity of the heme group and this active group. Moreover, concentration quenching studies, using KI as the quencher, have shown that the dye is incorporated and protected within the enzyme structure. These data have also been supported using molecular modelling.

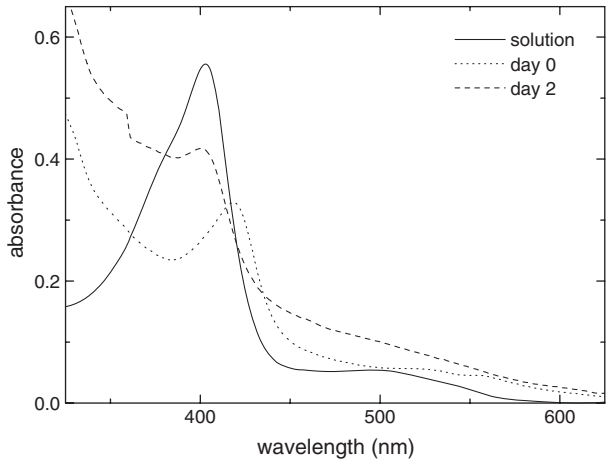

Fig. 1. Absorption spectrum of HRP in solution and on immediate incorporation into a sol–gel derived matrix, along with that in the matrix 2 days later.

3.2. Nile red–HRP in sol–gel derived SiO_2

The absorption spectrum of the NR–HRP system was measured, during incorporation into the sol–gel matrix, as shown in Fig. 1. This figure shows that there is a shift in the heme *Soret* band from 402 nm (in solution) to 419 nm on initial incorporation into the matrix. However the initial wavelength is recovered with time. This behaviour has been reported elsewhere as relating to changes in oxidation of the heme [20] and reversible absorption changes in this region have been previously noted with the incorporation of heme containing enzymes into sol–gel derived media [12]. Monitoring the wavelength of the *Soret* band over a 3 week period did not show any further change.

3.3. Effect of temperature

In order to access the molecular dynamics of NR–HRP system, when incorporated into the matrix, the fluorescence of NR was examined in the NR–HRP–SG system as a function of temperature (room temperature to 80 °C). Other studies have reported that protein confinement within a host medium enhances thermal stability [21,22] as well as safeguarding catalytic activity [6]. Fluorescence from the dye in NR–HRP–SG is depicted in Fig. 2. Throughout, the peak emission occurs at lower wavelengths than that observed for NR directly incorporated into a sol–gel matrix (Fig. 3.), which is indicative that no significant leaching of NR from the enzyme to the matrix occurs. The peak intensity for NR–HRP–SG increases with temperature (unlike that for NR in SG) up until 50 °C, after which a slight decrease is observed. The shift of the peak emission to shorter wavelengths (comparing measurements taken three days after initial study, for example) is similar to the behaviour found for NR–HRP system under a similar temperature study [16].

