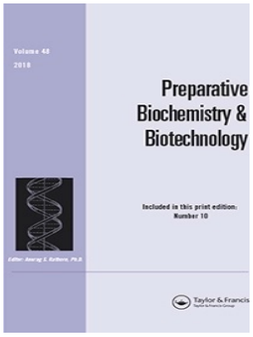

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Development of urea biosensor using non-covalent complexes of urease with aldehyde derivative of PEG and analysis on serum samples

Gokay Vardar[a], Azade Attar[b], and Melda Altikatoglu Yapaoz[a]

[a]Department of Chemistry, Faculty of Science and Letters, Yildiz Technical University, Istanbul, Turkey; [b]Department of Bioengineering, Faculty of Chemical & Metallurgical Engineering, Yildiz Technical University, Istanbul, Turkey

ABSTRACT

Non-covalent complexes of urease/polyethylene glycol (PEG)-aldehyde were synthesized using regular molar ratios of urease and PEG-aldehyde at room temperature. The physical properties of the non-covalent complexes were analyzed in order to investigate the impact of coupling ratio, temperature, pH, storage stability, and thermal stability. Urease activity was analyzed by UV–Vis spectrophotometer at 630 nm. The results showed that the strongest thermal resistance was obtained using n_U/n_{PEG}:1/1 (mg/mL) complex within all molar ratios tested. The enzymatic activity of n_U/n_{PEG}:1/1 complex doubled the activity of the free enzyme. Therefore, this complex was chosen to be used in the analyses. When coupled with PEG-aldehyde, urease exhibited improved activity between pH 4.0–9.0 and the optimum pH was found to be 7.0. The thermal inactivation results of the complex demonstrated that higher activity remained (40%) when compared with the free enzyme (10%) at 60 °C. The storage stability of the non-covalent complex was 4 weeks which was greater than the storage stability of the free enzyme. A kinetic model was suggested in order to reveal the mechanism of enzymatic conversion. Potentiometric urea biosensor was prepared using two different membranes: carboxylated poly vinyl chloride (PVC) and palmitic acid containing PVC. The potentiometric responses of both sensors were tested against pH and temperature and the best results were obtained at pH 7.0 and 20–30 °C. Also, selectivity of the suggested biosensors toward Na^+, Li^+ Ca^{2+}, and K^+ ions was evaluated and the reproducibility responses of the urea biosensors were measured with acceptable results.

KEYWORDS

Enzyme-polymer modifications; non-covalent complexes; PEG-aldehyde; urea biosensor; urease

Introduction

It is a common approach using physically or chemically modified biomolecules in biotechnology.[1] The economic and facile bioproduction of novel and improved enzymes are leading in this method by having an important role in health sector. There are several methods of bioprocessing of enzymes such as immobilization, encapsulation, non-covalent binding, and covalent binding.[2] The most studied is covalent modification however this method has some obstacles.[3–5] Covalent bonding may lower the activity of enzymes because of the conformational changes of the protein structure. The non-covalent modifications are formed by the interaction between electrons systems that cause self-assembly which is the common nature of organization of biological structures by H-bonding.[6] There are no major considerations about weak interactions on structural alterations of proteins. The molecular recognition on the non-covalent interactions between functional groups of proteins and polymers is a subject of interest, because of the reversible nature of the non-covalent complexation. The obtained biocomplex has superior properties compared with covalently modified ones due to the improved features such as dynamic structure, capability of annealing, healing, and adaptation.[7]

Urease is a prevalently found enzyme in the native environments such as living organisms and soil.[8] The importance of this enzyme depends on its catalytic activity on urea which is the end-product of nitrogen cycle. The hydrolysis of urea into ammonia and carbonate leads to an increase in the pH with undesirable results on agronomy and public health.[9] Urea can be detected in biological fluids in blood and urine; therefore, urease assay is essential in pharmaceutical, medical, agricultural, and environmental applications. There are several studies on the analysis of urease such as Raman and FTIR spectroscopy,[8] X-ray diffraction,[9] circular dichroism and fluorescence spectra,[10] and flow analysis.[11]

