Bio-Smart Materials: Kinetics of Immobilized Enzymes in p(HEMA)/p(Pyrrole) Hydrogels in Amperometric Biosensors

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Summary: Various strategies are being pursued to confer the highly specific molecular recognition properties of bioactive molecules to the transducer action of inherently conductive polymers. We have successfully integrated inherently conductive polypyrrole within electrode-supported, UV cross-linked hydroxyethyl methacrylate (HEMA)-based hydrogels. These electroactive composites were used as matrixes for the physical immobilization of several oxidase enzymes to fabricate clinically important biosensors. Measurements were made of the amperometric responses via H_2O_2 oxidation for each biosensor. Apparent Michaelis constants, $K_{m(app)}$, for glucose oxidase immobilized in p(HEMA) membranes and in p(HEMA)/p(Pyrrole) composite membranes were 13.8 and 43.7 mM respectively compared to 33 mM in solution. The inclusion of polypyrrole in the hydrogel network increased the thermal stability of the immobilized enzyme at 60°C by 30% and 40% compared to p(HEMA) membranes and solution phase respectively. The composite also yielded larger I_{max} values (19 $\mu A/cm^{-2}$) for glucose biosensors compared to similar glucose biosensors fabricated without the conducting polymer (15 μA). $K_{m(app)}$ values for cholesterol oxidase immobilized in the same composite films were ca. three orders of magnitude higher than the K_m for the soluble enzyme. The polypyrrole component is shown to reduce diffusive transport but to confer thermal stability to these biosensors.

Introduction

The widespread application of polymers in the area of biosensors is not surprising given their evolution and use in the analytical sciences for the separation and isolation of the components of complex mixtures by physical and/or chemical methods. The use of conducting polymers, particularly polypyrrole[1-3], polyaniline[4,5] and polythiophene[6], as enzyme entrapment matrixes has generated considerable interest because of the potential for integrated signal transduction and chemically stimulated controlled release[7-10]. While these electrically conducting polymers are particularly well suited to the immobilization of enzymes on electrode surfaces, the biocompatibility of these materials is still being explored [11,12]. In fact, one of the main challenges in using enzymes and other biological

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species in amperometric biosensors is the need to design the electrodes to be compatible with the biological component and at the same time to achieve interference shielding, facile diffusive transport and rapid electron transfer at the bio-membrane-electrode interface.

In order to achieve the above objectives, interpenetrating networks of inherently conductive polypyrrole were incorporated into cross-linked p(HEMA)-based hydrogel networks.[13,14] The resulting composite membrane served as the immobilizing matrix for physically entrapped oxidase enzymes and was fabricated as thin membranes on metallic electrodes to form amperometric biosensors. This was done in an attempt to establish the conducting polymer as a macromolecular redox mediator. The use of polypyrrole and modified derivatives to function in this capacity is well documented in the literature.[15-17] This approach seeks to achieve this requirement by capitalizing on the biocompatibility of p(HEMA), the hydration characteristics of p(HEMA) that support rapid diffusion and the potential for enhanced rate of electron transfer provided by polypyrrole. We have recently demonstrated the application of these novel electroactive hydrogel membranes in biosensor fabrication.[18-20] The present investigation is concerned with the effect of the incorporated polypyrrole component on the kinetics of oxidase enzyme biosensors for glucose, cholesterol and galactose.

Oxidase enzymes generally follow well established Michaelis-Menten kinetics, wherein $S + E \leftrightarrow ES \rightarrow E + P$ and where S and E are the molar substrate and enzyme concentrations respectively, ES is the concentration of the enzyme-substrate complex and P is the concentration of product. The formation and dissociation of the ES complex occur at specific rates defined by k_1 and $(k_2 + k_{cat})$ respectively. Its dissociation to product, generally fast, occurs at a rate defined by k_{cat}. Three of the best known equations that may be used to evaluate the kinetic parameters, K_m [the Michaelis-Menten constant, $(k_2 + k_{cat})/k_1$] and V_{max} (the maximum reaction rate) for enzyme catalyzed reactions are:

