Free and sol–gel immobilized alkaline phosphatase-based biosensor for the determination of pesticides and inorganic compounds

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Abstract

Alkaline-phosphatase (ALP) catalyses the hydrolysis of 1-naphthyl phosphate to fluorescent 1-naphthol ($\lambda_{ex} = 346$ nm, $\lambda_{em} = 463$ nm). This enzymatic reaction was investigated in presence of inhibitors: organochlorine (tetradifon), carbamate (metham-sodium) and organophosphorus pesticides (fenitrothion), heavy metal (Ag$^+$) and CN$^-$. The fluorescent signal, which is inversely dependent on the inhibitor concentration, is related to the amount of the inhibitor. Detection limits between 4.1 M for tetradifon and 91.2 M for metham-sodium were found. The relative standard deviation (R.S.D.) was between 2.6 and 6.2%.

Sol–gel matrices derived from tetramethyl orthosilicate were doped with ALP using microencapsulation. The response of the biosensor based ALP sol–gel encapsulated to 1-naphthyl phosphate was reproducible (R.S.D. = 6.6%). Inhibition plots obtained for test pesticides (metham-sodium and tetradifon) display linear calibration in the ranges 194–774 M and 3.5–28 M, detection limits of 4.9 and 292.3 M and R.S.D. of 3.9 and 7.3% for metham-sodium and tetradifon, respectively.

The results show that the system is able to detect class compounds such as pesticides and inorganic compounds.

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Keywords: Alkaline-phosphatase; Optic biosensor; Inhibitors; Sol–gel; Metal; Pesticides; Fluorescence

1. Introduction

Organochlorine pesticides are known to resist biodegradation, bioaccumulate due to their capacity to bind to lipids and therefore can be redistributed through the food chain. Organophosphorus and carbamates show low environmental persistence but display high acute toxicity. Cyanides are produced by many industrial processes such as electroplating, organic synthesis and metal mining and there is interest to develop methods for the determination of cyanide compounds in environmental samples. Silver enters the environment because the increasing use of silver-containing preparations in industry, medicine and because it often occurs as an impurity in copper, zinc, arsenic and antimony ores. Interaction of silver with nutrients, especially selenium, copper, Vitamin E and Vitamin B12, has focused attention on its potential toxicity. Analytical methods have been described for the determination of these organic and inorganic compounds in environmental samples. However, as a complement to traditional analytical methods there is a need for developing of rapid and simple screening methods useful for routine analysis of a large number of samples.
of samples. In this respect, enzyme-based biosensors represent potential alternatives to the analysis or screening in environmental samples.

Enzyme biosensors based on immobilized enzymes has advantages over soluble enzymes or alternative technologies. The properties of immobilized enzyme are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties [1].

Adsorption, covalent binding to solid surfaces and supported films, entrapment in polymer hydro gels, and microencapsulation have long been used to im mobilize enzymes [2–6]. The special features that the sol–gel entrapment provides over other used immobilization methods made this one of the fastest growing methodology for the immobilization of proteins [7–10]. Sol–gel technology has proved to be remarkably versatile for fabricating matrices of defined porosity in the form of monoliths, particulates and thin and thick films, for use in optics, sensors, catalytic coatings and specialty polymers. All these properties are especially useful in the optical biosensors development.

Direct monitoring of analytes (enzymes or substrates) has been the major application of optical biosensors. Alternatively, biosensors have been used for indirect monitoring of organic (e.g. pesticides) or inorganic substances (e.g. heavy metals) that inhibit biocatalytic properties of the biosensor [11–14]. The problem with biosensors based in enzymatic inhibition is that only a few enzymes [15] are sensitive simultaneously both pollutants: pesticides and heavy metals. One of these enzymes, alkaline phosphatase (ALP) react both organic and inorganic compounds. ALP is a metalloenzyme which contain in the active site Zn\textsuperscript{2+} and Mg\textsuperscript{2+} ions and compounds such as EDTA, 1,10-phenantroline, l-cysine, mercaptoethanol and dithiothreitol can lead to full loss of the enzyme activity, some inorganic salts such as Na\textsubscript{2}PO\textsubscript{4}, Na\textsubscript{2}HAsO\textsubscript{4}, Na\textsubscript{3}WO\textsubscript{4}, heavy metals (Hg\textsuperscript{2+}, Ag\textsuperscript{+}, Bi\textsuperscript{3+}, Cu\textsuperscript{2+}, Pb\textsuperscript{2+}, V\textsuperscript{4+} and excessive amounts of Zn\textsuperscript{2+}) [16] and organophosphorus pesticides are inhibitors of ALP. As data, inhibition of immobilized ALP on glass has been used for several metal ions and EDTA detection in a flow-injection system [17] and for pesticides [18–20] and metal ions [20–22] detection.