Polyethylene glycol (PEG) is a neutral polyether that shows interesting biological features of being either protein rejecting or biocompatible.[12] Various synthetic and natural polymers have been investigated for the modification and immobilization of proteins.[13] PEG is one of the most preferred polymers in biotechnological processes owing to its modification effect. Urease and PEG were studied as enzyme

CONTACT Melda Altikatoglu Yapaoz maltikatoglu@yahoo.com Department of Chemistry, Faculty of Science and Letters, Yildiz Technical University, Davutpasa campus, Istanbul 34210, Turkey.
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systems especially in medical applications.[14–16] There are also many reports on amperometric,[17,18] potentiometric,[19,20] conductometric,[21,22] and optical[23,24] urea biosensors. In this study, non-covalent complexes of urease and PEG-aldehyde were prepared in order to find the most stable and active couple, and potentiometric urea biosensor was developed using this complex with enhanced characteristics for urea determination in biological and environmental samples.

Materials and method

Materials

Urease from Jack beans *Canavalia ensiformis* (Mw∼460 kDa), urea and PEG (Mw∼20 kDa) were supplied by Sigma Aldrich (St. Louis, MO). The chemicals used in the study were analytical grade.

Preparation of urease-PEG aldehyde non-covalent complexes

The aldehyde derivative of PEG was obtained with $NaIO_4$ by our previous study.[25] Quantity of PEG-aldehyde required for the preparation of complexes was calculated depending on the formula written below for constant urease (U) concentration (0.2 mg/mL).

$$\frac{n_U}{n_{PEG}} = \frac{C_U \times M_{PEG}}{C_{PEG} \times M_U} = 1/1,\quad 1/5,\quad 1/10,\quad 1/20,\quad 5/1,\quad \times 10/1,\quad 20/1,\quad 30/1$$

where C is the concentration (mg/mL) and M is the molar weight.

Urease and PEG-aldehyde solutions were freshly equipped in phosphate-buffered saline (PBS) buffer (10 mM and pH 7.0). Afterward, the reaction was proceeded by combining PEG-aldehyde and enzyme solutions at room temperature and was stirred under constant magnetic stirring for about 60 min.

Urease activity assay

The ammonia formed was determined by the Berthelot assay method using a UV–Vis spectrophotometer at 630 nm wavelength.[25] The activities were calculated by using standard curve with ammonia in the range of 0–12.0 μmoL/mL. One unit of urease activity includes hydrolysis of 1.0 μmol urea/min at 25 °C, pH 7.0. Stabilities of the complexes and free urease were determined at different temperatures and pH values in keeping with the same method for activity determination. All experiments were made in triplicate.

Kinetics of the enzyme

Free enzyme and n_U/n_{PEG}:1/1 complex were evaluated for kinetic constants (K_m, V_{max}, and k_{cat}) using substrate concentration from 1 to 5 mM. Lineweaver–Burk plot was used to calculate kinetic constants. All experiments were made in triplicate.

Thermal and storage stability

The free enzyme and n_U/n_{PEG}:1/1 complex were incubated at 60 °C in 0.01 M PBS and pH 7.0. At specified time intervals, a certain amount of aliquots was taken out and the enzymatic activity was assayed as described before. For storage stability studies, the sample preparations containing 2 mg/mL free urease and urease-PEG aldehyde complex (n_U/n_{PEG}:1/1) were stored in PBS buffer (10 mM and pH 7.0) at 4 °C. The remaining activity was determined at regular time intervals using procedures described earlier. All the experiments were carried out in triplicate and the results represent mean values with less than 5% of error.

Production of ammonium-selective membrane electrode

All-solid-state contact electrodes were prepared with a mixture of 50% (*w/w*) graphite, 35% (*w/w*) epoxy, and 15% (*w/w*) hardener by dissolving in tetrahydrofuran (THF) solvent. The solution was allowed to stand for 20–30 min in air after mixing. A shielded copper wire (*ca.* 0.5–1 mm in diameter and 10 mm long) was polished and dipped into the solution several times to obtain a uniform coating when the suitable viscosity was attained and left overnight in an oven at 40 °C. Two different membranes were composed: (1) polyvinyl chloride (PVC) containing palmitic acid and (2) carboxylated PVC. The contents of ammonium-ion selective electrodes' membranes are shown in Table 1.

