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Kinetics of glucose oxidase immobilized in p(HEMA)-hydrogel microspheres in a packed-bed bioreactor

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Abstract

Glucose oxidase (GO_x) was immobilized via both physical entrapment and covalent linkage to crosslinked poly(hydroxyethyl methacrylate-*co*-dimethylaminoethyl methacrylate, (p(HEMA-DMEMA)) hydrogel microspheres (20–150 μm in diameter) that were synthesized by inverse suspension polymerization. Loading capacities of 7–8 mg GO_x per gram of hydrogel were achieved with physical entrapment, compared to <1.8 mg GO_x per gram of gel with the covalent technique. The microspheres containing physically entrapped enzyme were packed into bioreactors and the kinetics of the immobilized enzyme investigated under various flow conditions. Flow rate dependence of $K_{m(app)}$ and C_{max}, when extrapolated to near diffusion-free conditions, resulted in values of 13.2 mM and 2.7×10^{-3} mol min^{-1}, respectively, for the immobilized enzyme. Studies of pH-dependence of $K_{m(app)}$ and C_{max} suggest that the imidazolium and sulphydryl groups may be involved at the active site of the immobilized GO_x. Studies of the temperature dependence of C and C_{max} confirm lower activation energies for the oxidation of glucose at temperatures >35 °C, suggesting the influence of diffusional limitations within the hydrogel.

Keywords: p(HEMA); Hydrogel microspheres; Glucose oxidase; Packed-bed bioreactor; Enzyme kinetics

1. Introduction

The specificity of enzymes and their ability to catalyze reactions make them attractive for applications in biochemical, biomedical, industrial and bioanalytical fields. One major advantage of this specificity is that the yields are free of side products. However, the recovery and re-usability of free enzymes as catalysts are quite limited and this has led to the development of a wide variety of immobilization techniques. Immobilization also offers some other operational advantages over free enzymes, such as choice of batch or continuous processes, rapid termination of reactions, controlled product formation, ease of removal from the reaction mixture and adaptability to various engineering designs [1–3]. Among the available methods for enzyme immobilization, matrix entrapment seems to have advantages over the others, such as simplicity of the immobilization process and the fact that the enzyme is retained in its native state. With this technique, no reactive group of any amino acid residue of the protein is used to form specific covalent bonds with the matrix in order to achieve enzyme immobilization [4].

Currently, there is considerable interest in the preparation, properties and the use of solid-supported enzyme systems due to their potential applications in

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such areas as automated analyses [5–7], clinical investigations [8], syntheses in organic solvents [9,10] and as the recognition layer in biotransducers as part of biosensors [11,12]. In addition, these solid-supported enzyme systems can serve as model systems for the behavior of enzymes in vivo [13]. Polyacrylamide gels have been widely employed as the matrix material of choice for enzyme immobilizations by entrapment. However, the mechanical strength of these gels is low and there are frequent reports in the literature of enzyme leakage from these gels. The use of poly(hydroxyethyl methacrylate) (pHEMA) as a matrix for immobilization of enzymes was first reported in the early 1980s [14,15]. Since then, reports using p(HEMA) as the matrix for the entrapment and immobilization of glucose oxidase (GO_x) have all been aimed at producing glucose-responsive membranes [16,17], without much detailed consideration and discussion of the influence of the matrix on the kinetics of the immobilized enzyme.

In this manuscript, we present an insight into the role of this pH-responsive hydrogel matrix in influencing the mechanism of action of the immobilized enzyme. The present study is concerned with the preparation and characterization of pHEMA microspheres as an immobilization matrix for GO_x. The microspheres were rendered pH responsive by co-polymerization of dimethylaminoethyl methacrylate (DMEMA) within the p(HEMA) hydrogel network. The pH and temperature dependence of the immobilized enzyme kinetics were studied under flow conditions in a packed bed bioreactor. The results were analyzed in terms of the kinetic equations developed by Lilly et al. [18] for plug flow systems in packed-bed bioreactors.

2. Material and methods

2.1. Chemicals

GO_x (E.C. 1.1.3.4. from *Aspergillus niger*, 152,000 units g^{-1} solid), sorbitan sesquioleate and light white mineral oil were obtained from Sigma (St. Louis, MO). The monomer, hydroxyethyl methacrylate (HEMA) was obtained from Polysciences Inc. (Warrington, PA), while the dimethylaminoethyl methacylate monomer (DMEMA), crosslinker tetraethyleneglycol diacrylate (TEGDA) and inhibitor remover columns (cat no. 30,631-2) were purchased from Aldrich (Milwaukee, WI). These columns were used to remove the monomethyl ether hydroquinone inhibitor that is used as a preservative to prevent the methacrylate monomers from polymerization during storage. The HEMA monomer was vacuum distilled (1.3 mmHg, 80 °C) before use. All other reagents used were of the Analytical Reagent Grade and were obtained from BDH (Poole, UK).

