The Synthesis of Complex Polymer Electrolytes Based on Alginic Acid and Poly(1-vinylimidazole) and Application in Tyrosinase Immobilization

By Müjgan KARTAL,¹ Senem Kiralp KAYAHAN,¹ Ayhan BOZKURT,^{2,*} and Levent TOPPARE¹

In this study, proton conducting polymer electrolyte networks consisting of alginic acid (AA) and poly(1-vinylimidazole) (PVI) were prepared. The polymer networks were obtained by mixing AA and PVI with several stoichiometric ratios, x (with respect to monomers). Polymer networks were characterized by FT-IR spectroscopy and their compositions were investigated by elemental analysis (EA). Enzyme entrapped polymer networks (EEN) were produced by immobilization of tyrosinase in the AA/PVI matrix during complexation. The maximum reaction rate ($V_{\max}$) and Michaelis-Menten constant (K_m) were investigated for the immobilized tyrosinase. Also, the temperature and pH optimizations, operational stability and shelf life of enzyme immobilized in the polymer network were examined.

KEY WORDS: Alginic Acid / Poly(1-vinylimidazole) / Immobilized Enzymes / Biosensors / Proton Conductivity / Kinetic Parameters /

Proton conducting polymers have received growing interest in recent years since they are considered to be promising separator membranes during application in electrochemical devices. For that purpose, hydrated or anhydrous proton conducting polymeric systems were successfully employed in electrochemical devices at various temperature domains.^{1–4} Especially aromatic heterocyclic structures such as imidazole were considered as promising proton carriers that exhibit moderate intrinsic proton conductivity under anhydrous conditions. When they doped into acidic polyelectrolytes, these protogenic units form complexes through proton exchange reactions and proton transport occurs predominantly over azole units.^{5,6}

The use of azole comprising polymer systems for enzyme immobilization would be interesting where the acidic and basic/amphoteric polymers form polysalts by means of 'ionic crosslinking'⁷ and biological molecules can be physically entrapped during the process. The methodology of enzyme immobilization *via* polymer complexation is easier and more practical compared to chemical and electrochemical systems as well as the quantity of enzyme can be controlled during the polymer complex formation.⁷

Alginic acid, one of the elements of marine algae, is a natural polysaccharide containing linear chains of 1,4'-linked β -D-mannuronic acid and α -L-guluronic acid (Figure 1). Alginic acid is a biodegradable, biocompatible, non-toxic, and low cost polymer, which shows many interesting properties, such as wound healing, ion-exchange ability, and absorption of metal ion. Alginic acid can also be used as an electrical or optical material.^{8,9}

Tyrosinase or polyphenol oxidase (PPO) (E.C. 1.14.18.1) is a copper containing enzyme widely distributed in nature for the formation of natural macromolecule pigment in species.¹⁰

Tyrosinase contains two binuclear copper-containing active sites, presenting at least two distinct binding sites, one of which has an affinity to aromatic compounds (substrate site) and the other an affinity for a metal binding agent (oxygen site).¹¹ In its first reaction (monooxygenase (cresolase) activity), PPO hydroxylates a phenolic substrate at the ortho position into a hydroxyl group. In the second reaction (oxidase (catecholase) activity), the *o*-dihydroxy compound is oxidized to *o*-quinone derivative. *O*-Quinone takes part in a series of chemical and further enzymatic reactions and finally polymerizes to a macromolecule (pigment).¹²

Phenolic compounds are widely used in chemicals and released into the environment by a large number of industries including the ones utilizing resins and plastics, wood preservation, petroleum refining, dyes, chemicals and textiles.¹³ They are a class of polluting chemicals, easily absorbed by animals and human through the skin and mucus membranes. A variety of methods including gas chromatography for the analysis of phenolic compounds can be utilized. In addition to such conventional methods, application of enzymes allows considerable enhancement of specificity decreasing the expenditure for sample pretreatment. Immobilized enzymes are preferred over the native ones owing to their multiple and repetitive use. In addition, the reaction product is not contaminated with the enzyme. Furthermore, the immobilized enzyme has a longer life time and predictable decay rate.

The immobilization of enzymes into complex electrolytes based on Alginic Acid (AA) and poly(1-vinylimidazole), PVI would be interesting where the biopolymer matrix can maintain the activity of enzymes under different conditions. The complexation phenomenon is shown in Figure 2. In this context, we prepared a novel biosensor by immobilizing tyrosinase into a biological polymer network obtained by

¹Department of Chemistry, Middle East Technical University, 06531 Ankara, Turkey

²Department of Chemistry, Fatih University, 34500 Büyükcçekmece, Istanbul, Turkey

*To whom correspondence should be addressed (Tel: +90-212-8663300, Fax: +90-212-8663402, E-mail: bozkurt@fatih.edu.tr).

