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Inhibitive determination of Hg^{2+} ion by an amperometric urea biosensor using poly(vinylferrocenium) film

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Abstract

A new approach based on amperometric urea biosensor for the inhibitive determination of Hg^{2+} ion using immobilized urease in poly(vinylferrocenium) film was developed. A $PVF^{+}ClO_4^{-}$ film was coated on Pt electrode at +0.7 V versus an Ag/AgCl by electrooxidation of poly(vinylferrocene) in metylene chloride containing 0.1 M tetrabutylammonium perchlorate (TBAP). The enzyme modified electrode $PVF^{+}E^{-}$ was prepared by anion-exchange in an enzyme solution in 50 mM phosphate buffer at pH 7.0. The catalytic oxidation current at +0.7 V (versus SCE) was monitored as a response of this immobilized enzyme electrode. The response of the urease enzyme electrode was effected by the presence of metal ions in the solution. Hg^{2+} ion was found to be the most dominant interfering species. The presence of Hg^{2+} ions in the samples inhibited the urease activity, resulting a decrease in oxidation current. The amperometric urease inhibition biosensor developed in this study provided linearity to Hg^{2+} ions in the 2.5 $\mu g\,mL^{-1}$ (9.2×10^{-6} M) to 115 $\mu g\,mL^{-1}$ (4.2×10^{-4} M) concentration range. The detection limit under the optimum working conditions was determined as 2.0 $\mu g\,mL^{-1}$ (7.4×10^{-6} M) for Hg^{2+} ion. The recovery of the biosensor was studied. Application of the biosensor to battery samples gave reliable results when compared to atomic absorption spectrometric findings. The interference effect of Cu^{2+}, Fe^{3+}, Cr^{3+}, Cd^{2+}, Zn^{2+}, and Pb^{2+} ions were also investigated under the same working conditions.
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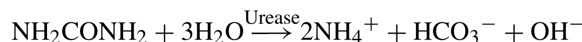
Keywords: Hg^{2+} ion; Urease inhibition; Poly(vinylferrocenium) modified electrode; Urea biosensor; Amperometric urease electrode

1. Introduction

Many substances alter the activity of an enzyme by combining with it in a way that influences the binding of the substrate. These substances are known as inhibitors [1]. Heavy metals are well known to inhibit the activity of enzymes and application of these hazardous toxic elements offers several advantages. Enzymes are often specific to inhibitor and in many cases the inhibition effect of investigated pollutant is related to its biological toxicity [2].

Certain metal ions are highly toxic. The determination of traces of toxic heavy metals in biological material, natural waters, soil and air has become very important. This is because the environment is vulnerable for this class of pollutants. Heavy metals are accumulated and stored in living organisms. Among them the influence of Hg^{2+} ion on the environment is particularly serious due to its strong toxicity and increasing level of its extended use in industrial processes [3–5].

Numerous enzymes such as urease, peroxidase, glucose oxidase, invertase, xanthine oxidase, butyrylcholinesterase, isocitric dehydrogenase and alkaline phosphatase have been used for the determination of Hg^{2+} ions [6]. Urease is the most used enzyme for this purpose. The effect of Hg^{2+} ions on urease enzyme is due to their binding to thiol groups present near the active center [7]. The enzyme urease, which is often present in most biological systems, plays a very important role by catalyzing the decomposition reaction of urea as follows:



$$NH_2CONH_2 + 3H_2O \xrightarrow{\text{Urease}} 2NH_4^{+} + HCO_3^{-} + OH^{-}$$

In the case of conventional urea biosensors there are several types of electrochemical biosensors. Numerous works have been carried out on the preparation of urease-based sensors using amperometric enzyme electrodes [8–12], potentiometric ammonium ion-selective electrodes [13–17], ammonia gas electrodes [18], and pH electrodes [19–22]. In most of studies Hg^{2+} ion was the most inhibiting species. Amperometric biosensors, which have emerged as the most commonly used biosensors, are considered promising for urea determination because of their effectiveness and simplicity. They measure the changes in the current on the

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working electrode due to direct oxidation or reduction of the products of a biochemical reaction. These biosensors can also be used in turbid media [9–11].