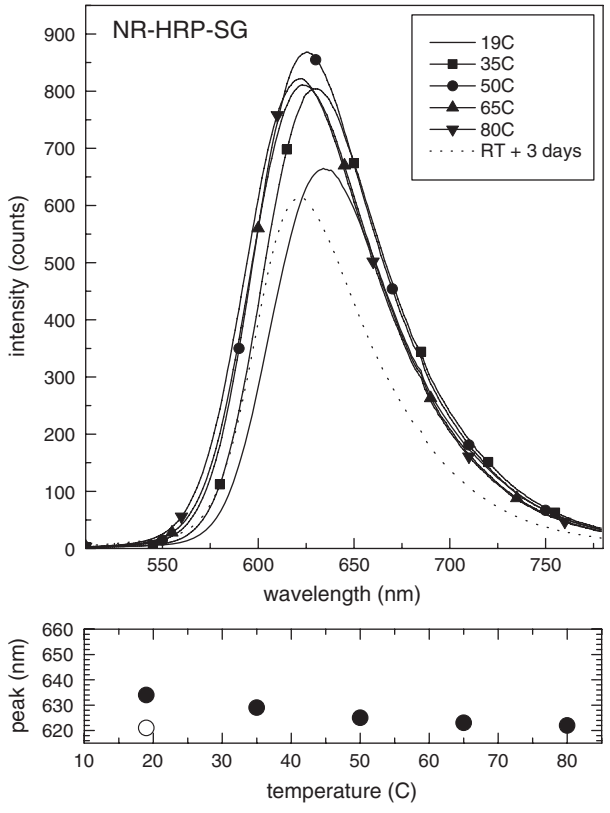

Fig. 2. Fluorescence emission of nile red (excitation 490 nm) labelled horseradish peroxidase in a sol–gel derived matrix at different temperatures (upper panel, symbols designate different spectra). The lower panel shows the position of the peak emission, with the open symbol indicating a measurement 3 days later.

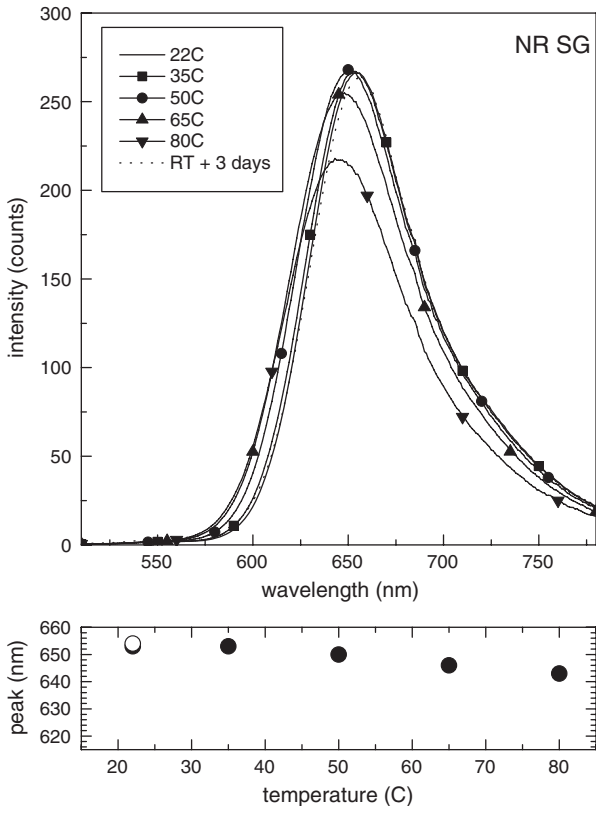

Fig. 3. Fluorescence emission of nile red (excitation 490 nm) in a sol–gel derived matrix at different temperatures (upper panel, symbols designate different spectra). The lower panel shows the position of the peak emission, with the open symbol indicating a measurement 3 days later.

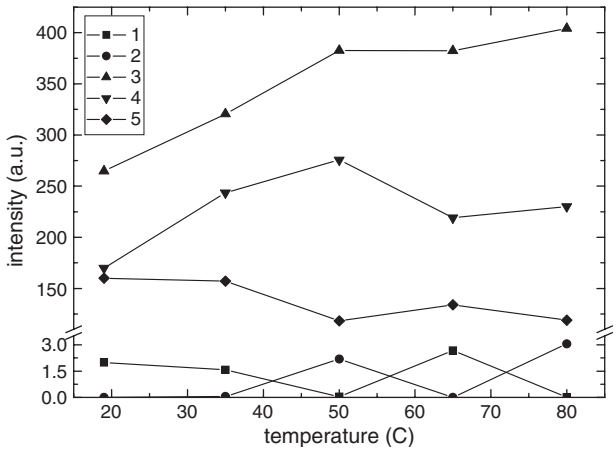

Fig. 4. Change in intensity of NR fluorescence of the 5 Gaussian components with temperature for NR–HRP–SG.