Thus the ability of ALP of react with numerous compounds, organic and inorganic could make this system useful as biomarker of sample toxicity. Although ALP has been successful immobilized into sol–gel matrices [7] this system has not been applied to pollutants analysis. In this work, the inhibition of free and immobilized ALP by pesticides and inorganic compounds is studied to estimate the potential use of the ALP sol–gel immobilized as biosensor screening.

Inhibitors such as organochlorine (tetradifon), carbamate (metham-sodium) and organophosphorus pesticides (fenitrothion), heavy metals (Ag\textsuperscript{+}) and CN\textsuperscript{−} have been studied. The analytes detected are used as a model compounds in further developments of non-specific inhibition of a range pollutants.

2. Experimental

2.1. Reagents and instruments

Alkaline-phosphatase (EC 3.1.3.1) from bovine Intestine Type I 4.3 U/mg (Sigma) was prepared in NaHCO\textsubscript{3} buffer 0.1 M pH 9.5. 1-Naphthyl phosphate (Sigma) was prepared in bidistilled water to give final concentration of 0.01 M. The inhibitors fenitrothion (96%), metham-sodium (96%) and tetradifon (96%) were obtained from Dr. S. Ehrenstorfer (Augsburg, Germany); potassium cyanide and silver nitrate were provided from Merck (Darmstadt, Germany) and Panreac (Barcelona, Spain), respectively. Tetramethyl orthosilicate (TMOS) was provided from Aldrich (grade 99%). The disposable poly(methyl methacrylate) (PMMA) cuvette, 4.5 ml, four clear sides (P.N. 1961) was obtained from Dispolab-Kartell. Emission measurements were made in a Perkin-Elmer LS50 luminescence spectrophotometer equipped with a xenon discharge lamp (9.9 W) pulsed at line frequency. An agitation system was used for the study in solution. However, this agitation would damage the gel, thus no agitation was applied with immobilized ALP.

2.2. Immobilization of the enzyme

The sol–gels were prepared in a similar manner to that described previously [23]. The acid-catalysed silica sol was prepared by the sonication, in a crystal vial,
for 20 min of TMOS (1.5 ml), deionised H₂O (320 µl) and 0.01 M HCl (30 µl).

For the gel formation 40 µl of NaHCO₃ buffer, 40 µl aliquot of enzyme 4.3 U/ml (0.172 U in gel), and 26.7 µl of sol were added into a disposable PMMA cuvette, and the gel was formed after 2 min. The buffer was needed to avoid losses of enzyme activity by de-naturalization during the sol–gel process (since the pH of the sol is acid), thus making the enzyme remain at its pH optimum [8].

The gel was prepared daily by producing monolith blocks with dimensions of 1 cm × 1 cm × 0.5 cm, conservation and storage was carried out at 4 °C before measurements. Because the TMOS gelation occurs at basic pH the stored gel was rinsed and conserved in water to avoid possible interactions of gel with buffer. The vial containing the gel was sealed with parafilm to prevent cracking, through rapid evaporation during aging. The gel in water was stable during two months of storage with zero leach ability of the enzyme.

2.3. Procedures

For measurements with free ALP in solution, the appropriate volumes of 0.1 M NaHCO₃ buffer at pH 9.5, 0.43 U/ml alkaline-phosphatase, inhibitors when necessary and 0.01 M 1-naphthyl phosphate up to 2 ml final volume were added, in the indicated order, to a disposable PMMA cuvette. The variation of the fluorescence intensity, Δ(RFI), per unit of time, Δ(RFI)/Δt (∆t = 0–300 s) was used to determine the initial rate (V).

For measurements with immobilized ALP, the appropriate volumes of 0.1 M NaHCO₃ buffer pH 9.5, inhibitors when necessary and 0.01 M 1-naphthyl phosphate up to 2 ml final volume and 0.01 M 1-naphthyl phosphate, were added to a cuvette containing the gel with ALP immobilized. For the calibration plots of inhibitors the relative fluorescence intensity (RFI) 5 min after substrate addition was determined. The percentage inhibition was calculated as follows:

\[
\% \text{ INH} = \frac{\text{RFI}_0 - \text{RFI}}{\text{RFI}_0} \times 100
\]

where RFI₀ is the fluorescence without inhibitor, and RFI is the fluorescence with inhibitor [24].