In this study, an amperometric urea biosensor which was previously developed in this laboratory was utilized for the inhibitive determination of Hg^{2+} ions using poly-(vinylferrocenium) (PVF^+) modified electrode [12]. Application of the biosensor to commercial battery samples and the influence of possible interferents were also examined.

2. Experimental

Phosphate buffer solutions were prepared from $NaH_2PO_4{\cdot}2H_2O$ (Merck) and $Na_2HPO_4{\cdot}2H_2O$ (Merck) using triple distilled water. Urea (Sigma) solutions were prepared using phosphate buffer solutions. Urease (E.C.3.5.1.5, Sigma) enzyme solutions with 16.000 U/g solid enzyme activity were prepared using phosphate buffer solutions at a concentration of 0.1 mg protein mL^{-1} which was then diluted. During the preparation of enzyme solution, ultrasonic bath was used. Enzyme solution was kept in refrigerator at +4.0 °C when it was not used. The pH of the buffer solutions was adjusted to the working pH of 7.0 by adding 0.1 M NaOH (Merck) solutions. For the interference studies $HgCl_2$ (BDH, Analar), $Cu(NO_3)_2{\cdot}3H_2O$ (Analar), $FeCl_3{\cdot}6H_2O$ (Merck), $Cr(NO_3)_3{\cdot}9H_2O$ (Merck), $Pb(NO_3)_2$ (Analar), $Cd(NO_3)_3{\cdot}4H_2O$ (Merck) and $ZnCl_2$ (Fisher) were used.

The chemical polymerization of PVF and the electroprecipitation of $PVF^+ClO_4^-$ have been described in previous publications [12,15]. Methylene chloride (Aldrich), was purified according to a procedure published elsewhere [23]. (TBAP) was prepared by the reaction of tetra-*n*-butyl ammonium hydroxide (40% aqueous solution, Merck) with perchloric acid (BDH), recrystallized from the water and ethyl alcohol mixture (1:9 by volume) several times. It was then dried at 120 °C under vacuum for 12 h. This salt was always kept under nitrogen atmosphere.

The preparation of the enzyme electrode was accomplished by immersing the $PVF^+ClO_4^-$ coated Pt electrode in a solution of urease enzyme with stirring. The enzyme is held electrostatically in the polymeric structure. The pH of the enzyme solution was kept above the isoelectric point (5.5) of the enzyme [20]. The enzyme molecule exists in the form of an anion (E^-) under working conditions, facilitating its ion exchange interaction with the oxidized polymer (PVF^+). The enzyme-attached electrode was rinsed with 50 mM phosphate buffer solution of pH 7.0 to remove the excess enzyme, which was not held electrostatically.

The response of an enzyme electrode was measured as the steady-state current value. In order to determine the steady-state background current of the enzyme electrode, a potential of +0.7 V (versus SCE) was applied to the urease enzyme electrode, which was kept in 50 mM phosphate buffer solution (pH 7.0) that did not contain urea solution. After the steady-state current value was reached, known amounts of the urea solution, which contained Hg^{2+} ions were added to the cell and the solution was stirred for 5 s. The response of the electrode was measured at an applied constant potential of +0.7 V (versus SCE). +0.7 V versus SCE, which was set in all our previous measurements as the optimized potential for the oxidation of PVF to PVF^+ [24–25]. The enzyme electrode was kept in 50 mM phosphate buffer solution at pH 7.0 and +4.0 °C when not in use.

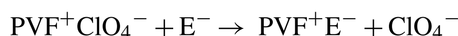
Application of the biosensor was carried out with commercial battery samples. Firstly battery samples were dissolved with aqua regia (HCl Sigma–Aldrich, HNO_3 Sigma–Aldrich). After this step, the samples were waited in hot water bath. Then their solutions were prepared with 0.01 M urea solution at pH 7.0. Certain amount of the dissolved battery sample was added to the electrochemical cell for the determination of Hg^{2+} ion inhibition.