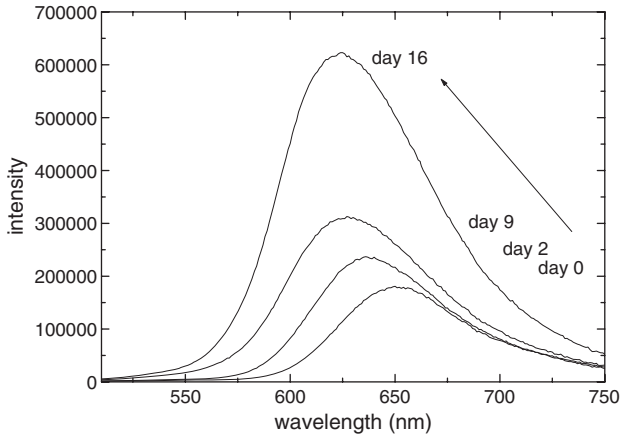

Fig. 5. Fluorescence emission of nile red (excitation 490 nm) labelled horseradish peroxidase in a sol–gel derived matrix at different times after enzyme incorporation.

However, it should be noted that in that study a significant increase in intensity was observed for the higher temperatures.

The results of the spectral decomposition, performed on the spectra of Fig. 2, are shown in Fig. 4 and the relative contributions of each band are given in Table 1. It is found that bands 1 and 2, pertaining to the vibronic structure of NR in LPM, represent a minor contribution to the overall emission, in agreement with the studies of NR–HRP system in buffer. Band 3 is ascribed to a superimposition of a band (vibronic) from NR emission in LPM and another band from NR emitting from a polar environment (PM). In agreement with this assumption, band 3 behaviour should not follow any of the other bands, as confirmed by the data of Fig. 4. Bands 1 and 2 always represent minor contributions to the total emission, thus confirming that efficient RET is observed in NR–HRP–SG, similarly to the behaviour observed in buffer solution. Bands 4 and 5 are ascribed to two vibronic bands of NR molecules emitting in a PM.

HRP is a resistant enzyme with an apparent melting temperature of 74 °C [23] and reported to undergo a two stage melting of the secondary structure. A first stage (below 40 °C) of melting involves local changes in the conformation of the active (heme) site accompanied by small changes in the overall HRP secondary structure. The second stage (50 to 93 °C) exhibits larger changes in the secondary structure and can be associated with the removal of the heme from the active site. The same work reports changes in the tertiary structure close to the heme (in the range 35 to 55 °C) and proposes the presence of pre-molten globule folding intermediates [23]. Our observations for the NR–HRP–SG system confirm an increase in the fluorescence intensity for temperatures up until 50 °C (Figs. 2 and 4), in agreement with that work. However, after this temperature no further increase is observed (unlike what we have reported for a solution study [16]) indicating that the matrix is conferring some stability to the enzyme.

Table 1
Percentage of nile red fluorescence attributed to each band in NR–HRP in a sol–gel derived medium at different temperatures

Band	Temperature (°C)				
	19	35	50	65	80
1	0	0	0	0	0
2	0	0	0	0	0
3	45	44	50	52	54
4	28	34	35	30	30
5	27	22	15	18	16

3.4. Effect of aging

During the aging process the sol–gel derived medium goes from being a soft, but supportive matrix to becoming a rigid, glass-like host. It is clear that these mechanical changes can affect the encapsulated enzyme. To obtain information on both the enzyme structure and its ability to catalyse reactions, the fluorescence emission from NR, incorporated within the enzyme in a host matrix (NR–HRP–SG system) and also the catalytic activity of HRP were examined as a function of time. The fluorescence spectrum of the dye was found to change significantly over a 2 week period, as seen in Fig. 5. Here the NR emission was found to increase and appear to shift to the blue with time. Decomposition of the spectra into its Gaussian components yielded the data presented in Fig. 6 and Table 2.