3. Methods

3.1. General method for the preparation of hydrogel microspheres by inverse suspension polymerization

Inverse suspension polymerization gives rise to almost perfect spherical particles which when packed into column bioreactors allow work to be conducted at high flow rates with minimum pressure loss. The detailed procedure for the synthesis of hydrogel miocrospheres is described in previous work [19]. Briefly, appropriate volumes of HEMA (H), DMEMA (D) and TEGDA (T) that were all previously de-inhibited were dissolved in an aliquot of the buffer (phosphate/citrate, 25 mM each, pH 6.8) so as to produce a monomer:aqueous ratio of 1:1 (the discontinuous phase). The continuous phase consisted of sorbitan sesquioleate (2.50 ml) dissolved in paraffin oil (50.0 ml). Both the continuous and discontinuous phases were purged with nitrogen for 30 min. The purged monomer solution, to which the redox initiator ammonium peroxydisulphate (APS, 0.140 g dissolved in 0.50 ml buffer) was added, was then poured into the polymerization flask containing the continuous phase. This mixture was maintained under a nitrogen atmosphere at 15 °C and at a stirring rate of 1500 rpm using a paddle stirrer. The polymerization process was initiated by injecting *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED, 0.50 ml) into the reaction mixture and the polymerization allowed to proceed for 10 min. The polydisperse hydrogel microspheres produced were centrifuged (1200 × *g*, 1 min) and then washed with acetone followed by phosphate/citrate buffer (working buffer). Finally, the microspheres were sequentially wet-sieved with mesh sizes of 150 and 20 μm. These dimensions corresponded to the upper

and lower diameters of the microspheres that were used in the bioreactor, respectively. Microspheres were then stored in the working buffer at 4 °C.

3.2. Immobilization of enzymes to hydrogel microspheres

Two methods, physical entrapment and covalent coupling, were used to immobilize GO_x to the various microspheres synthesized.

3.2.1. Physical entrapment

In this method, varying quantities of GO_x were dissolved in buffer (0.5 ml) and mixed with the required volumes of the monomer to produce a final monomer:aqueous ratio of 1:1. These were then polymerized as described above. The resulting beads were stored in phosphate/citrate buffer (25 mM each, pH 6.8) in the presence of the antibacterial agent, thimerosal (0.1% w/v), at 4 °C.

3.2.2. Covalent immobilization

GO_x was covalently linked to the preformed hydrogel microspheres by covalent coupling of the enzyme to the available hydroxyl groups of p(HEMA). Hydrogel microspheres (2.0 g) were washed with the working buffer followed by sequential washing with 10, 20, 40 and finally 60% acetone:water mixtures. Activation of the hydrogel microspheres was achieved using cyanogen bromide and triethylamine (TEA) as follows. A cyanogen bromide (CNBr) solution (0.4 ml, 1 M in acetone) was added dropwise to the microspheres followed by the addition of a TEA solution (0.4 ml, 1.5 M in 60% acetone). The entire reaction mixture was then poured into 5.0 ml of an ice-cold washing medium (10 °C) of acetone:0.1 M HCl (1:1, v/v). The CNBr activated hydrogel microspheres were then washed sequentially with cold 60, 40, 20 and 10% acetone:water mixtures and finally with Na_2CO_3 (coupling buffer, 0.1 M, pH 8.5, 4 °C).

The GO_x (1.5–30 mg), dissolved in 2.0 ml of the coupling buffer, was then added to the activated microspheres and allowed to tumble for 16 h at 18 °C. At the end of this time, the microspheres were washed with the Na_2CO_3 buffer followed by this same buffer containing 1 M NaCl, pH 8.5. Ethanolamine solution (2.0 ml, 1 M in Na_2CO_3, pH 9.0) was then added to react with any uncoupled activated –OH groups of the hydrogel and the mixture tumbled for a further 2 h at 18 °C. The hydrogel microspheres were then washed with Na_2CO_3 buffer containing NaCl as described above, then with the working buffer (NaH_2PO_4, 25 mM, pH 6.8) until the wash was free of any GO_x activity. The enzyme-coupled microspheres were finally stored in the working buffer containing thimerosal (0.1% w/v) at 4 °C.

3.3. Characterization of the GO_x-hydrogel microspheres using packed-bed bioreactors

The amount of enzyme immobilized to the hydrogel microspheres was determined from differences between the concentration of enzyme (protein) offered for coupling and the amount of protein present in the washings after coupling using the Lowry method [20]. Six different quantities of GO_x (1.5–30.0 mg) were utilized in the preparation of batches of enzyme-linked hydrogel microspheres (2.0 g) via both physical and chemical methods of immobilization. The enzymatic activities of the various batches of these microspheres were determined at 35 °C using glucose (5.0 mM in 0.1 M NaH_2PO_4 solution, pH 6.8) as substrate. The quantity of H_2O_2 liberated as a result of oxidation of glucose by immobilized GO_x was determined spectrophotometrically using peroxidase (0.5 mg ml^{-1}) and the chromogenic system of dimethylaminobenzoic acid (DMAB, 12 mM) and 4-aminoantipyrine (4-AAP, 0.4 mM) [21].

