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A bio-imprinted urease biosensor: Improved thermal and operational stabilities

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Abstract

Despite the increasing number of applications of biosensors in many fields, the construction of a steady biosensor remains still challenging. The high stability of molecularly bio-imprinted enzymes for its substrate can make them ideal alternatives as recognition elements for sensors. Urease (urea aminohydrolase, EC 3.5.1.5), which catalysis the hydrolysis of urea to ammonia and carbon dioxide, has been used in immobilized form in artificial kidney for blood detoxification. According to one report approximately half a million patients worldwide are being supported by haemodialysis.

In this study, the enzyme of urease was first complexed by using a substrate analogue, thiourea, in aqueous medium and then this enzyme was immobilized on gelatin by crosslinking with glutaraldehyde on a glass electrode surface. Similarly, urease noncomplexed with thiourea was also immobilized on a glass electrode in the same conditions. The aim of the study was to compare the two biosensors in terms of their repeatability, pH stability and thermal stability, and also, linear ranges of two biosensors were compared with each other.

Keywords: Bio-imprinted biosensors; Urease biosensor; Biosensor stability; Molecular imprinting technology

1. Introduction

In the past "Molecular Imprinting" was mainly confined to the separation of organic molecules and to nonenzymatic catalysis. With advancement in this technology, its potential and utility in field of biotechnology was also realized [1]. In order to improve it applicability, new variations in the conventional imprinting technique have emerged, such as "bioimprinting", metal chelation imprinting, affinity imprinting and a combination of immobilization and "bioimprinting" [2–8]. Several application areas have been envisaged for imprinted matrices the use of molecularly imprinted polymers in separation and isolation, the use of molecularly imprinted polymers as antibody and receptor mimics in immunoassay-type analyses, the use of molecularly imprinted polymers as enzyme mimics in catalytic applications, and the use of molecularly imprinted polymers in biosensor-like devices [9–13]. The memory of effect of enzymes caused by bio-imprinting without a further immobilization step was found to depend on the water content of medium and was completely lost when the reaction was carried out in the presence of a certain amount of water [14–18]. However, water is an indispensable milieu for most enzymatic reactions. Here we demonstrate that the combinatorial crosslinked imprinting approach. Keyes et al. employed a different kind of methodology to alter the catalytic properties of enzymes [19,20]. In their strategy, the enzyme complexed with ligands was crosslinked by using glutaraldehyde. This whole process was carried out in aqueous buffer, probably because the present imprinting concept in organic solvents was not known to that time.

In our contribution, we discussed potential application of the technique of molecular imprinting with emphasis on an enzyme with its competitive inhibitor and its using for the construction of a biosensor.

Below, schematic illustration of imprinting methodology is showed. The enzyme of urease was first complexed by using thiourea (Scheme 1).

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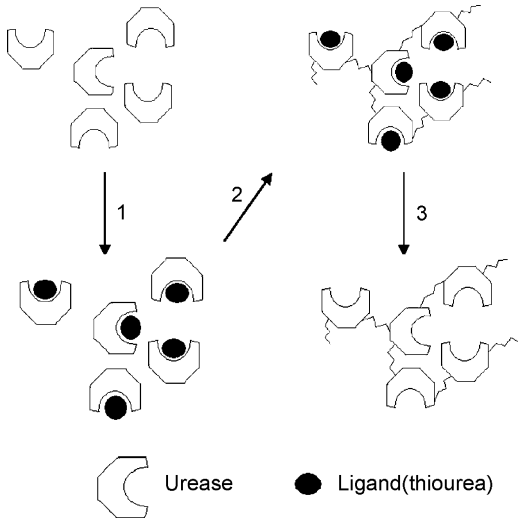

Scheme 1. Schematic diagram of the imprinted process (1, modification of the enzyme with thiourea; 2, immobilization and crosslinking with glutaraldehyde; 3, removal of thiourea from the active sites of the enzymes).

And then the enzyme–thiourea complex was immobilized with gelatin by the help of glutaraldehyde on a pH electrode tip. The influence of the imprinting of urease by its competitive inhibitor on the analytical performance such as pH and temperature and operational stabilities, of the resulting biosensor is discussed in this paper.

2. Experimental

2.1. Chemicals

Urease (E.C. 3.5.1.5, Type III: from Jack Beans 29,500 units/g solid), gelatin (Type 3, 225 Bloom), glutaraldehyde (Grade II, 25% aqueous solution) were purchased from Sigma (USA), Tris–HCl, urea, thiourea, were purchased from Merck (Germany).