The Lineweaver-Burk equation

$$1/V = (K_m/V_{max})\,(1/[S]) + 1/V_{max} \qquad (1)$$

The Eadie-Hofstee equation

$$V/[S] = V_{max}/K_m - V/K_m \qquad (2)$$

The Hanes equation

$$[S]/V = [S]/V_{max} + K_m/V_{max} \quad (3)$$

where V is the reaction rate at a given molar substrate concentration, [S]. However, the most commonly used equation and the one adopted in this study is the Lineweaver-Burk plot. This approach has the advantage that the variables V and [S] are plotted on separate axes. Further, by assuming that the output anodic current, I, is proportional to V, then V and V_{max} in the Lineweaver-Burk equation (eq. 1 above) could be substituted by I and I_{max} respectively. This allows a convenient conversion of amperomtric biosensor response to enzyme kinetic data.

Experimental

The amperometric enzyme biosensors were fabricated by a simple, two-step protocol.[19] Briefly, enzyme was dissolved into the monomer formulations (HEMA, pyrrole, tetraethyleneglycol diacrylate [TEGDA] and photoinitiator) and the deaerated mixture applied to the working area (0.25 cm^2) of a platinum electrode. The hydrogel network was UV polymerized (2.3 watts/cm^2, 366 nm, Spectroline Model 330844) for 40 minutes followed by electrochemical polymerization (+ 0.85 V *vs.* Ag/AgCl for 100 seconds) of the pyrrole component in phosphate buffered KCl solution (0.1M NaH_2PO_4 containing 0.1M KCl, pH 7.0) that was saturated with pyrrole monomer (ca. 0.4M). The bioactive membranes were further extensively oxidized at +0.70 V in pH 7.0 phosphate buffered potassium chloride until the background current decayed to a steady low (< 1μA) current density of 4 μA/cm^2.

The immobilized enzyme electrodes (glucose oxidase [GOx], cholesterol oxidase [ChOx] or galactose oxidase [GalOx]) were each made the working electrode in a three-electrode electrochemical cell that also comprised a coiled platinum counter electrode and a miniature Ag/AgCl, 3M Cl^- (RE803, ABTECH Scientific) reference electrode. All three electrodes were placed in the cell containing phosphate buffer (0.1M, pH 7.0) and connected to a potentiostat (Bioanalytical Systems – BAS 100B Electrochemical Analyzer, West Lafayette, Indiana). A constant potential of +0.70 V was applied to the three-electrode cell with stirring (450 r.p.m.). Aliquots (1mL) of enzyme substrate (glucose, cholesterol or galactose) were then injected into the phosphate buffer (3 mL)

and the steady state current produced as a result of oxidation of the enzymatically generated H_2O_2 was recorded (eq. 4)

$$\text{Substrate} + O_2 \xrightarrow{\text{Oxidase}} \text{Product} + H_2O_2 \xrightarrow{-e} H_2O + 1/2O_2 \qquad (4)$$

Results and Discussion

The apparent Michaelis-Menten constant, $K_{m(app)}$, gives an indication of the kinetics of the enzyme-substrate reaction. Generally, immobilized enzymes confined to pores or other low dimensional surfaces display an associated decrease in the rate of enzymatic reaction and a corresponding increase in their Michaelis-Menten constant.[21] This increase in the Michaelis-Menten constant relative to solution-borne enzyme is generally attributed to diffusional limitations associated with the formation and dissociation of the enzyme–substrate complex. As illustrated in Fig. 1, where $K_{m(app)}$ is represented by the substrate concentration at half maximal velocity, $V_{max}/2$, a high $K_{m(app)}$ value implies a lower initial enzyme catalysis rate for the same specified substrae concentration. A high value for $K_{m(app)}$ also implies that the rate of dissociation of the enzyme-substrate complex (ES) back to its reactants is faster than the rate of its formation, thus making the formation of ES complex the rate limiting step.