Plug-flow bioreactors were prepared by packing small lengths of Tygon® tubing (25 mm × 3 mm i.d.) with the immobilized GO_x hydrogel microspheres. Porex spheres were used to plug both ends of the bioreactor to prevent loss of the microspheres. The GO_x bioreactor was studied under conditions of varying substrate concentrations (10^{-4} to 10^{-2} M), flow rates (0.3–1.4 ml min^{-1}), pH's (4.0–8.5) and temperatures (10–50 °C). Under each set of conditions investigated, the steady state current (μA) produced at an amperometric detector (Model LC4C Electrochemical Analyzer, Bioanalytical Systems, West Lafayette, IN) as a result of the oxidation of H_2O_2 (Eq. (1)), generated by the oxidation of the substrate by the immobilized enzyme, was monitored at +700 mV versus Ag/AgCl.

$$H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-, \quad E = +700\,\text{mV (versus Ag/AgCl)} \tag{1}$$

4. Results and discussion

The conversion of glucose to gluconolactone and H_2O_2 by GO_x is schematically illustrated in Fig. 1 [22]. The cyclic ketone that is first produced is spontaneously hydrolyzed to gluconic acid.

4.1. Activity-loading capacity relationships

Both the quantity and activity of the GO_x immobilized within the hydrogel microspheres were determined spectrophotometrically and the values compared with those of the enzyme introduced into the monomer formulation prior to microsphere polymerization. The latter quantity is regarded as enzyme offered for immobilization. The activity-loading relationship for each composition of hydrogel synthesized (containing physically and chemically immobilized GO_x) is displayed in Fig. 2A–F. On the left axis is plotted the normalized activity of the enzyme immobilized within the microspheres. On the right axis is plotted the quantity of enzyme bound as determined by the Lowry method. For hydrogel microspheres containing physically entrapped GO_x and with compositions 100:0:03, 90:10:03 and 80:20:03 mol% H:D:T (Fig. 2A–C, respectively), a general trend can be observed across all three compositions. The activity profiles (number of micromoles of H_2O_2 produced per gram of gel at pH 6.8 and $T = 35\,^{\circ}C$) were all found to increase, rising to a maximum corresponding to 15–20 mg GO_x offered per gram of gel. The activity then leveled off, where after increased amounts of enzyme offered to the hydrogel support resulted in no further increase in enzyme activity. As expected, the mass of enzyme immobilized shows similar profiles as the activity. The quantity of GO_x immobilized per gram of hydrogel increased rapidly across all three compositions (A, B and C) and then leveled off as the enzyme concentration offered to the gel increased. For physically entrapped GO_x, the optimum loading/activity was achieved when about 20 mg of enzyme per gram of gel was offered for coupling. At this enzyme concentration, a loading capacity of between 7 and 8 mg GO_x per gram of gel was obtained. The resulting specific activities were between 100 and 110 $\mu mol\,min^{-1}\,mg^{-1}$ enzyme (U) per gram of gel.

With respect to the covalently immobilized systems having hydrogel compositions of 100:0:03, 90:10:03 and 80:20:03 mol% H:D:T (Fig. 2D–F, respectively), there was again a similar rapid increase in quantity of enzyme immobilized per gram of gel for all compositions. The activity profiles also exhibited similar trends, increasing and then leveling off as the enzyme concentration offered increased. However, unlike the physically immobilized enzyme, optimum loading/activity for the covalently immobilized systems was observed to vary with the monomer composition of the hydrogel. For the p(HEMA) microspheres containing no DMEMA (100:0:03 mol%—H:D:T), (Fig. 2D), the activity showed a rapid initial increase,

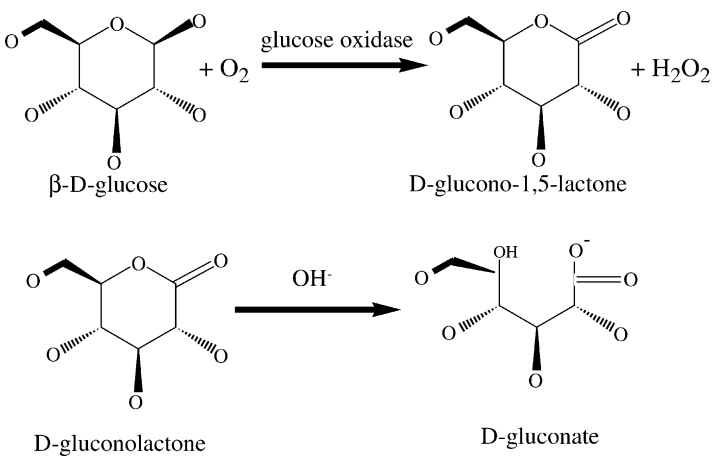

Fig. 1. Enzymatic reaction between glucose and glucose oxidase.