2.2. Apparatus

pH electrode and pH meter were used for the potentiometric pH measurements (Hanna Instruments, Portugal). A water bath was used for preparation of bioactive material (Stuart scientific Linear Shaker bath SBS 35)(UK). All the measurements were carried out of constant temperature using a thermostat (Haake JF, Germany) Magnetic stirrer (IKA-Combimag, RCO) and pH meter with electrode (WTW pH 538, Germany) for preparing buffer solutions were used. The temperature was maintained at constant in the reaction cell by circulating water at appropriate temperature around the cell compartment during the experiment.

2.3. Procedure

2.3.1. Bio-imprinting of urease with thiourea

In the preparation of bio-imprinting biosensor, firstly, the enzyme urease (30 U/50 μL) and its competitive inhibitor thiourea (1.5 mg/50 μL) were incubated together for 1 h. If urease is added to a mixture of urea and thiourea the activity of the enzyme is strongly diminished. Like urea thiourea is bound to the active centre of the enzyme. Thus the active site of the enzyme is temporarily blocked by the 'false substrate'. The inhibition is called competitive because urea and thiourea compete on equal terms for the binding site of urease. Characteristically increasing the concentration of substrate reduces the effect of the inhibitor. Inhibition can be overcome. Consequently, in our system, by the help of thiourea, the active site of urease can be protected from the negative effects of the immobilization procedure during this process.

2.3.2. Preparation of the imprinted biosensor

Two milligrams per 50 μL of gelatin was added to urease–thiourea mixture mentioned above. This mixture was spread over the glass pH electrode. The electrode was allowed to wait for 30 min for drying. At the end of the preparation step, the bioactive layer of the biosensor was crosslinked by glutaraldehyde (2.5%). Finally, thiourea in the bioactive layer was removed for 1 h by waiting in the distilled water. The other biosensor was prepared in the same way. But there was no pretreatment with thiourea before the immobilization process. The enzyme and gelatin amount, and glutaraldehyde percentage were same as the bio-imprinted biosensor.

2.3.3. Measurement procedure

The bio-imprinted biosensor based on urease was put in to the thermostatic reaction cell containing working buffer (pH 6, 50 mM Tris–HCl buffer) and the magnetic stirrer was fixed at a constant speed. A few minutes later, the pH was equilibrated because of the diffusion between working buffer and pH probe. At this time, urea was injected into the thermostatic reaction cell. pH started to increase and a few minutes later it reached constant value due to the enzymatic reaction equilibration below:

$$\text{urea} + H_2O \xrightarrow{\text{urease}} CO_2 + 2NH_3$$

Measurements were carried out by the change of pH related to urea concentration added to the reaction cell. For all urea concentrations 10 min reaction period was chosen. The enzymatic reaction above was equilibrated within 10 min at all urea concentrations. So, response time was kept constant as 10 min.

3. Results and discussion

The bio-imprinted urease biosensor was compared with urease biosensor in their stability characteristics because the aim of this work was to improve the stabilities of urease biosensor by imprinting technique. For this purpose, for both biosensors optimum temperature and pH, thermal, pH and operational stability investigations were carried out. And all data were compared with each other for each parameter tested.

3.1. Optimum temperatures of the biosensors

The results obtained with two biosensors showed that the best activities were monitored at the same temperature, 30 °C. So,

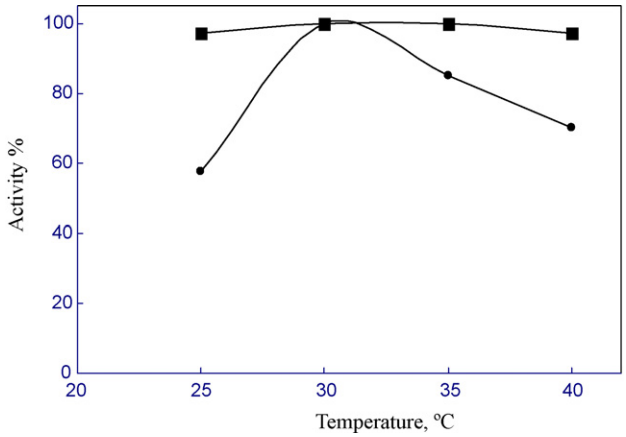

Fig. 1. Temperature dependence of the biosensors ((■) imprinted urease biosensor; (●) urease biosensor. Working conditions: the substrate urea concentration was injected to a final concentration of 1 mM. Tris–HCl buffer, 50 mM and pH 6.0).