Electrochemical measurements were carried out in a three-electrode cell. A Pt disc electrode (area: 7.85×10^{-3} cm^2) was used as the working electrode in electrochemical studies. SCE was used as a reference electrode and a Pt wire electrode (area: 2 cm^2) was used as the counter electrode. The reference electrode was Ag/AgCl during polymer electrooxidation in methylene chloride. The electrochemical studies were carried out with PAR system, which consists of Model 173 Potentiostat and Model 179 Digital Coulometer. Current-time curves were recorded on a Cole-Parmer 60648 X-t recorder. An atomic absorption spectrometer (AAnalyst 800-Perkin-Elmer) was used for the atomic absorption spectroscopy method. An electrochemical cell, which had five inlets, was used for the electrochemical studies. Three of these were used for the electrodes and the other two were used for nitrogen gas inlet and gas outlet.

Each point measured for each graph in this work was repeated three times and no significant deviations have been found. Furthermore, in some cases the measurements had to be repeated by another electrode fabricated at different times and gave almost the same response under the same conditions.

3. Results and discussion

It was reported in previous studies from this laboratory that $PVF^+ClO_4^-$ matrix is sensitive to the anions present in the solution [26–29]. The enzyme urease (E) is negatively charged at a pH value over its isoelectric point. After modification of Pt electrode with PVF^+, negatively charged urease enzyme (E^-) was immobilized into the polymer matrix electrostatically due to the following equation:



$$PVF^+ClO_4^- + E^- \rightarrow PVF^+E^- + ClO_4^-$$

The immobilization of urease enzyme into the polymeric film was proven by FTIR and UV techniques [15]. During urea hydrolysis, immobilized urease in the PVF^+ matrix produced a local pH change. A pH change significantly influences the formal redox potential of the $PVF^+ClO_4^-$ film. At a suitable constant potential, the pH change of the system is accompanied by a detectable current change. When the local pH on the electrode surface changes as a consequence of the biocatalytic reaction, the current value resulting from the electrochemical reaction of the pH-sensitive redox compound also changes to an extent, which is correlated to the substrate concentration [30]. The interference effect of the Hg^{2+} ions in the urea solution appears as a decrease in the oxidation current.

For the interference studies optimum working conditions of the prepared urea biosensor at room temperature were used. These conditions were 1.0 mC polymeric film thickness, 0.025 mg mL^{-1} urease enzyme concentration, 10 min immersion time and 50 mM pH 7.0 phosphate buffer solution [12].

3.1. pH dependence of the polymer

A blank experiment (without the urease enzyme) was carried out for the $PVF^+ClO_4^-$ film. The pH dependence of the polymer was determined using buffer solutions containing 50 mM total phosphate ions at a pH range of 6.0–8.0. The maximum current value was obtained at pH 7.0, which decreases linearly beyond this pH. pH 7.0 has been cited as an optimum value for the catalytic activity of urease in the decomposition reaction of urea [13]. The optimum working pH of the medium was also found as pH 7.0 in the previous study [12].

3.2. Inhibition effect of Hg^{2+} ion

The activity of urease enzyme is effected by the presence of metal ions in the solution. Hg^{2+} ions effect the urease enzyme due to their binding to thiol groups present near the active center of the enzyme. The inhibitory effect of Hg^{2+} ion on the response of the urea biosensor was investigated by adding

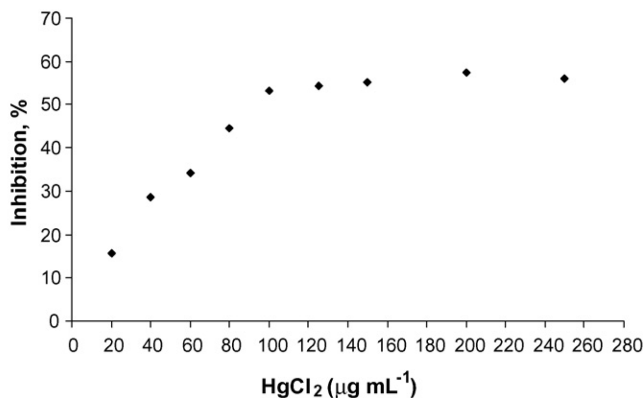


Fig. 1. % Reduction in the response of the enzyme electrode caused by Hg^{2+} ions (1.0 mC polymeric film thickness, 0.025 mg mL^{-1} urease enzyme concentration, 10 min immersion time, 50.0 mM pH 7.0 phosphate buffer solution, room temperature).