47

To determine activity of immobilized PPO, different concentrations of catechol were prepared (3.0 mL) and put in water bath at 25 °C. EEPN was immersed in the solution and shaken for 3 min. After removing polymer, 1 mL sulfuric acid and 1 mL acetone were added for a total volume of 6 mL. After mixing, absorbances were measured at 495 nm.

Determination of Kinetic Parameters

For determination of the maximum reaction rate ($V_{\max}$) and the Michaelis-Menten constant (K_m), the activity assay was applied for different substrate (catechol) concentrations. Catechol solutions (8, 10, 30, 50, 80, 100, 300, 500, 800 μM) were prepared in citrate buffer (pH 6.5) and kept in a water bath at 25 °C for 5 min, and the enzyme immobilized network was added to the test tubes and shaken for incubation times of 3, 6, and 9 min.

Determination of Optimum Temperature and pH

Optimum temperature was determined by changing temperature between 10 °C and 80 °C while keeping the substrate concentration constant ($10 K_m$). In addition to temperature, pH optimization is also important for improvement of a biosensor. The AA/PVI polymer network was synthesized with a stoichiometric ratio of $x = 1$. In the present work, pH optimizations were carried out by changing the pH between 2 and 11 at constant temperature (25 °C) and constant substrate concentration ($10 K_m$).

Operational Stabilities and Shelf Life

The operational stability of the enzyme biosensor was determined at optimum activity conditions using the electrodes in 40 activity assays per day. The shelf life of the electrodes was investigated by performing activity measurements within 25 d.

RESULTS AND DISCUSSION

FT-IR Results

The FT-IR spectra of the samples were obtained with a Varian 1000 FT-IR spectrophotometer. FT-IR results of AA and AA/PVI polymer electrolytes were examined. (Figure 4) A strong absorption appears at 1742 cm^{-1} due to C=O stretching. Absorbances 1247 cm^{-1} and 1419 cm^{-1} represent the C-O-H stretching of carboxylic acid groups of the AA. After obtaining polymer network by mixing AA and PVI in different ratios, a new peak was observed at 1604 cm^{-1} , and the intensity of the carbonyl stretching at 1742 cm^{-1} decreased due to protonation of PVI. This occurs by the transfer of the acidic proton of carboxylic acid to the 'free' nitrogen side of PVI to form imidazolium ion. A broad peak centered at 3433 cm^{-1} was observed due to stretching vibration of the isolated non-hydrogen bonded NH group and the broad peak approximately located between 3050 and 3300 cm^{-1} was assigned to the stretching vibration of hydrogen bonded NH groups.¹⁷ Percent protonation was evaluated for the peaks that are located at 1742 cm^{-1} and 1604 cm^{-1} .

Figure 4. FT-IR results of AA and AA/PVI networks at different compositions.

Before immobilization of enzyme, the degree of protonation was evaluated. Presence of two acidic protons on Alginic Acid rise the expectations of highest protonation with $x = 2$. (molar ratio of the monomer repeat units). The percent protonation of the complex electrolyte (AA: PVI) was 96% for 1:1, 83% for 1:2, 89% for 1:3 and 87% for 1:4. Clearly, the highest protonation was with $x = 1$. The reason can be attributed to the structure of AA. AA is a linear copolymer with homopolymeric blocks of (1-4)-linked β -D-mannuronate (M) and its C-5 epimer α -L-guluronate (G) residues, covalently linked together in different sequences or blocks. The monomers can appear in homopolymeric blocks of consecutive G-residues (G-blocks), consecutive M-residues (M-blocks), alternating M and G-residues (MG-blocks) or randomly organized blocks. Hence, intermolecular proton exchange reactions between imidazole and carboxyl groups may take place in the 1:1 ratio of the monomeric units.

Elemental Analysis

Table I shows the feed and the final compositions of the complex polymer electrolytes. Stable gels were collected from the solution and dried under vacuum for EA analysis. While the PVI content in the feed was varied from 50% to 80%, the PVI composition of the polymer electrolyte was changed between 48% and 69%.