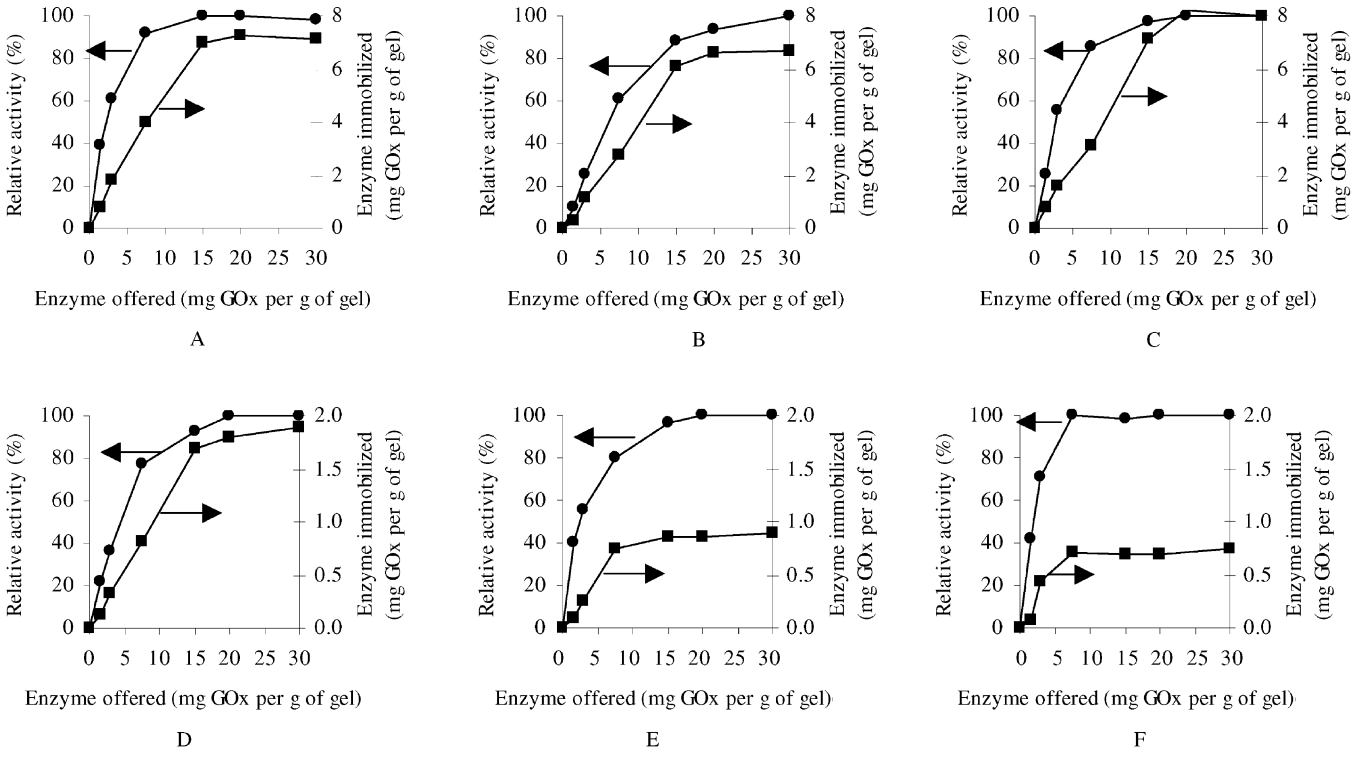

Fig. 2. Activity/loading relationships for glucose oxidase immobilized onto hydrogel microspheres via physical entrapment (A, B and C) and covalent methods (D, E and F). The two series A, B and C, and D, E and F both correspond to microsphere compositions of 100:0:03, 90:10:03 and 80:20:03 mol% H:D:T, respectively.

reaching a maximum at about 20 mg GO_x offered per gram of gel. At this enzyme concentration, a loading capacity of about 1.8 mg GO_x per gram of gel was obtained resulting in a specific activity of 70 U per gram of gel. As the percentage of DMEMA in the microsphere increased from 0 to 20% and the corresponding percentage of HEMA decreased from 100 to 80%, the optimum enzyme loading was found to decrease from 1.8 to 0.7 mg GO_x per gram of gel. Similarly, the specific activity of the bound enzyme was found to decrease from 70 to 31 U per gram of gel.

To explain these observations, one has to examine the method employed to immobilize the GO_x enzyme. Immobilization via physical entrapment results in a highly crosslinked polymer network that forms in the presence of the enzyme. Consequently, the enzyme is contained within the interstitial spaces of the polymeric network. As all three monomer formulations contained the same quantity of cross-linking agent (3 mol%), the resulting polymer network will likely possess similar crosslink densities [23]. Each hydrogel network that was formed should, thus, be capable of accommodating/entrapping similar amounts of the enzyme within the interstitial spaces of its matrix. Consistent with the foregoing, all three hydrogel formulations were found to exhibit loading capacities of 7–8 mg GO_x per gram of hydrogel. Similar results were reported by Arica and Hasirci [24] who observed an increase in the activity of GO_x entrapped within p(HEMA) membranes upon increasing the enzyme offered from 2 mg GO_x per gram of gel up to 20 mg GO_x per gram of gel. Sheppard and Lesho [25] reported an optimum loading of 166 U GO_x per milligram of gel during the synthesis of a photopatterned conductimetric glucose biosensor utilizing a H:D ratio of 90:10 mol%, while Pishko et al. [26] utilized a loading of 2 mg GO_x per milligram of redox hydrogel for the construction of amperometric glucose microelectrodes.

In the case of GO_x covalently immobilized via endogenous hydroxyl groups of the preformed hydrogel microspheres, as the concentration of the HEMA monomer in the gel decreases (i.e. as the DMEMA content increases) there would be fewer available hydroxyl groups for activation by CNBr/TEA and for subsequent coupling to the enzyme. Thus, for the hydrogel formulation of composition 80:20:03 mol% (H:D:T), which contained the lowest concentration of HEMA, and hence, the fewest available hydroxyl groups for activation, the polymer is expected to become saturated at a lower enzyme concentration than that for the other compositions. The results in Fig. 2D–F support this conclusion. For comparison, Hall et al. [27] reported up to 9.5 mg GO_x covalently immobilized per gram of methyl methacrylate copolymers. Because a greater loading capacity was achieved via physical entrapment of GO_x into the hydrogel microspheres, this method of immobilization was selected for the following flow rate/concentration studies using hydrogel microspheres of composition 90:10:03 mol% (H:D:T).