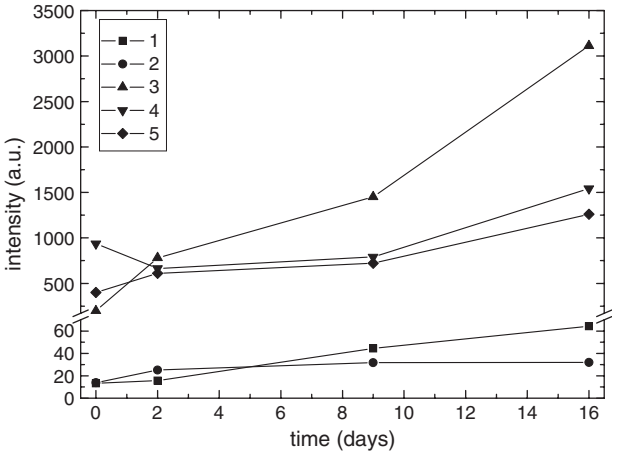

Fig. 6. Change in intensity of NR fluorescence of the 5 Gaussian components with time for NR–HRP–SG.

Table 2
Percentage of nile red fluorescence attributed to each band in NR–HRP in a sol–gel derived medium at different times after incorporation

Band	Time (days)			
	0	2	9	16
1	1	1	1	1
2	1	1	1	1
3	13	37	48	51
4	59	32	26	26
5	26	29	24	21

These data show a slight increase in bands 1 and 2 and a more pronounced enhancement of bands 3, 4 and with time.

Time-resolved measurements (see Table 3), taken 26 days after incorporation of NR–HRP into the matrix, showed that the decay required three exponential components to obtain a satisfactory fit to the data. The decrease in the fractional intensity of the shortest lived fluorescence is consistent with a decrease in the efficiency of NR–heme energy transfer, manifest by both an increase in fluorescence yield of the less polar environments and a decrease in the quantity of the quenched (fastest) lifetime component. It is possible that the location of the NR molecules in the vicinity of the heme may suffer alterations, as compared to the NR–HRP system in solution, brought upon by the geometrical restrictions imposed on the NR–HRP system by the sol–gel matrix. A time-resolved anisotropy measurement taken at the same time showed no significant change in the rotational correlation time, as compared to that in solution, although the rotational freedom measured by the semi-cone angle was found to reduce to 11°, from 35°. The restricted rotational freedom is to be expected in view of the shrinkage process and the rigidity of the SiO_2 matrix.

3.5. Enzyme activity

A clear objective of the inclusion of enzymes into rigid supporting media is that, after incorporation, they should exhibit catalytic activity. Therefore, a comparative study was made between NR–HRP and NR–HRP–SG samples using ABTS as a substrate, as described in the Experimental section. It should be noted that slices were taken from the complete sol–gel derived monolith in this study and used as such. A different strategy has been reported in other studies [6,18], where samples were reduced to powder. In order to ascertain the effect of different quantities of HRP (as might be encountered with the sol–gel derived matrices) a study in solution was undertaken monitoring the formation of the ABTS radical over time with fixed amounts of ABTS and H_2O_2. The outcome is given in Fig. 7 and shows that increasing the amount of HRP causes a quickening in the reaction rate plus an enhancement of the yield of the radical. It should be noted that at longer times there is a decrease caused by a back reaction. Percentage yield was used in order to attempt comparison with the sol–gel system and other work [18].

Table 3
Time-resolved decay data from NR in HRP in solution and after incorporated in a sol–gel derived matrix for 26 days

System	τ_1	τ_2	τ_3	f_1	f_2	f_3	χ^2
Solution	5.36±0.24	3.49±0.57	0.63±0.02	30%	28%	42%	1.09
Matrix	6.03±0.48	3.32±0.10	1.06±0.18	6%	84%	10%	1.13

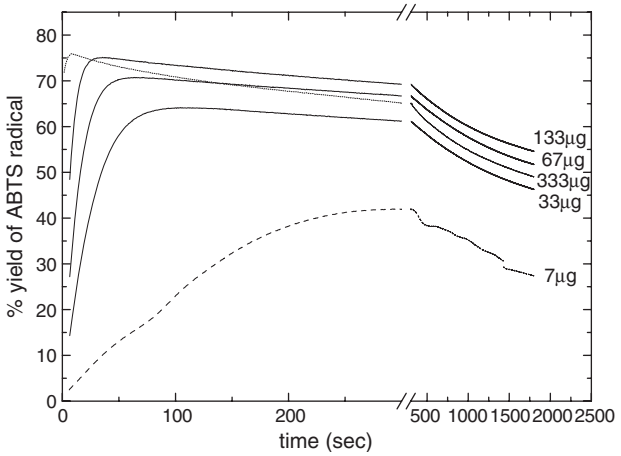

Fig. 7. Catalytic activity for different quantities of HRP in solution, obtained by monitoring the production of ABTS radical at 414 nm with time.