Table I. PVI composition in the feed and in the complex electrolytes

Sample (AA: PVI)	% VI in the feed (mol)	% VI in the complex (mol)
1:1	50	48
1:2	66	55
1:3	75	67
1:4	80	69

Table II. Kinetic parameters for free and immobilized tyrosinase

	K_m (mM)	V_{max} ($\mu\text{mol}/\text{min} \cdot \text{mg}$ protein)
Free tyrosinase	4	11.2
AA/PVI/tyrosinase	2.9×10^{-2}	1.5

Kinetic Parameters

For determination of kinetic parameters K_m and V_{max} enzyme biosensor Lineweaver-Burk plots were utilized at constant temperature and pH while changing the substrate concentration.^{18,19} Free PPO has a maximum reaction rate of $11.2 \mu\text{mol}/\text{min} \cdot \text{mg}$ protein and K_m of 4 mM. The Michaelis constant (K_m) of an enzyme is a measure of the affinity of the enzyme to its substrate. The value of K_m for a particular enzyme is defined as the substrate concentration at which half of the enzyme molecules are reacted with substrate. The kinetic parameters K_m and V_{max} of this matrix are 2.9×10^{-2} mM and $1.5 \mu\text{mol}/\text{min} \cdot \text{mg}$ protein ($0.585 \mu\text{mol}/\text{min} \cdot \text{mL}$) respectively (Table II). The tyrosinase enzyme is biocompatible with AA. For that reason; there is a drastic decrease in K_m values compared to that of the free enzyme. The decrease in K_m value means higher affinity. The microenvironment of immobilized enzyme is more biocompatible and it feels more comfortable than that of free tyrosinase. The decrease in V_{max} value compared to the one for free enzyme results from good enzyme substrate interaction.

Temperature Optimization of the Enzyme Biosensor

The temperature optimization process is very important in enzyme immobilization and biosensor construction. Temperature is an important parameter for the enzyme activity in a polymer network. Free tyrosinase showed a maximum at 40°C . In the present matrix, the maximum activity was also observed at 40°C (Figure 5). Free PPO lost its activity completely at 50°C . This behavior resulted due to temperature dependent change in the structure of polymer network.

pH Optimization of the Enzyme Biosensor

In enzymatic assays pH optimization is important. Especially high and low pH media can denature the enzyme. The

Figure 5. Optimum Temperature of Enzyme Biosensor. **Figure 6.** Optimum pH for Enzyme Biosensor.

variation of tyrosinase activity for a pH range between 2 and 11 was investigated for the AA/PVI matrix. The results are presented in Figure 6. The maximum activity of free tyrosinase was observed at pH 5, and immobilized tyrosinase in the AA/PVI polymer matrix showed a maximum activity at pH 9. No activity was observed between 2 and 4. Since in low pH medium the polymer electrolyte can be disintegrated to AA and PVI, enzyme leaks to the solution and gets denatured in the acidic medium; as a result enzyme loses its activity. There is an increase in the enzyme activity from pH 5 to 9. Clearly, the pH of maximum activity was shifted towards the alkaline side compared to that of free tyrosinase. The reason can be attributed to the protonated azole units in AA/PVI network which may protect the enzyme against high concentration of OH^- , in other words this matrix reveals high pH stability for the enzyme in this matrix.

Operational Stability and Shelf Life of Enzyme Biosensor

One of the most important parameter is the operational stability. The operational stability was obtained by running 40 measurements with the same matrix in the same day at constant temperature, concentration and pH. Experiments were done between pH = 6.5 and 9 to check the pH effect on operational stability. At pH = 6.5 (Figure 7) the activity loss of 20% was observed after the fourth use. At pH = 9 where the highest enzymatic activity was found at constant temperature and substrate concentration (Figure 8), again an activity loss of 20% was observed after the fourth use. Both experiments showed that this behavior is related to the stability of tyrosinase.

The activity of AA/PVI/tyrosinase sensor was measured every 5 d for 25 d. This matrix lost 50% of its activity in the

Figure 7. Operational Stability of Enzyme Biosensor (pH = 6.5).

Figure 8. Operational Stability of Enzyme Biosensor (pH = 9).

Figure 9. Shelf Life of Biosensor.

first 2 d, 60% after 5 d and completely lost its activity within the next 20 d (Figure 9). The sharp decrease in enzyme activity can be attributed to diffusional effects and structural changes due to possible complexation of copper with PVI in the enzyme during the storage period. It can be concluded that a biosensor constructed with this matrix on an enzyme shows good stability for a short time.

CONCLUSIONS

Novel complex polymer electrolytes based on AA and PVI were prepared at several stoichiometric ratios with respect to monomer repeating units. The proton exchange reactions between PVI and AA were studied by FT-IR spectroscopy.

Maximum protonation occurs with $x = 1$. The composition of the materials was studied by elemental analysis revealing that PVI content of the polymer electrolytes was ranging from 48% to 69%. Tyrosinase can be successfully immobilized in AA/PVI polymer network. The AA/PVI matrix has high temperature resistance with reasonable operational stability, and shelf life. The decrease in enzyme activity in several days can be attributed to diffusional effects as well as structural changes due to possible complexation reaction of copper in the enzyme with imidazole units.

Received: May 28, 2008

Accepted: September 25, 2008

Published: November 6, 2008

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