4.2. Flow rate/concentration studies

The influence of flow rate on the kinetic parameters, K_m and V_{max}, of physically entrapped GO_x-hydrogel microspheres have been previously described [19]. Based on the Lilly et al. [18] model for packed-bed bioreactors (Eq. (2)),

$$f[A_0] = \frac{C}{Q} + K_{m(app)} \ln(1 - f) \quad (2)$$

a plot of $f[A_0]$ against $\ln(1 - f)$ (where f is the fraction of substrate converted during passage over the reactor and $[A_0]$ the initial substrate concentration), resulted in a straight line with a slope, and hence, $K_{m(app)}$, equal to 13.2 mM for the physically entrapped GO_x. This compares closely to that reported for free GO_x ($K_m = 10$ mM) [28]. The above model also allows a calculation of V_{max}, or the maximum reaction capacity, C_{max}, of the bioreactor. This parameter was found to also vary with flow rate [19], indicating, as did the $K_{m(app)}$ values, that over the flow rates examined, the immobilized GO_x bioreactor's kinetics were influenced by diffusional effects. A plot of $1/C$ against $1/Q$ (Fig. 3), the inverse flow rate, yielded a C_{max} value of 2.7×10^{-3} mol min^{-1}.

4.3. pH studies

Detailed kinetic studies of the influence of pH on an enzyme-catalyzed reaction can reveal important information on the nature of the amino acid residues that influence the active site of the enzyme. In this study, glucose solutions of varying concentrations (10^{-4} to 10^{-2} M) prepared over a pH range of 5.6–8.3

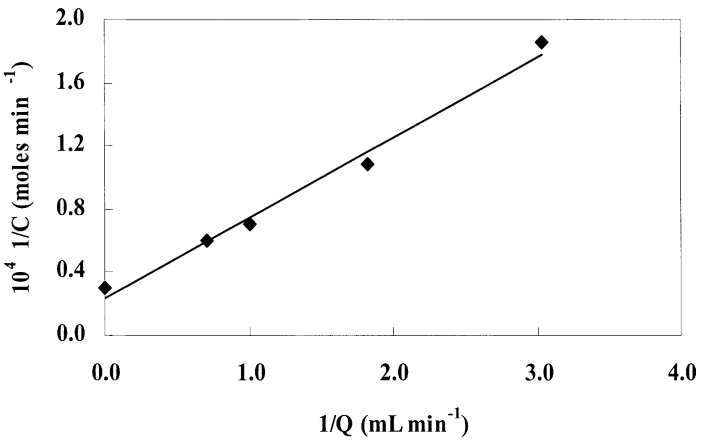

Fig. 3. Plot of 1/C (reactor capacity) vs. 1/Q (flow rate) for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase. Conditions were: hydrogel composition = 90:10:03 mol% (HEMA:DMEMA:TEGDA); buffer = citrate/phosphate (25 mM in each component); pH = 6.5; temp = 35 °C.

were allowed to flow over the GO_x-hydrogel bioreactor at various flow rates (0.1–1.4 ml min^{-1}). The amperometric responses resulting from the oxidation of enzymatically generated H_2O_2 under these varying conditions were monitored at each pH. Primary plots of $f[A_0]$ against $\ln(1-f)$ were used to generate K_m and C_{max} values. Secondary plots of $1/K_m$ and $1/C$ against $1/Q$ in turn generated K_m and C_{max} values. These latter values should be essentially free of diffusional effects. These diffusion free values enable a more meaningful comparison between the native, solution-borne enzyme and the hydrogel-immobilized enzyme system and, thus, reveal any influence of the hydrogel on the performance of the enzyme.

The pK values of the ionizable groups at the active site of the enzyme can be obtained from $\log C_{max}$ and $\log C_{max}/K'_{m(app)}$ versus pH plots, according to a procedure adapted from that detailed by Segel [29] for systems which have a ΔpK of greater than 2pH units i.e. well separated pK's. From the pH optimum and the pH's at which the curve intersects a horizontal line drawn at half the maximum, values for pK_a and pK_b may be determined (Figs. 4 and 5). Fig. 4 shows the pH dependence for C_{max}/K_m with a pH optimum of around 7.0, a pK_a of 5.8 and a pK_b of 8.1. The influence of pH on C_{max} reveals a pK'_a of 5.7, a pK'_b of 8.1 and similarly, an optimal pH of between 6.8 and 7.0 (Fig. 5). The variation of $K'_{m(app)}$ for the immobilized GO_x with pH is displayed in Fig. 6, with an observed minimum value for $K'_{m(app)}$ at the pH optimum. Between pH 6.5 and 8.0, the $K'_{m(app)}$ remains relatively constant, with a mean value of 9.0 mM. Since the pK of most side chains of the amino acid residues differ appreciably, such a pK assignment can, in many cases, lead to tentative identification of an amino acid group. The two pK values observed possibly correspond to the imidazolium group (p$K_a \sim 5.8$) of a histidine amino acid residue and to a sulphydryl group (pK_b between 8.0 and 9.0) of a cysteine residue. The approximate values expected for the intrinsic pK's of the imidazolium and sulphydryl groups are 6 and 8–10, respectively [30]. Swoboda and Massey [22] have previously confirmed that histidine residues were involved in the catalytic oxidation of glucose by GO_x. Hence, by immobilizing the GO_x within the hydrogel microspheres the functional group chemistry of the active site of the enzyme was not altered.