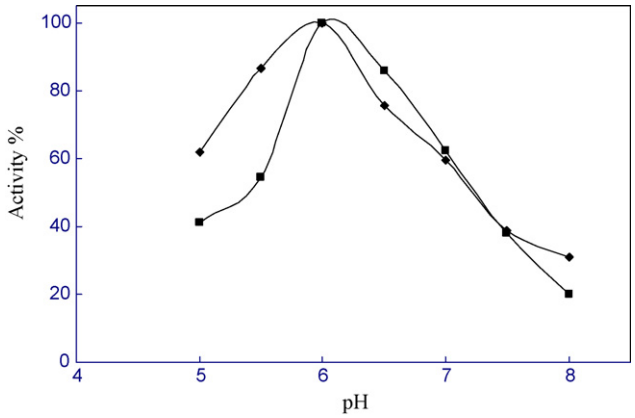

Fig. 2. pH dependence of the biosensors ((■) imprinted urease biosensor; (●) urease biosensor. Working conditions: urea concentration was injected to a final concentration of 1 mM. Tris–HCl buffer with different pH values, 50 mM and 30 °C).

there was no shifting on the optimum temperature of the biosensor by complexation with thiourea. Beside, by the biosensor complexed with thiourea before immobilization, it was possible to work in the range of 25–40 °C without activity decrease.

As seen in Fig. 1, bio-imprinted biosensor showed better temperature range for working than the other biosensor. Of course working temperature of a biosensor is determined after its thermal stability studies. These results are discussed below.

3.2. Thermal stabilities of the biosensors

Thermal stability was studied by incubation of the biosensors at 30 °C before the measurements for 3 h. The biosensor unmodified lost 61.4% of its initial activity. However, the biosensor modified with thiourea saved 65% of its original activity. This result is summarized in Table 1.

It should be concluded that, temporary blocked active site of urease by thiourea decreased the possibility of deactivation of the enzyme in the step of crosslinking by glutaraldehyde. Consequently, because of the resistant to immobilization conditions, the modified biosensor was more stabile than unmodified one.

3.3. Optimum pHs of the biosensors

In order to compare optimum pHs of two biosensors buffer solutions with different pH values were used in the experiments. The results showed that optimum pHs of both biosensors were same as 6 (Fig. 2). However, at more acidic pHs than 6, there was a dramatical decrease in the activity of the modified biosensor.

Table 1
Thermal stabilities of the biosensors

	Initial activity	Incubation period (h)	Residual activity (%)
Biosensor 1[a]	100	3	38.6
Biosensor 2[b]	100	3	65

[a] The biosensor with unmodified.
[b] The biosensor modified with thiourea.

This effect may be caused from the blockage of –COOH groups by hydrogen bond forming between the $–NH_2$ groups of the thiourea and –COOH groups of the enzyme. Besides, as can be seen in the figure, imprinted biosensor was more sensitive to change in the pH value than the other biosensor. The studies in the next step pH stability of the imprinted biosensor supported these results.

3.4. pH stabilities of the biosensors

The pH stability is one of the most important properties of any immobilized enzyme in biosensors. For investigation of pH stabilities of the biosensors both of them were incubated in pH 6, 50 mM Tris–HCl buffer for 3 h.

According to the optimum pH study, the biosensor activity is optimum at pH 6. The stability of the bio-imprinted biosensor was agreed with the results obtained thermal stabilities studies. The results showed that bio-imprinting of the urease increased the pH stability of it. As seen from Table 2 the initial activity of bio-imprinted biosensor decreases at the end of the 3 h incubation period at pH 6 Tris–HCl buffer. The residual activity of the bio-imprinted biosensor was 75.4%. However, the urease biosensor unmodified with thiourea was kept 64.1% of its initial activity.

3.5. Calibration graphs for the biosensors

In this study, the calibration graphs of two biosensors were also investigated. As shown from the figure, calibration graphs

Table 2
pH stabilities of the biosensors

	Initial activity	Incubation period (h)	Residual activity (%)
Biosensor 1[a]	100	3	64.1
Biosensor 2[b]	100	3	75.4

pH stabilities of the biosensors were carried out at pH 6 Tris–HCl buffer.
[a] The biosensor with unmodified.
[b] The biosensor modified with thiourea.