3. Results and discussion

3.1. Study of 1-naphthyl-phosphatase/alkaline-phosphatase/inhibitors system in solution

ALP catalyses the non-specific hydrolysis of orthophosphoric monoesters to alcohols. In the present work, ALP catalyses the 1-naphthyl phosphate hydrolysis to 1-naphthol, a highly fluorescent product (λ_ex = 346 nm, λ_em = 463 nm) and phosphate. The inhibition by OP pesticides and heavy metals are known to inhibit the activity of ALP. The inhibition by OP pesticides or inorganic ions of the 1-naphthyl phosphate hydrolysis by ALP was concentration dependent. The kinetic curves in presence of metham sodium, fenitrothion, tetradifon, CN⁻ and Ag⁺ are given in Fig. 1. Kinetic curves in presence of metham sodium, fenitrothion, tetradifon, CN⁻ and Ag⁺ are given in Fig. 1. Inhibitors not competitive influence the enzymatic reaction in such way that the inhibitor decreases the observed Vmax and also increase the observed K_i, this was the behavior for metham sodium, fenitrothion, tetradifon and Ag⁺. The inhibition constant, K_i value was calculated by using the equation:

\[
\frac{1}{V} = \left(1 + \frac{[I]}{K_i}\right) \frac{V_{max}}{V_{max}[S]} + \frac{1}{V_{max}}\frac{1}{V_{max}}
\]

A plot of 1/V versus 1/[S] should be linear, and K_i can be determined from the slope and intercept of the
Fig. 1. Enzyme kinetics as a function of substrate concentration fitted to Michaelis–Menten equation. [ALP] = 8.6 U/l, NaHCO₃ 0.1 M buffer pH 9.5. Without inhibitor, curve (■). With inhibitor, curves: (▲) 77.40 µM metham sodium, (▼) 14.05 µM tetradifon, (♦) 108.30 µM fenitrothion, (♦) 307.20 µM CN⁻, (◊) 53.51 µM Ag⁺.

Table 1 show the type of inhibition and the $K_I$ values. Non-competitive inhibition by tetradifon gives the lower $K_I = 5.26$ µM while by fenitrothion gives the greater $K_I = 90.21$ µM.

According to the Michaelis–Menten treatment of a reversible, competitive inhibitor, $V_{max}$ is unaffected by the inhibitor, but increases the observed $K_m$, this behavior can be ascribed to CN⁻. These results were expected because ALP is a metalloenzyme containing metal ions (Zn²⁺, Mg²⁺) in their active site and inactivation can occur by complexation upon removal the metal ion [16]. The inhibition constant, $K_i$, value was calculated by using the equation:

$$\frac{1}{V} = \left(\frac{1}{V_{max}}\right) \frac{K_a}{K_i} \frac{1}{[S]} + \frac{1}{V_{max}}$$

A plot of $1/V$ versus $1/[S]$ should be linear, and $K_i$ can be determined from the slope and intercept of the line. The results are in Table 1.

### Table 1

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Type of inhibition</th>
<th>$[I]$ (µM)</th>
<th>$K_i$ (µM)</th>
<th>DL (µM)</th>
<th>Linear range (µM)</th>
<th>R.S.D. (%)</th>
<th>r²</th>
</tr>
</thead>
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<tr>
<td>Metham-sodium</td>
<td>Non-competitive</td>
<td>77.40</td>
<td>81.3</td>
<td>36.5</td>
<td>75–480</td>
<td>6.2</td>
<td>0.992</td>
</tr>
<tr>
<td>Tetradifon</td>
<td>Non-competitive</td>
<td>14.05</td>
<td>53</td>
<td>4.1</td>
<td>5–35</td>
<td>2.6</td>
<td>0.997</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>Non-competitive</td>
<td>108.30</td>
<td>90.2</td>
<td>45.5</td>
<td>135–270</td>
<td>3.1</td>
<td>0.988</td>
</tr>
<tr>
<td>CN⁻</td>
<td>Competitive</td>
<td>307.20</td>
<td>252.4</td>
<td>91.2</td>
<td>230–614</td>
<td>4.2</td>
<td>0.994</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>Non-competitive</td>
<td>53.51</td>
<td>24.6</td>
<td>10.1</td>
<td>15–152</td>
<td>4.4</td>
<td>0.994</td>
</tr>
</tbody>
</table>

[ALP] = 8.6 U/l, NaHCO₃ 0.1 M buffer pH 9.5.

* Inhibitor concentration.

$K_i$ Inhibition constant.

DL Detection limit = 3 S.D. of the blank (n = 4)/calibration slope.

R.S.D. Relative standard deviation.

r² Regression coefficient of calibration curve.
possible. Thus, a working concentration of substrate of 100 \( \mu \)M was chosen from the linear zone of the kinetic curve.