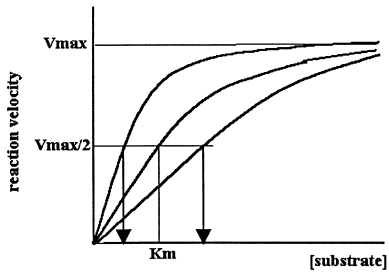

Figure 1. Schematic illustration of the dependence of K_m on initial reaction rate.

The apparent Michaelis-Menten constants, $K_{m(app)}$, for the three immobilized enzyme biosensors utilized in this study were obtained from the slopes and intercepts of Lineweaver-Burk plots (Fig. 2) and compared to those of the solution-borne enzyme system (Table 1). To examine the effect, if any, of the polypyrrole component on enzyme kinetic parameters, a fourth electrode consisting of GOx entrapped in a p(HEMA) matrix without any conducting polymer was also fabricated and used as a control. For the Pt | p(HEMA)/GOx biosensor, a $K_{m(app)}$ value of 13.8 mM compares very favourably to the value of 13.33 mM previously reported for GOx immobilized within p(HEMA) membranes[22] and 13.0 mM for Pt | Nafion/GOx electrodes.[23] This calculated K_m value is much lower than the literature value of 33 mM for the solution-borne enzyme[24] (0.1M NaH_2PO_4, pH 7.0) in air saturated solution.

The lowered $K_{m(app)}$ value for GOx in the Pt | p(HEMA)/GOx biosensor relative to solution-borne GOx possibly indicates that the ES complex is more stable. This manifests as an increase in initial reaction rate at a particular substrate conentration compared to the solution-borne GOx, thus making the saturation of the enzyme active site a fast process. Since $K_{m(app)}$ is also the substrate concentration at which the sensor reaction kinetics begin to change from being first order to zero order, $I_{max}/2$ is the current corresponding to the concentration for the kinetic order switch. The calculated maximum current density of 61.4 $\mu A\ cm^{-2}$ is significantly higher than I_{max} values reported for polypyrrole-GOx sensors by Foulds and Lowe[25] (40 $\mu A\ cm^{-2}$) and Fortier and Belanger [23] (21 $\mu A\ cm^{-2}$). The combined higher I_{max} and lower $K_{m(app)}$ values suggest that the microenvironment of the p(HEMA) favors the formation of the enzyme–substrate complex and overall increased turnover rate. The p(HEMA) microenvironment may be offering increased conformational stablity for the ES complex compared to water.

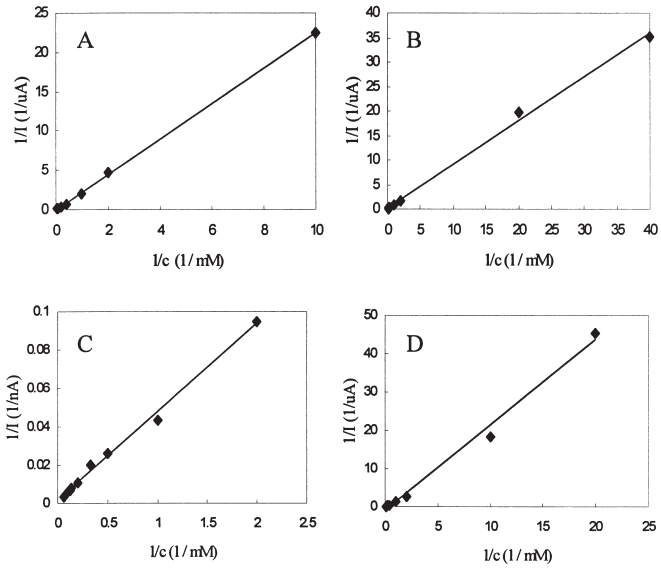

Figure 2. Lineweaver-Burk plots for the various immobilized enzyme biosensors:

A = p(HEMA)/p(pyrrole)/GOx , B = p(HEMA)/GOx

C = p(HEMA)/p(pyrrole)/ChOx , D = p(HEMA)/p(pyrrole)/GalOx

I = steady state current, c = concentration of analyte

Table 1. Apparent Michaelis-Menten constants for p(HEMA)/p(pyrrole) biosensors.