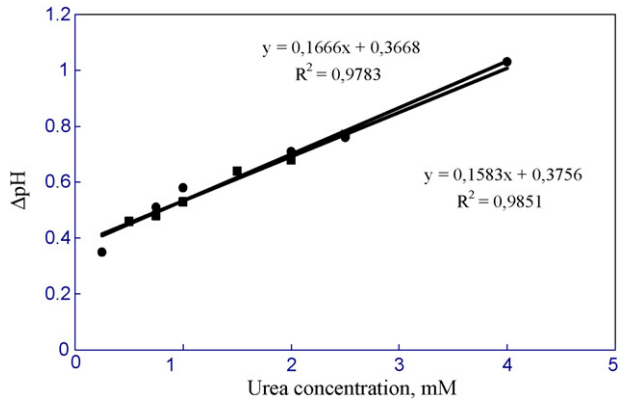

Fig. 3. Calibration graphs for the biosensors ((■) imprinted urease biosensor; (●) urease biosensor. Working conditions: Tris–HCl buffer pH 6.0, 50 mM and 30 °C).

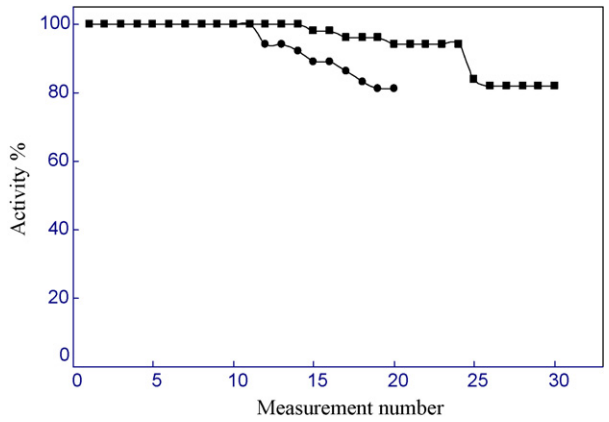

Fig. 4. Operational stabilities ((■) imprinted urease biosensor; (●) urease biosensor. Working conditions: the substrate urea concentration was injected to a final concentration of 1 mM. Tris–HCl buffer, 50 mM and pH 6.0, 30 °C).

were almost same. Moreover, linear ranges of both ones were identical, in the linear concentration range from 0.25 mM to 4 mM urea (Fig. 3).

The results showed that bio-imprinting of urease by its competitive inhibitor, thiourea, did not alter the linear range and detection limits of the biosensor.

K_m values for the bio-imprinted urease and urease biosensors were also calculated. According to the results the K_m values were 1.6 mM and 2.1 mM, respectively. As seen, K_m values supported the idea of bio-imprinting of urease could be advantageous for the biosensor applications.

3.6. Repeatability

The repeatabilities of the biosensors were also studied for 1 mM urea concentration ($n = 15$). The average value (x), the standard deviation (S.D.) and variation coefficient (CV) were 1.1, ±0.12 mM, and 11% for the biosensor unmodified with thiourea, 1.1, ±0.1 mM, and 9% for the bio-imprinted biosensor, respectively. It can be said that there was no effect of complexation with thiourea of the urease on the biosensors repeatabilities and analytical characteristics such as S.D., CV, etc.

3.7. Operational stabilities

The final studies of this investigation were operational studies. In these studies several measurements were carried out by two biosensors. And results showed that, modified biosensor was more stable than the other one in terms of the operational period (Fig. 4).

There was no activity decrease at the end of the 14th measurement with the modified biosensor. And, at the 15th measurement, it was just lost 2% of its initial activity, however, the biosensor unmodified was lost about 11% of its initial activity. This result should support the effect explained in the thermal stability section.

Moreover, the self-life of the biosensors were also determined and compared. Both biosensors were incubated at the working conditions for 5 h. pH measurements before and after incubation were very similar with each other. These results showed that 5 h incubation period was not affected the self of the bioactive layer and its components.

4. Conclusion

In this study, the molecular bio-imprinting technology has been used for the biosensor development. We have developed a novel biosensor for urea determination based on molecularly imprinted of urease. Moreover, this application of bio-imprinting technology was one of the first samples in literature. The sensor exhibits good sensitivity, selectivity and repeatability for urea. Moreover, stability studies showed that molecular bio-imprinted urease biosensor more stable than the other biosensor. Consequently, this way to improve the stabilities of the biosensors should be used by the other enzymes and biosensor. This bio-imprinting method is more convenient and inexpensive and of course simplier than the other imprinting methods. On this basis, the technique should provide a significant easy procedure for the preparation of biosensor system with the desired stabilities for many classes of biologic substance.

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