Calibration plots for inhibitors were made showing the extent of enzyme inactivation with variable concentration of the each inhibitor. Plots were obtained correlating the percentage inhibition 5 min after substrate addition with inhibitor concentration. In Table 1 the analytical parameters such as linear range, detection limit, relative standard deviation (R.S.D.) and regression coefficient \((r)\) for inhibitors determination are indicated. Detection limits were between 4.1 (tetradifon) and 91.2 \( \mu \)M (metham-sodium), the relative standard deviation was between 2.6 and 6.2%.

### 3.3. Sol–gel alkaline-phosphatase biosensor for inhibitors determination

Separate experiments were conducted to measure the activity of ALP in different conditions, in aqueous solution, immobilized in gel without inhibitor and immobilized in gel in the presence of an inhibitor. Fig. 2 shows the RFI versus the time reaction in the indicated conditions. In contrast with the Michaelis–Menten behavior of ALP in solution, the activity ALP in xerogel does not fit a Michaelis-Menten model showing a different distribution of activity efficiencies of the individually trapped molecules [7]. In the present work the increasing of RFI accompanying the hydrolysis of the substrate (1-naphthyl phosphate) was taken as a measure of enzyme activity. To compare the enzymatic activity in aqueous medium and in the gel, and as a measurement of the enzyme accessibility in the gel (100% accessibility in water) the relative activity was calculated using the expression:

\[
\% \text{ relative activity} = \frac{\text{RFI immobilized}}{\text{RFI in aqueous solution}} \times 100
\]

For immobilized ALP the relative activity without inhibitor was 21, 47 and 96% at 5, 10 and 120 min, respectively. The same for ALP immobilized and in presence of 50 ppm of \( \text{CN}^- \) was 12, 27 and 95% at 5, 10 and 120 min, respectively. It was deduced that the immobilization affect to the initial reaction rate, with a significantly fluorescence intensity decrease in the presence of inhibitor, but does not significantly decrease the enzymatic activity from 30 min or longer times. The decrease in ALP activity observed when the enzyme was immobilized in sol–gel can be accounted for the diffusion barrier of sol–gel.

For inhibitors determination the measurements were taken as fluorescence intensity at fixed time instead of the initial rate of enzymatic reaction because an agitation system damages the gel. A time of 5 min was adequate because enzyme retains sufficient activity, it produces a noticeable analytical signal, and permit adequate manipulation of the samples.

The biosensor responses depend on the proportion of the initial volume (buffer + enzyme)/sol used to obtain the gel, because this proportion determines the enzyme denaturation and the size pores of the gel [25]. Fig. 3 shows the relationship between the RFI and the ratio (buffer + enzyme)/sol used for the gel formation. On increasing this proportion the enzyme remained in a medium similar to a biological medium; it retains more activity and increases the RFI. On decreasing this proportion the denaturation increases, the activity and RFI decreases. A ratio of 3 was selected as optimum as a compromise between high RFI values and low detection limits for inhibitors determination. This proportion corresponds to volumes 80 \( \mu \)l of
Fig. 3. Effect of the proportion volumes initial (buffer + enzyme)/sol on RFI at 5 min. [ALP] in gel 0.086 U. Substrate concentration 250 μM.

Fig. 4. Calibration plot for the substrate, 1-naphthyl-phosphate. Ratio (buffer + enzyme)/sol = 3; [ALP] in gel = 0.172 U.

The repeatability of measurements of the biosensor with ALP immobilized in sol-gel was demonstrated calculating the precision of measurements as relative standard deviation, giving 6.6% (n = 6) for a working concentration of substrate of 200 μM which was selected from the linear zone of the calibration curve. The inhibitors metham-sodium and tetratadifon have been analyzed with the ALP biosensor, correlating the percentage inhibition with the pesticide concentration. To obtain a linear calibration of metham-sodium the time incubation was 8 min. Inhibition plots obtained for test pesticides (metham-sodium and tetratadifon) display linear calibration in the ranges 194–774 μM and 3.5–28 μM, detection limits of 4.9 μM and 292.3 μM and R.S.D. of 3.9 and 7.3% for metham-sodium and tetratadifon, respectively.

4. Conclusions

A fluorimetric, sol-gel immobilized ALP-based biosensor has been shown for the determination of inhibitors of the enzymatic activity. The application is related to the interaction between the ALP and the target pollutants, in order to develop a simple device for rapid screening of selected organochlorine (tetratadifon), carbamate (metham-sodium) and organophosphorus pesticides (emtrorphon), heavy metal (Ag⁺) and CN⁻ or similar compounds. Other advantageous characteristics for operation with biological systems such as enhanced stability is observed; moreover, the gel can be rapid and easily produced and discarded after a single use. The present method detects classes of compounds that cause the inhibition; this could the system make useful for environmental evaluation of the total toxicity of a sample.

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References