various amounts of $HgCl_2$ to 0.01 M urea solution. The interference effect of Hg^{2+} ions was carried out under the conditions of 1.0 mC polymeric film thickness, 0.025 mg mL^{-1} urease enzyme concentration, 10 min immersion time, 50.0 mM pH 7.0 phosphate buffer solution at room temperature. It was shown previously that there was a linear decrease of response of the electrode in the presence of Hg^{2+} ions in solution up to a concentration value of 115 μg mL^{-1} [12]. Above this value there was no significant change in current values.

The percent reduction in the response of the electrode is given in Fig. 1. The presence of 20 μg mL^{-1} Hg^{2+} ions resulted in a 15% decrease in the measured current and the addition of 125 μg mL^{-1} Hg^{2+} ions produced a 54% suppression of the initial enzyme electrode response.

3.3. Hg^{2+} ion calibration of the enzyme electrode

When the concentration of substrate was fixed as 0.01 M, the measured current values only depend on the activity of the urease enzyme. The calibration curve for Hg^{2+} ion is presented in Fig. 2 after inhibition. It can be seen from the figure enzyme electrode developed provided linearity to Hg^{2+} ion in the 2.5 μg mL^{-1} (9.2×10^{-6} M) to 115 μg mL^{-1} (4.2×10^{-4} M) concentration range. The detection limit under the optimum working conditions was determined as 2.0 μg mL^{-1} (7.4×10^{-6} M) for Hg^{2+} ion. The response time to Hg^{2+} ions was 60 s.

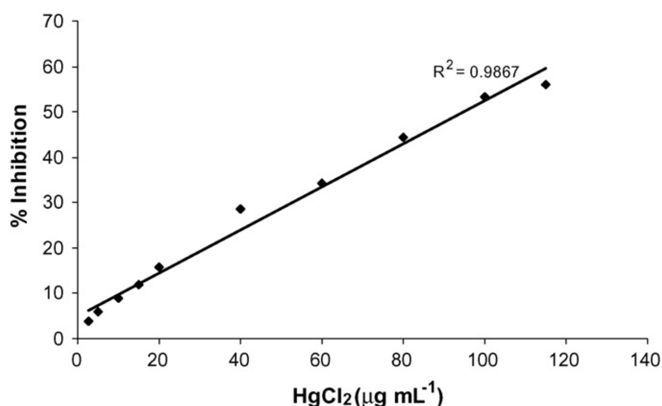


Fig. 2. Calibration curve for Hg^{2+} ion obtained after inhibition (1.0 mC polymeric film thickness, 0.025 mg mL^{-1} urease enzyme concentration, 10 min immersion time, 50.0 mM pH 7.0 phosphate buffer solution, room temperature).

Table 1
Comparison of inhibitory effects of various metal ions at 1000 μg mL^{-1} concentration

Ion	Inhibition (%)
Hg^{2+}	57.4
Fe^{3+}	29.1
Cu^{2+}	28.7
Cr^{3+}	21.7
Pb^{2+}	9.2
Zn^{2+}	7.3
Cd^{2+}	2.3

3.4. Interferences

Selectivity is an important parameter in the performance of an inhibition based biosensor. Cu^{2+}, Fe^{3+}, Cr^{3+}, Cd^{2+}, Zn^{2+}, and Pb^{2+} ions also inhibit the activity of urease enzyme. The interference effect of these ions was also investigated under the same working conditions. The comparison of inhibitory effects caused at a concentration level of 1000 μg mL^{-1} for each cation was found to be less than that of Hg^{2+} ion (Table 1).

3.5. Recovery studies by proposed method

In order to establish the reliability and suitability of the proposed method recovery experiments were perpormed. After the addition of 15.00 μg mL^{-1} Hg^{2+} ion three times into the electrochemical cell, the % recoveries were found as 90.00, 91.67 and 98.33. The % relative standard deviation was also found as 4.72 for these three recoveries. The results are given in Table 2.