Each membrane was prepared by dissolving about 200 mg membrane components in 2.5 mL of THF. The all-solid-state contacts were dipped into each membrane solutions. Electrodes dried for 24 hr at room temperature. The all-solid-state contact membrane electrodes were immersed in a 0.1 M solution of ammonium chloride for 5 hr before use. Performance characteristics of the suggested electrodes were tested by measuring ammonium chloride in a concentration range between 10^{-1} and 10^{-5} M.

Preparation of urea biosensor based on urease/PEG-aldehyde complex

Urease/PEG-aldehyde complex was chemically immobilized on all ammonium selective membrane electrodes' (I and II compositions) surfaces in three steps:

1. The complex with a n_U/n_{PEG} ratio of 1/1 (10 mg/mL) and 5 mg carbodiimide were dissolved in 1 mL distilled water. About 50 μL of this solution was dropped onto the membrane surface of ammonium-selective electrodes and left overnight.

Table 1. Membrane compositions of prepared ammonium-selective electrodes.

	Membrane compositions (%)	
Membrane components	I	II
PVC	30	–
Palmitic acid	6.8	–
Carboxylated PVC	–	32.5
Nonactin	3	3
Bis-(2-ethyl)hexylsebacate	64	64.5

2. Then 50 µL of glutaraldehyde solution (2.5%) was placed on the surface of electrodes and left to dry for 30 min. Next, the electrode surface was rinsed with distilled water in order to get rid of the residual glutaraldehyde.
3. An amount of 50 µL of urease/PEG-aldehyde complex (10 mg/mL) was spotted onto the membrane surface of ammonium-selective electrodes which then left to dry overnight.

All types of biosensors were washed with distilled water to get rid of the residual unbound enzymes. Biosensors were stored at 4 °C in 0.01 M Tris buffer when not in use.

Analysis of serum samples by urea biosensor

The accuracy of urea determination by the suggested urea biosensor was checked. The serum samples were analyzed in a diagnostic laboratory by Blood Urea Nitrogen Test. The analyses were constructed by the control method using the commercial analyzer Vitros 250, a dry chemistry technology to investigate the precision of urea determination of the biosensor system. Urea amount was determined by urease reaction based on colorimetric detection of ammonia produced.

Results and discussion

Physical properties of non-covalent complexes

The effect of temperature

Non-covalent complexes of urease were prepared in order to investigate the effect of the aldehyde derivative of PEG on the stability of the enzyme. It was previously demonstrated that polymers are effectively used in enzyme stabilization by non-covalent interactions.[25–27] Therefore, in this study, the activity of urease/PEG-aldehyde complexes and urease enzyme were analyzed and compared. Enzyme activity results of the solutions prepared at different molar ratios at different temperature values are given in Figure 1. Enzyme activity of sole urease and complexes at various ratios showed thermal stability between 20 and 90 °C, at pH 7.0. Sole enzyme has a maximum activity at 40 °C while n_U/n_{PEG}:30/1 and 20/1 showed maximum activity at 50 °C and kept their activity till 90 °C. n_U/n_{PEG}:10/1 and 5/1 exhibited higher activity when compared with sole enzyme, 30/1 and 20/1 ratios. The urease/PEG-aldehyde complex prepared at 1/1 ratio is the most active sample (14,000 U/mL). It also has the highest activity above 80 °C, hence n_U/n_{PEG}:1/1 complex represents strongest thermal resistance. As can be seen from the graph, all the complexes exhibited higher activity than the sole enzyme except for the complexes of 1/5 and 1/10 at all temperatures. Obtained result was better than a previous report on urease in alginate beads, because it showed higher activity in a broader temperature range.[28] The use of intermolecular non-covalent interactions for the preparation of enzyme–polymer complexes has been demonstrated to be a handy approach to the design of thermally stable highly active enzymes.[7] Therefore, the difference in activity between the complexes prepared in different ratios