The catalytic activity measured over a 16 days period is displayed in Fig. 8. This clearly shows that the NR–HRP–SG sample exhibits catalytic activity. However, the amount and rate are lower than those detected even for the smallest amount of HRP used in solution. This can easily be explained by the fact that the reactants have to diffuse in and out of the matrix pores and the possibility that not all the HRP is accessible (or observation of the ABTS radical is not possible because of retention within the matrix). However, our samples appear to exhibit similar activity to that obtained for a powdered SiO_2 sol–gel derived matrix containing HRP, where a comparative yield ca. 30% was obtained [18]. It is reassuring to know that the encapsulated HRP displays catalytic activity as the matrix forming reaction could damage the enzyme, in terms of structure or removal of the heme group. The latter has been shown to occur in acidic conditions (below pH 3) [24]. The

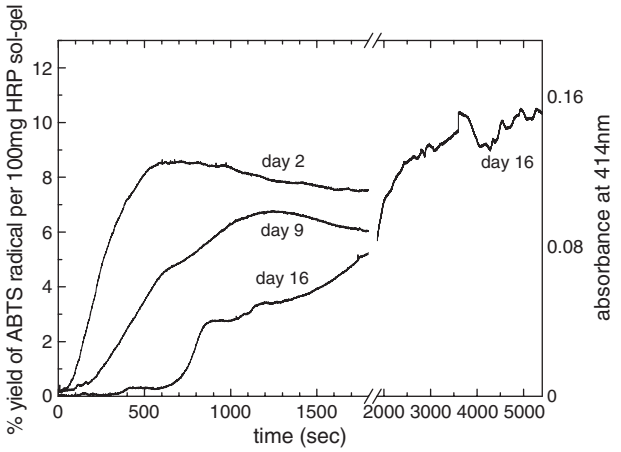

Fig. 8. Catalytic activity for HRP in sol–gel derived medium, obtained by monitoring the production of ABTS radical at 414 nm with time, at different times after incorporation.

form of the curves reflects the fact that as the matrix shrinks the pore interconnections narrow and diffusion of the reactants becomes harder, hence the longer time taken to achieve maximum activity. The "bursts" of activity may relate to the pore distribution as a function of distance from the exterior of the matrix slice.

4. Conclusion

In this work we have analysed the emission of nile red incorporated into horseradish peroxidase when this system is incorporated into a SiO_2 sol–gel matrix. Spectral analysis of NR emission, in steady and time resolved modes, has shown that NR was found to be well incorporated within the HRP network and did not leach on entrapment within the host, while the enzyme continued to exhibit catalytic activity in the silica host.

The emission studies also confirmed the presence of a specific interaction between NR and HRP, through resonance energy transfer between NR (donor) molecules located in the vicinity of the heme group and this active group (acceptor), similarly to what was observed by the authors in a previous study in a buffer solution [16], albeit somewhat less efficient. Furthermore, these data confirm that a fraction of NR is located in the vicinity of the active site but NR can also be found within the enzyme network, in agreement with a modelling study of the enzyme conformation. Fluorescence quenching using KI also indicates that the NR is protected from the aqueous environment. The studies on aging and on the effect of temperature confirm that the NR–HRP system is quite robust within the sol–gel matrix. The activity of the enzyme persists over a period of 16 days, but the time response of ABTS radical formation is mostly determined by mass transport control within the pores of the sol–gel structure.

Finally it is worth noting that a detailed spectral analysis of the emission of nile red, has revealed features that would otherwise be overlooked. One such case is the mechanism of energy transfer between NR and the heme active site of HRP.

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