3.6. Application of the enzyme electrode

This biosensor based on urease inhibition was applied to two commercial battery samples. The same samples were also analyzed with atomic absorption spectrometry (AAS). Application of the biosensor to battery samples gave reliable assesments when compared to those obtained by atomic absorption spectrometry (AAS). The results are given in Table 3.

Comparison was made between two sets of replicate measurements by *t* test. For the first battery sample the standard deviation was found as ±0.353. According to the *t* test the calculated *t* value was 6.588 and this value did not exceed the tabulated *t*

Table 2
Recovery studies by proposed method at PVF^{+} modified electrode

Added (Hg^{2+} μg mL^{-1})	Found (Hg^{2+} μg mL^{-1})	Recovery (%)	R.S.D. (%) ($n = 3$)
15.00	13.50 ± 0.13	90.00 ± 2.35	
15.00	13.75 ± 0.18	91.67 ± 1.17	4.72
15.00	14.75 ± 0.53	98.33 ± 3.54	

Table 3
Comparison of two methods (amperometric and AAS methods) by *t* test

Amperometric method (Hg^{2+}, $\mu g\,mL^{-1}$)	AAS method (Hg^{2+}, $\mu g\,mL^{-1}$)
Sample 1	
12.80	10.87
13.50	10.78
Sample 2	
86.75	82.53
88.00	82.69

value (9.925) at 99% confidence level, which confirms that there is no significant difference between the two methods. For the second battery sample the standard deviation was found as ±0.631 and the calculated t value was 7.552 and this value didn't exceed the tabulated *t* value (9.925) at 99% confidence level either which confirms that there is no significant difference between the two methods.

4. Conclusions

It can be concluded that the previously developed urease biosensor, which has an advantage of easy preparation and fast response can also be used for the inhibitive determination of Hg^{2+} ions. The enzyme inhibition based biosensor showed good linear concentration range to Hg^{2+} ions when compared with similar studies in the literature.