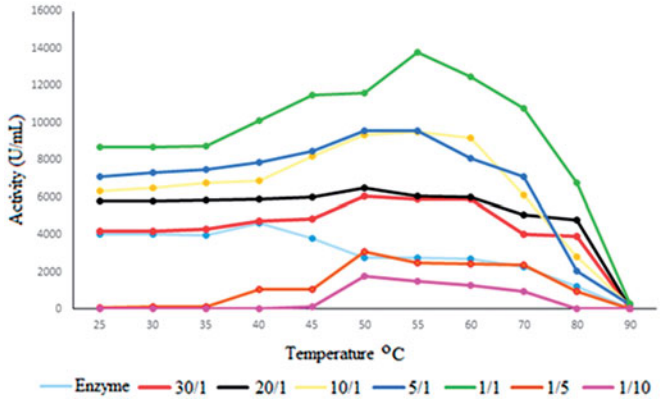

Figure 1. Thermal stability results of the free enzyme and the complexes with different molar ratios (n_U/n_{PEG}:30/1, 20/1, 10/1, 5/5, 1/1, 1/5, 1/10) at pH 7. All data points were achieved by the average of three repeated measurements.

is due to the alteration of the structure of the PEG-aldehyde bound to the enzyme.[29] Therefore, the difference in activity between the complexes prepared in different ratios is due to the alteration of the structure of the PEG-aldehyde bound to the enzyme. This alteration originates from non-covalent interactions such as steric exclusion, hydrophobic interactions, electrostatic and hydrogen bonds between the enzyme and polymer. The hydroxyl groups of PEG can be modified in order to form aldehyde groups and modified PEG including aldehyde groups performs as a good macromolecular crosslinker for free amino groups.[30] Generally, PEG-aldehyde environs the surface of the enzyme molecule when the subject is non-covalent interactions. Thus, the water layer around the enzyme surface is conserved and more rigid enzymes obtained by this way.[31]

The effect of coupling ratio

PEG-aldehyde, which is an amine reactive derivative, catalyzes the reaction between aldehyde group with α-amine of lysine residues and the α-amine at the N-terminus. The results of the activity of free urease and its PEG-aldehyde complexes in various molar ratios at 25 °C pH 7.0 were shown in Figure 2. These results indicated that the best ratio is 1/1, thereby supporting the results shown in Figure 1. The free enzyme had 4000 U/mL activity which was very close to the results of n_U/n_{PEG}:30/1 ratio. Total activity was increased

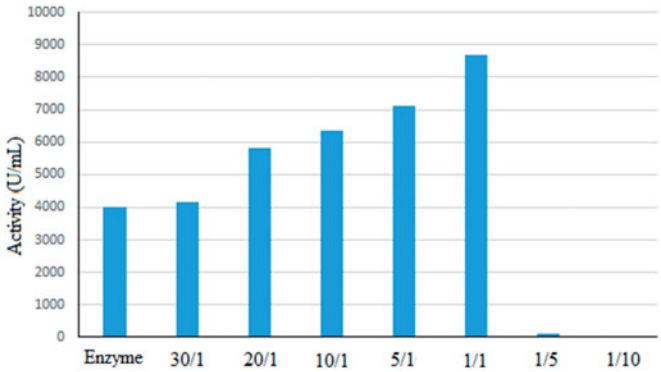

Figure 2. The activity results of the free enzyme and the complexes as a function of coupling ratio at pH 7.0; 25 °C; C_{urease}:0.2 mg/mL (n_U/n_{PEG}: 30/1, 20/1, 10/1, 5/5, 1/1, 1/5, 1/10).

as the urease/PEG-aldehyde ratio decreased, therefore n_U/n_{PEG}:1/1 complex exhibited maximum activity. According to the literature, the modification efficiency decreases with the increase of the amount of urease added.[32] Therefore, our results are parallel with the current literature. Also, no yield was claimed by the complexes of 1/5 and 1/10. This situation can be explained by the multiple point binding of urease which causes blockage of the enzyme active side. It was reported that immobilization of urease by a polymer lowers the activity when compared with native urease.[33] There are several studies on covalent modification of urease using different polymers reporting the decrease in enzyme activity because of the conformational changes and reduce of accessibility active sites in the protein structure.[34,35] However, in this case, the equality of urease and PEG-aldehyde concentration brings peak enzyme activity.