4.4. Temperature studies

The reaction of glucose within the bioreactor containing GO_x physically immobilized within hydrogel microspheres, presents the situation of coupled mass transport and enzyme reaction wherein transport occurs within the bulk aqueous medium as well as within the gel. This may be summarized in the mass balance

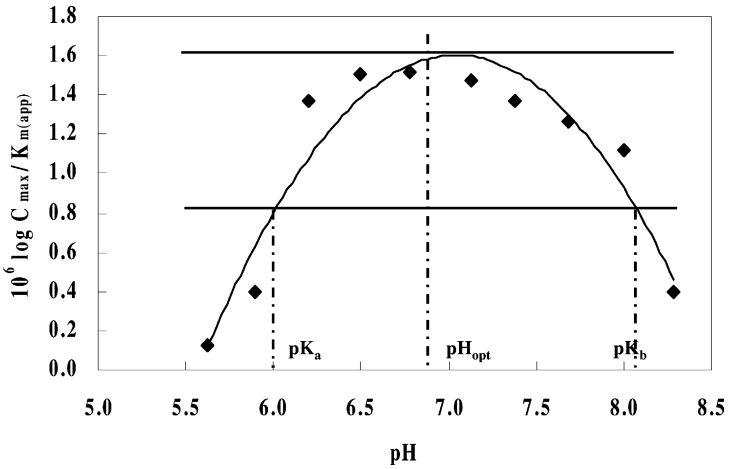

Fig. 4. Relationship between $\log C_{max}/K_{m(app)}$ and pH for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at 35 °C. C_{max} and $K_{m(app)}$ values were obtained from plots of $f[A_0]$ vs. $-\ln(1-f)$ and from Fig. 3 for the oxidation of glucose by the packed-bed bioreactor at various flow rates.

Eqs. (3) and (4) [31].

$$\frac{\partial C_P^i}{\partial t} = D_P^i \frac{\partial^2 C_P^i}{\partial x^2} + R_P^i \tag{3}$$

$$R_P^i = \nu_{max} \frac{C_P^i}{C_P^i + K_m} \tag{4}$$

where D_P is the diffusion coefficient for the product of glucose oxidation (H_2O_2), R_P the rate of product appearance and K_m the Michaelis constant of the familiar Michaelis–Menten kinetic expression. Temperature is capable of influencing both diffusivity and reactivity and, therefore, insight into the role of the hydrogel in influencing the mechanism of action of the enzyme may be inferred from the temperature dependence of the reaction rate and the shape of the appropriate Arrhenius plots. Flow influences the apparent diffusivity as it lends a convective contribution

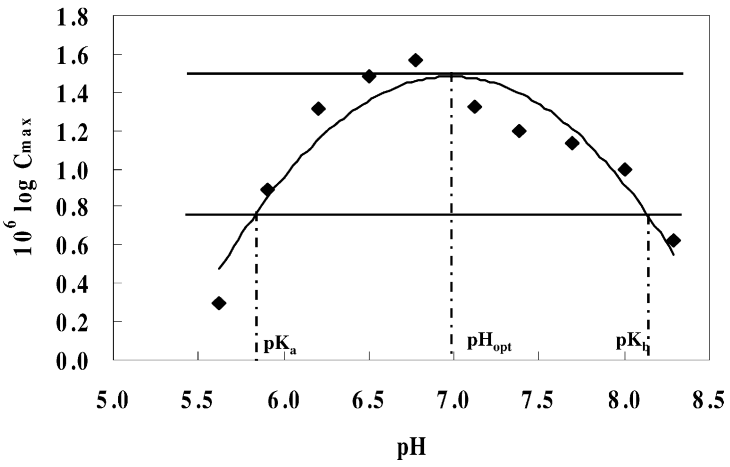

Fig. 5. Relationship between $\log C_{max}$ and pH for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at 35 °C. C_{max} values were obtained at various pH's from plots of $f[A_0]$ vs. $-\ln(1-f)$ and from Fig. 3 for the oxidation of glucose by the packed-bed bioreactor at various flow rates.

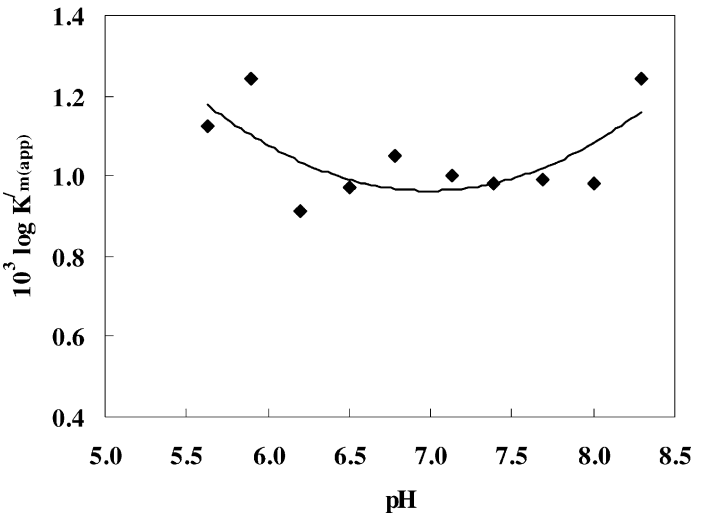

Fig. 6. Relationship between $\log K'_{m(app)}$ and pH for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at 35 °C. $K_{m(app)}$ values were obtained at various pH's from plots of $f[A_0]$ vs. $-\ln(1-f)$ for the oxidation of glucose by the packed-bed bioreactor at various flow rates.