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References

[1] Voet D, Voet JG. Biochemistry. New York: John Wiley and Sons Inc.; 1995. p. 332.
[2] Krawczynski vel Krawczyk T, Mosczynska M, Trojanowicz M. Inhibitive determination of mercury and other metal ions by potentiometric urea biosensor. Biosens Bioelectron 2000;15:681–91.
[3] Bello Rodriguez B, Bolbot JA, Tothill IE. Development of urease and glutamic dehydrogenase amperometric assay for heavy metals screening in polluted samples. Biosens Bioelectron 2004;19:1157–67.
[4] Mohammadi H, Amine A, Cosnier S, Mousty C. Mercury-enzyme inhibition assays with an amperometric sucrose biosensor based on a trienzymatic-clay matrix. Anal Chim Acta 2005;543:143–9.
[5] Malitesta C, Guascito MR. Heavy metal determination by biosensors based on enzyme immobilised by electropolymerisation. Biosens Bioelectron 2005;20:1643–7.
[6] Y. Yang, Z. Wang, M. Yang, M. Guo, Z. Wu, G. Shen, R. Yu, Inhibitive determination of mercury ion using a renewable urea biosensor based on self-assembled gold nanoparticles, Sens Actuators B, in press.
[7] Prakash O, Upadhyay LSB. Effect of thiols on the activity of urease from dehusked seeds of watermelon (*Citrillus vulgaris*). Plant Sci 2003;164:189–94.
[8] Adeloju SB, Shaw SJ, Wallace GG. Pulsed-amperometric detection of urea in blood samples on a conducting polypyrrole-urease biosensor. Anal Chim Acta 1997;341:155–60.
[9] Yakamoto Y, Senda M. Amperometric ammonium ion sensor and its application to biosensors. Sens Actuators B 1993;13:57–60.
[10] Vostiar I, Tkac J, Sturdik E, Gemeiner P. Amperometric urea biosensor based on urease and electropolymerized toluidine blue dye as a pH-sensitive redox probe. Bioelectrochemistry 2002;56:113–5.
[11] Pizzariello A, Stredansky M, Stredanska S, Miertus S. Urea biosensor based on amperometric pH-sensing with hematein as a pH-sensitive redox mediator. Talanta 2001;54:763–72.
[12] Kuralay F, Özyörük H, Yıldız A. Amperometric enzyme electrode for urea determination using immobilized urease in poly(vinylferrocenium) film. Sens Actuators B 2006;114:500–6.
[13] Adeloju SB, Shaw SJ, Wallace GG. Polypyrrole-based potentiometric biosensor for urea. Anal Chim Acta 1993;281:621–7.
[14] Lakard B, Herlem G, Lakard S, Antoniou A, Fahys B. Urea potentiometric biosensor based on modified electrodes with urease immobilized on polyethylenimine films. Biosens Bioelectron 2004;19:1641–7.
[15] Kuralay F, Özyörük H, Yıldız A. Potentiometric enzyme electrode for urea determination using immobilized urease in poly(vinylferrocenium) film. Sens Actuators B 2005;109:194–9.
[16] Soldatkin AP, Montoriol J, Sant W, Martelet C, Jaffrezic-Renault N. a novel urea sensitive biosensor with extended dynamic range based on recombinant urease and ISFETs. Biosens Bioelectron 2003;19:131–5.
[17] Chen JC, Chou JC, Sun TP, Hsiung SK. Portable urea biosensor based on the extended-gate field effect transistor. Sens Actuators B 2003;91: 180–6.
[18] Macsini M, Guilbault GG. Urease coupled ammonia electrode for urea determination in blood serum. Anal Chem 1977;49:795–8.
[19] Wang JQ, Chau JC, Sun TP, Hsiung SK, Hsiung GB. pH-based potentiometrical flow injection biosensor for urea. Sens Actuators B 2003;91: 5–10.
[20] Koncki R, Leszczyński P, Hulanicki A, Glab S. Urea sensors based on glass pH electrodes with physically immobilized urease. Anal Chim Acta 1992;257:67–72.
[21] Koncki R, Chudzik A, Walcerz I. Urea determination using pH-enzyme electrode. J Pharm Biomed Anal 1999;21:51–7.
[22] Pandey PC, Singh G. Tetraphenylborate doped polyaniline based novel pH sensor and solid-state urea biosensor. Talanta 2001;55:773–82.
[23] Perin DD, Armarego WLF, Perin DR. Purification of Laboratory Chemicals. Oxford: Pergamon Press; 1980. p. 265.
[24] Aydın G, Çelebi SS, Özyörük H, Yıldız A. Amperometric enzyme electrode for L(+)-lactate determination using immobilized L(+)-lactate oxidase in poly(vinylferrocenium) film. Sens Actuators B 2002;87: 8–12.
[25] Gülce H, Özyörük H, Çelebi SS, Yıldız A. Amperometric enzyme electrode for aerobic glucose monitoring prepared by glucose oxidase immobilized in poly(vinylferrocenium). J Electroanal Chem 1995;394:63–70.
[26] Gülce H, Çelebi SS, Özyörük H, Yıldız A. Amperometric enzyme electrode for sucrose determination prepared from glucose oxidase and invertase co-immobilized in poly(vinylferrocenium). J Electroanal Chem 1995;397:217–23.
[27] Gündoğan-Paul M, Özyörük H, Çelebi SS, Yıldız A. Amperometric enzyme electrode for hydrogen peroxide determination prepared with horseradish peroxidase immobilized in poly(vinylferrocenium) (PVF^+). Electroanalysis 2002;14:505–11.
[28] Gündoğan-Paul M, Çelebi SS, Özyörük H, Yıldız A. Amperometric enzyme electrode for organic peroxides determination prepared from horseradish peroxidase immobilized in poly(vinylferrocenium) film. Biosens Bioelectron 2002;17:875–81.
[29] Gülce H, Özyörük H, Yıldız A. Electrochemical response of poly(vinylferrocenium)-coated Pt electrodes to some anions in aqueous media. Electroanalysis 1995;7:178–83.
[30] Stredansky M, Pizzariello A, Stredanska S, Miertus S. Amperometric pH-sensing biosensors for urea, penicillin and oxalacetate. Anal Chim Acta 2000;415:151–7.