The effect of pH

One of the most important coupling conditions on the activity of urease is pH. n_U/n_{PEG} = 1/1 complex of urease/PEG-aldehyde and free urease were studied at 25 °C between pH 4.0 and 9.0. The activity of bound urease increased with increasing pH of coupling media from 4.0 to 7.0 which can clearly be seen in Figure 3. According to the literature, PEG-aldehydes react with amine groups at an optimal pH of 5.0–6.0 and higher pH will result in multiple pegylation with both terminal and lysine groups.[36] On the basis of our results, the optimal pH for urease/PEG-aldehyde complexes was observed at a neutral pH 7.0, which is consistent with the reaction mechanism of PEG-aldehyde. PEG-aldehydes are commonly used for modification of biomolecules, the reaction they catalyze results in an intermediate Schiff base which can generate a stable bond between carbon and nitrogen by additional reduction.[37] Therefore, PEG-aldehyde is a conventionally utilized PEG reagent for N-terminus pegylation in enzymes.[38] The free enzyme maximized its activity between pH 7.0 and 8 to 3965 U/mL, even so this result is not close to the complexes of urease/PEG-aldehyde result which was 8792 U/mL. The optimum pH for both samples was determined to be 7.0 and no activity was obtained at pH 9.0. The figure shows that n_U/n_{PEG} =1/1 complex has higher activity than free urease at all pH values. No shift was observed at the pH optimum in the presence of PEG-aldehyde because of the neutral composition of the PEG-aldehyde.

Determination of thermal stability

The activities of free urease and urease/PEG-aldehyde complex n_U/n_{PEG}:1/1 were determined at 60 °C by sampling every 30 min (Figure 4). Free urease lost its activity from 100 to 40% in 30 min and decreased to a minimum after 6 hr. However, the activity of urease/PEG-aldehyde complex remained for 3 hr at 60 °C and 50% of its activity remained after 5 hr. Residual activity of the complex was 40% at the end of the analysis (6 hr). Previous reports implied that complexation or immobilization improve thermal stability of urease.[39–41] The time dependent thermal inactivity studies evaluated that the n_U/n_{PEG}:1/1 complex had higher activity than the free enzyme by exhibiting improved stability.

Determination of storage stability

Storage lifetimes of free enzyme and the complex with n_U/n_{PEG}:1/1 were studied for a period of 4 weeks (data not shown). The solutions were stored in phosphate buffer (0.01 M, pH 7.0) at 4 °C. The free enzyme lost its initial activity after 11 d. Whereas, complex preserved its initial activity more than 35% at the end of 4 weeks of storage period. These results indicated a better storage stability of complex with PEG-aldehyde than free urease. Consequently, this complex had both higher activity and longer storage lifetime when compared with free urease and a previous report.[28] It has been considered as a superior characteristic for usage in practice.

Kinetics of the enzyme

To study the mechanism of enzymatic conversion, a kinetic model was adapted to the experimental data. The catalytic variables of free urease and complex concerning substrates (urea) were calculated with regard to typical Michaelis–Menten behavior. The modification of enzyme with PEG raised the catalytic constant (k_{cat}) and lowered the values of the Michaelis–Menten constant (K_m) was shown in Table 2.

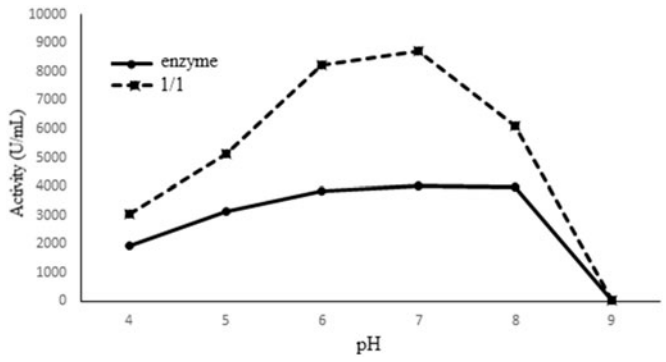

Figure 3. Activities of free urease and n_U/n_{PEG}: 1/1 complex depending on pH, at 25 °C. All data points were achieved by the average of three repeated measurements.