to the transport of substrate to the immobilized enzyme. In the present studies, thermostated solutions of glucose (in 0.1 M NaH_2PO_4, pH 6.8) were pumped through bioreactors containing GO_x physically immobilized within hydrogel microspheres. Varying flow rates (0.1–1.2 ml min^{-1}) and varying substrate concentrations (0.1–20 mM) were studied over the temperature range 10–40 °C. The activation energies, detailed in Table 1, were determined from plots of the logarithm of the oxidation current of enzymatically generated H_2O_2 under these conditions versus $1/T$ in accordance with the Arrhenius equation, $k = Ae^{-E_A/RT}$. A plot of the logarithm of the effective rate constant ($\log C_{max}$ or $\log I_{max}$) versus $1/T$ should, therefore, yield a straight line with slope equal to E_A.

The logarithm of the maximum reaction capacities, C_{max}, (obtained from the y-intercept of plots similar to Fig. 3) was plotted versus $1/T$. This plot showed marked curvature (Fig. 8) indicating that C_{max} is a complex function and is likely affected by several velocity constants [32]. Instead of these plots therefore, values of C at three different flow rates corresponding to low, intermediate and high flow rate regimes, were examined and were used to determine activation

Table 1
The apparent activation energies for the oxidation of glucose by a covalently immobilized GO_x-hydrogel bioreactor

S. no.	[Glucose] (mM)	Flow rate Q (ml min^{-1})	Temperature (°C)	Activation energy E (kJ mol^{-1})	Correlation coefficient, r^2
1	0.1	0.1	10–25	29.10	0.999
2	0.1	0.1	30–40	10.38	0.995
3	0.1	1.2	10–20	33.26	0.995
4	0.1	1.2	25–45	11.09	0.990
5	20	0.1	10–25	26.33	0.992
6	20	0.1	30–40	3.75	0.972
7	20	1.2	10–20	12.47	0.996
8	20	1.2	25–40	4.90	0.995
9	0.1–20	0.1	10–40	10.72	0.985
10	0.1–20	0.4	20–40	13.77	0.999
11	0.1–20	1.2	20–40	6.05	0.996

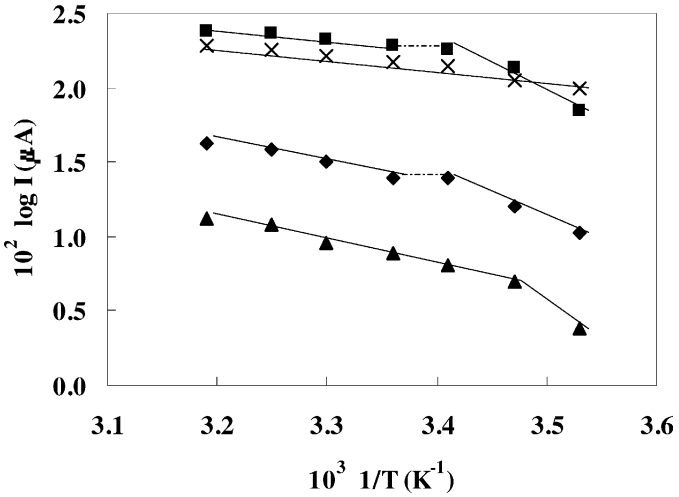

Fig. 7. Plots of log I of H_2O_2 oxidation vs. $1/T$ at various flow rates and substrate concentrations for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at pH 6.8. Low [substrate], low flow rate (◆); high [substrate], high flow rate (■); low [substrate], high flow rate (▲); high [substrate], low flow rate (×).

energies (entries 9–11 in Table 1). Such plots are shown in Fig. 9 and the activation energies obtained from their slopes are presented in Table 1 and compared to activation energies obtained from the slopes of product-generated currents versus $1/T$ (entries 1–8, Table 1 and Fig. 7). Overall there appears to be little or no effect of flow rate on the activation energies obtained.