Electrode type	$K'_{m(app)}$ / mM	I_{max} / μA	K_m soluble enzyme (mM)
P(HEMA)/GOx	13.8	15.34	33
p(HEMA)/p(pyrrole)/GOx	43.7	19.42	33
p(HEMA)/p(pyrrole)/ChOx	23.2	0.42	0.025
p(HEMA)/p(pyrrole)/GalOx	2.6	1.18	38

Incorporating polypyrrole into the immobilization membrane matrix resulted in an increase in the $K_{m(app)}$ value from 13.8 mM to 43.7 mM. The increase in $K_{m(app)}$ from 13.8 mM (for the p(HEMA)/GOx system) likely reflects a slowing down in initial reaction rate due to increased or more pronounced diffusional limitations brought about by using the two-polymer composite membrane for the immobilization of GOx. This value is somewhat higher than that of the solution-borne enzyme and not significantly larger than calculated $K_{m(app)}$ values for GOx immobilized within redox hydrogels[26] (37.5 mM), polypyrrole-GOx sensors[25,23](31 mM), and GOx immobilized within polyethyleneoxide layers[27] (36 mM). Compared to the solution-borne GOx however, there may be no such pronounced diffusional constraints. This is gleamed from the linearity of the Lineweaver-Burk plot (Fig. 1A) for the Pt | p(HEMA)/PPy/GOx electrode. The presence of the polypyrrole component also resulted in a slight increase in the I_{max} value (from 61.4 to 77.7 $\mu A\ cm^{-2}$) for our electrodes. This compares favourably with a mean value of 74 $\mu A\ cm^{-2}$ for Au | glutaraldehyde/GOx amperometric electrodes of Kuwabata et al.[28], but which was significantly higher than corresponding I_{max} values for polypyrrole/GOx (25 $\mu A\ cm^{-2}$) and redox-hydrogel/GOx electrodes of Pravda et al.[29] (11.5 $\mu A\ cm^{-2}$).

The calculated $K_{m(app)}$ value of 23.2 mM for the Pt | p(HEMA)/PPy/ChOx biosensor is in good agreement with the reported $K_{m(app)}$ value for a screen-printed amperometric cholesterol biosensor[30] (25 mM). This $K_{m(app)}$, however, is 2 and 4 orders of magnitude larger than that reported for other amperometric cholesterol biosensors[31,32] (0.53 mM and 7.0×10^{-3} mM in air-saturated 0.1M citrate, pH 5.9). The immobilized cholesterol oxidase in the present biosensor exhibits a much higher $K_{m(app)}$ value than that of the solution-borne enzyme[32] (0.025 mM), in accordance with the generally observed increase in K_m on going from aqueous media to crosslinked enzyme or enzyme-immobilized polymer membrane electrodes under diffusion control. This result supports the findings from previous diffusion studies[20], that the present amperometric cholesterol biosensor operated under diffusion limited conditions.

The Lineweaver-Burk plot (Fig. 61D) for the Pt | p(HEMA)/PPy/GalOx biosensor was a straight line consistent with an enzyme system conforming to Michaelis-Menten kinetics. No deviations arising from enzyme inhibition or restricted transfer of substrate within the polymer were detected, the enzymatic reaction ([ES] $\rightarrow$ [E] + [P]) was always the rate-

determining step. The apparent Michaelis-Menten constant and maximum current estimated from the Lineweaver-Burk equation were 2.6 mM and 1.2 μA, respectively. This $K_{m(app)}$ value is an order of magnitude lower than that for soluble galactose oxidase (38 mM).[33]

Thermodeactivation Kinetics of Entrapped Glucose Oxidase

Comparative studies of thermodeactivation of the solution-borne and both forms of the membrane-imobilized GOx [-p(HEMA) and p(HEMA)/p(pyrrole)] at temperatures of 50°C and 60°C are presented in Figs. 2 and 3 respectively. Both immobilized enzyme biosensors were incubated at the mentioned temperatures and their residual enzyme activities were periodically evaluated by measuring the amperometric current generated in the presence of 20 mM glucose.