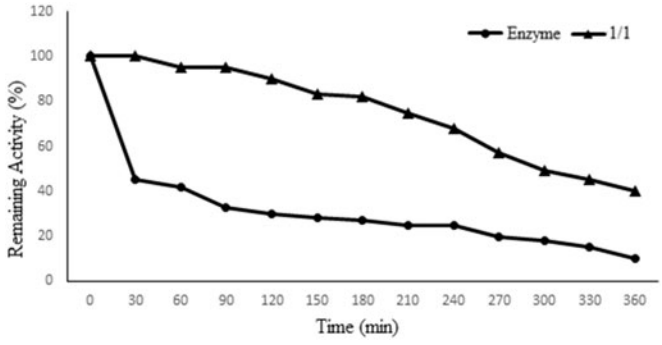

Figure 4. Thermal inactivation of free urease and n_U/n_{PEG}:1/1 complex at 60 °C. 100% activity is approved as the maximum absorbance. All data points were achieved by the average of three repeated measurements.

Table 2. Kinetic constants of enzyme and complex (n_U/n_{PEG}:1/1).

Parameter	Urease	1/1 Complex
K_m (g dL^{-1})	2.35 ± 0.03	0.85 ± 0.01
V_{max} (μmol min^{-1})	0.757 ± 0.01	0.781 ± 0.04
k_{cat} (s^{-1})	37.85 ± 0.3	39.05 ± 0.5
K_{cat}/K_m (dL g^{-1} s^{-1})	16.11 ± 0.1	45.94 ± 0.8

±SD, $n = 3$.

Table 3. Potentiometric performance features of all-solid-state contact ammonium-selective membrane electrodes.

	NH^+_4 selective electrode	
Parameter	Membrane I	Membrane II
Slope (mV/decade)	43 ± 5	48 ± 5
Correlation coefficient (ϰ)	0.9911	0.9965
Limit of linear range (mol/L)	10^{-1}–10^{-5}	10^{-1}–10^{-5}
Response time (s)	<20	<20
Limit of detection (mol/L)	2×10^{-6}	3×10^{-6}

Therefore, the catalytic efficacies (k_{cat}/K_m) of PEG-modified urease improved when compared with free enzyme.

Characteristics of urea biosensor

According to the results of the activity determination, the best result was obtained by n_U/n_{PEG}:1/1 ratio therefore, urea biosensor was prepared by using this complex. Two different membranes were prepared with palmitic acid containing PVC and carboxylated PVC, respectively membrane I and II. The results of potentiometric performances obtained by the proposed ammonium-selective electrodes are given below (Table 3).

Effect of pH

About 10 mM phosphate buffer at pH values between 6.0 and 8.5 was used in measurements in order to investigate the effect of pH on the analytical signal of the urea biosensors. Figure 5 demonstrates potentiometric responses of the suggested biosensor *versus* pH. The output signals of the potentiometric urea biosensors raised up to pH 7.0 and reduced over pH 7.0. This result implied that pH 7.0 is the critical pH for the enzyme urease which might be gradually denaturized at pH values except 7.0.

Effect of temperature

The effect of temperature on the response of the urea biosensor was demonstrated in Figure 6. The responses remained quite constant around 20–25 and 20–30 °C for the urea biosensors based on PVC containing palmitic acid (membrane I) and carboxylated PVC membrane (membrane II) ammonium-selective electrodes, respectively. The potentiometric responses gradually declined by the elevation of temperature for both of the biosensors which can be explained by decrease in the activity of urease at high temperatures.

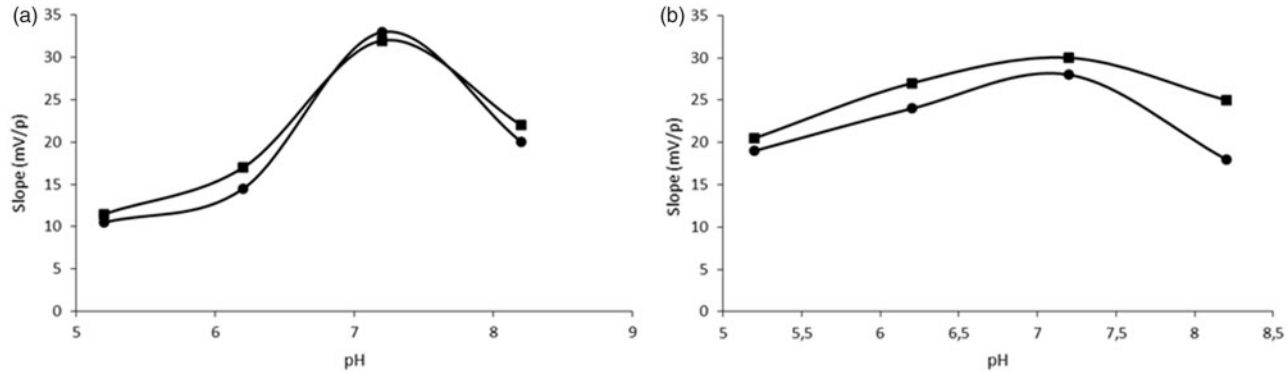

Figure 5. The response of urea biosensor prepared with free urease (—■—) and PEG-aldehyde modified urease (—●—) on the carboxylated PVC membrane ammonium-selective electrode [membrane II] (A) and on the PVC containing palmitic acid membrane ammonium-selective electrode [membrane I] (B) against pH.

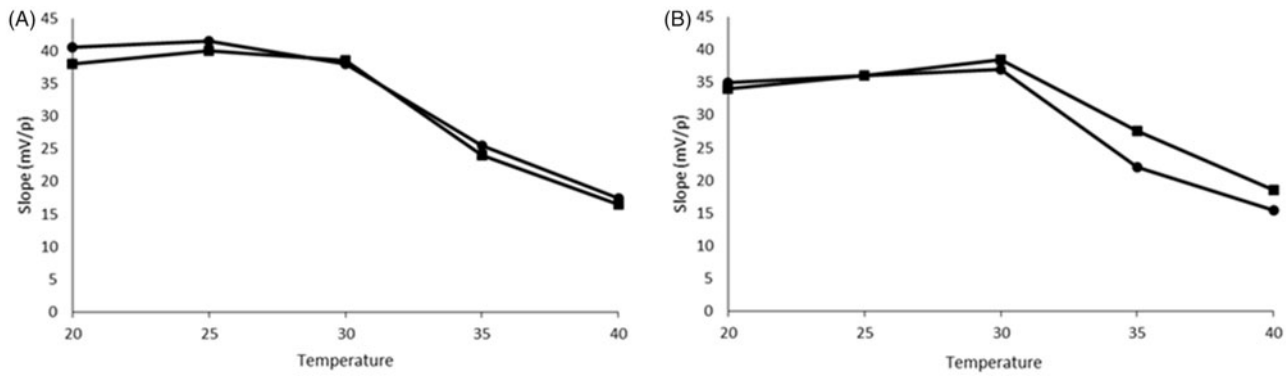

Figure 6. The response of urea biosensor prepared with free urease (—■—) and PEG modified urease (—●—) on PVC containing palmitic acid membrane ammonium-selective electrodes (A) and on the carboxylated PVC membrane ammonium-selective electrodes (B) against temperature.

Table 4. The selectivity coefficients of urea biosensors.

	The selectivity coefficient, $K_{NH_4,X}$	
Ions	Membrane I	Membrane II
K^+	1.6×10^{-2}	2.3×10^{-2}
Na^+	2.7×10^{-3}	5.1×10^{-3}
Li^+	7.4×10^{-3}	6.7×10^{-3}
Ca^{2+}	1.2×10^{-2}	1.4×10^{-2}

Selectivity of the biosensor

Prepared urea biosensors were analyzed on account of sensitivity toward sodium, potassium, calcium, and lithium ions (as chlorides, pH 7.0). The separate solution method was employed for the calculation of selectivity coefficients according to the IUPAC recommendation.[42] As indicated in Table 4, interference effect of Na^+, Li^+, and Ca^{2+} ions, on the response of the urea biosensors was very low, but K^+ ions showed the highest interference among all cations.