At 50°C, there was no significant difference in the residual enzymatic activity among the solution-borne and membrane-immobilized enzymes over the time period studied (Fig. 2). All systems showed a loss of ca. 25-30%. This is in sharp contrast to the observations made by Fortier and Belanger[23] for their polypyrrole/GOx membrane system for which, after 150 minutes of incubation at 50°C, they recorded a 25% loss of activity for the solution-borne enzyme (similar to our observations) but a loss of only 10 % for the PPy-immobilized enzyme. Clearly, in their case the pure polypyrrole membrane conferred considerable thermal stability to the enzyme. In further contrast, a Nafion/GOx membrane system[34] displayed a similar 25-30% loss of enzymatic activity as the present study of p(HEMA)/GOx and p(HEMA)/PPy/GOx membranes.

At 60°C however, there is considerable difference in the residual enzyme activities among the three membrane systems of the pesent study (Fig. 3). The soluble enzyme loses 70% of its enzyme activity in less than 30 minutes compared to a loss of a mere 30% and 40% for the p(HEMA)/Ppy/GOx and p(HEMA)/GOx immobilized-enzyme membrane systems respectively. After 150 minutes at 60°C, the p(HEMA)/PPy/GOx membrane system retains as much as 50% of its original enzyme activity, while the activities of the p(HEMA)/GOx matrix and solution-borne GOx retain only 30% and 20% respectively. The polypyrrole component of the p(HEMA)/PPy/GOx membrane system

appears to contribute the thermal stability observed by Fortier and Belanger[23] even when it is but a component of a composite hydrogel matrix. On the other hand, the Nafion matrix[34] did not protect the enzyme from thermal deactivation at 60°C.

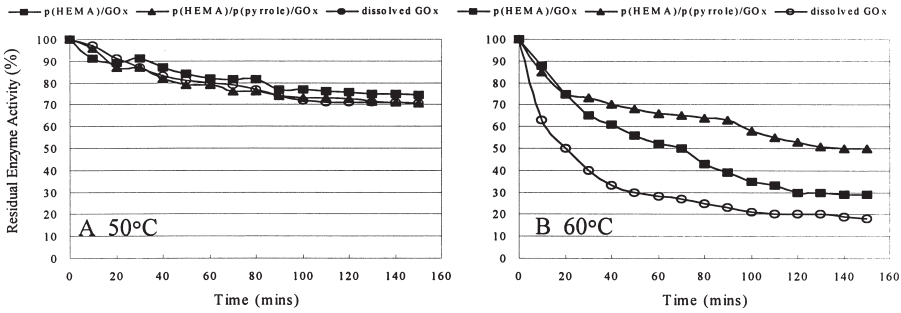

Figure 3. Thermodeactivation kinetics of soluble GOx and entrapped GOx in p(HEMA) and p(HEMA)/p(pyrrole) films at (A) 50°C and (B) 60°C.

Conclusions

From these studies it can be concluded that the introduction of polypyrrole within p(HEMA) networks seems to increase the diffusional limitations to immobilized enzyme catalyzed reactions. This is most probably due to the inclusion of a second diffusional polymeric barrier to substrate and product. On the other hand, the inclusion of the conducting polymer component increases the thermal operational stability of these biosensors at elevated temperatures compared to the pure p(HEMA) membranes. The small increase in I_{max} values observed with the p(HEMA)/PPy composite may also suggest some slight enhancement of amperometric current, produced as a result of oxidation of enzymatically generated H_2O_2, by the inclusion of the conducting polypyrrole component.

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