Response reproducibility of the biosensor

Reproducibility is the most important characteristics of biosensors. To determine the reproducibility of biosensor, the responses to the same urea concentrations (10^{-1}–10^{-3} M) were measured over one working day with 30 min intervals, the biosensor being remained between measurements in the working buffer with constant stirring. The relative standard deviation was 2.11% ($n = 15$) (for PEG modified urease on the carboxylated PVC membrane) and 3.14% ($n = 15$) (for PEG modified urease on the PVC containing palmitic acid membrane), which is quite acceptable; therefore, theoretically, the biosensor can be used to determine urea in biological samples (Figure 7).

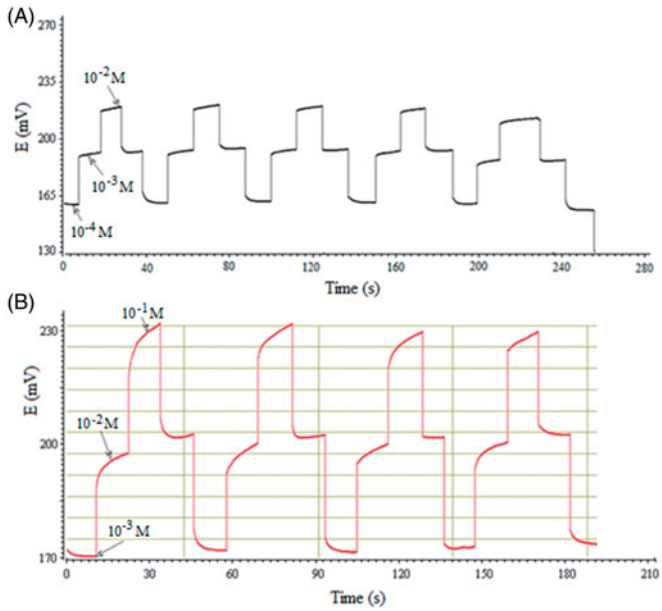

Figure 7. Reproducibility test for urea biosensor prepared by using PEG modified urease on the carboxylated PVC (A) and PVC containing palmitic acid (B) ammonium-selective membrane electrodes (B) in phosphate buffer (pH 7.0). The results are directly printed out from the measurement system maintained in our work.

Table 5. Diagnostic results of urea biosensor.

Sample	Control method (mg/dL)	Proposed biosensor (mg/dL)
1	27	27.2
2	139	139.3
3	19	16.5
4	33	21.2
5	57	45.4

Analysis of serum samples

The concentration of urea was analyzed in five samples of serum in order to verify functionality of the suggested urea biosensor. The samples belonged to patients with chronic kidney disease and had urea content in a broad variety of concentrations. The samples were included and the response of the biosensor system was measured and checked with the previously plotted calibration curves (Table 5).

Conclusions

Complexes of urease and aldehyde derivative of PEG were prepared by non-covalent modification. The urease activity of the complexes was determined by the Berthelot assay and compared with free urease. It was observed that non-covalent complexes of urease/PEG-aldehyde showed greater activity than free urease. Thermal and storage stability were significantly enhanced with the inclusion of PEG-aldehyde. The enzyme is active in a wider pH range when modified with PEG-aldehyde. All activity and stability results of the complex were improved compared to the results of the free enzyme, therefore, the characteristics of the complex indicated superior enzymatic results. Two different membranes were suggested for the ammonium-ion selective electrode preparation using PVC containing palmitic acid and carboxylated PVC. The urease/PEG-aldehyde complex immobilized electrodes were selective and response reproducible. The potentiometric urea biosensor results on serum samples indicated that the suggested urea biosensor could be used in real samples with high accuracy and reliability. This simple and effective non-covalent complex immobilized urea biosensor is useful in enzyme technology.

Acknowledgments

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