The present study has found that at the higher temperature range (25–40 °C; entries 2, 4, 6, 8 of

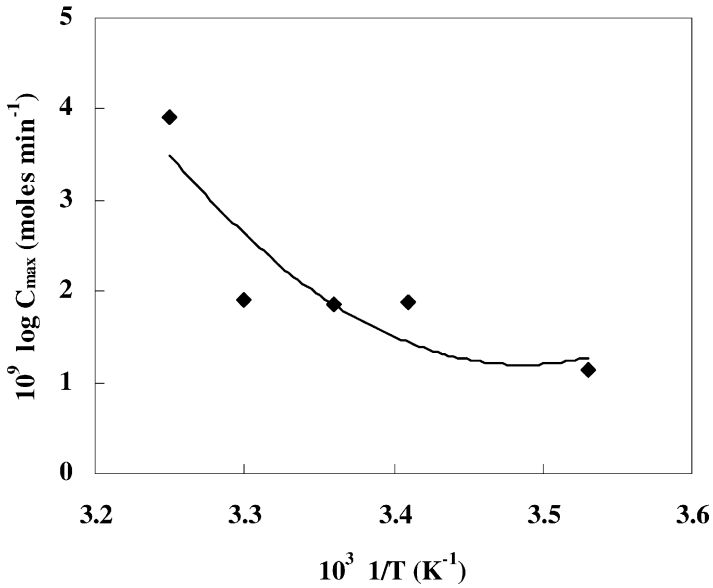

Fig. 8. Relationship between log C_{max} and $1/T$ for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at pH 6.8. C_{max} values were obtained from plots similar to those used for Fig. 3.

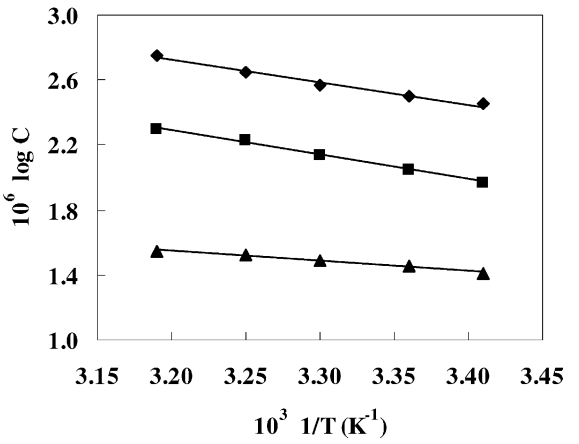

Fig. 9. Plots of $\log C$ vs. $1/T$ at low (0.1 ml min^{-1}, ◆), intermediate (0.4 ml min^{-1}, ■) and high (1.2 ml min^{-1}, ▲) flow rates for the packed-bed bioreactor containing hydrogel microspheres with physically immobilized glucose oxidase at pH 6.8.

Table 1), regardless of the substrate concentration, the activation energies obtained are all quite low, between 3.75 and 11.09 kJ mol^{-1}. In contrast, at the lower temperatures (10–25 °C; entries 1, 3, 5 of Table 1) and for the substrate concentrations used, activation energies were considerably higher, in the range 26.33–33.26 kJ mol^{-1}. The effects of temperature changes on the kinetics of immobilized enzymes have been examined by several researchers [32,33]. Within the low substrate concentration region, such that $K_{m(app)} \gg [S]$, the following approximation to the Michaelis–Menten equation should apply, provided that there are no diffusional limitations:

$$v = \frac{k_c[E]_m[S]}{K_{m(app)}} \quad (5)$$

where k_c is the diffusion-free catalytic constant modified by conformational and environmental effects; $[E]_m$ the concentration of immobilized enzyme; $[S]$ the substrate concentration in bulk solution and $K_{m(app)}$ the apparent Michaelis constant, influenced by conformational, environmental and diffusional effects. When diffusional effects are strongly influencing the kinetics of an enzyme-catalyzed system, it has been proposed [34] that the activation energy determined is more accurately represented by Eq. (6):

$$\tfrac{1}{2}(E_c + \Delta E_m + E_D) \quad (6)$$

where E_D is the activation energy for the diffusion step and ΔE_m and E_c, the activation energies for binding and the breakdown of the enzyme-substrate complex, respectively.

Equating the average of this high temperature region, but low range of activation energies (7.2 kJ mol^{-1}) to $1/2(E_c + \Delta E_m + E_D)$ and taking $E_c = E_a$ for solution-borne GO_x ($\sim$14 kJ mol^{-1}) [35] results in a value of 0.2 kJ mol^{-1} for $E_m + E_D$. This would make the sum of E_D and $E_m \cong 0$ and suggest that the reaction is entirely under enzymatic control. When the average of the low temperature region but high range of activation energies (25.3 kJ mol^{-1}) is similarly compared to $1/2(E_c + \Delta E_m + E_D)$, the result is a value of 36.6 kJ mol^{-1} for $(E_m + E_D)$. This suggests the possibility that the apparent kinetics may be influenced by diffusion.

Narinesingh et al. [36] reported similar results for the evaluation of a β-galactosidase covalently immobilized onto a fractogel^{TM} support. They concluded, however, that at high temperature the lower activation energy corresponded to diffusion limiting conditions and that at the lower temperatures, the higher activation energy corresponded to kinetically controlled conditions. Ngo and Laidler [33] have studied the behavior of acetylcholine esterase entrapped in polyacrylamide slices with the enzyme attached to the inner surface of nylon tubing. These authors reported similar observations for the activation energies as a function of temperature. They demonstrated that under high substrate—low temperature conditions the calculated activation energy for the gel slices could be attributed to the breakdown step of the enzyme—substrate complex to form product. They showed that in the higher temperature ranges, where diffusion of substrate into the gel would contribute significantly to the apparent kinetics, lower activation energies were obtained.

5. Conclusions

Hydrogel microspheres of crosslinked p(HEMA-*co*-DMEMA) were used for the physical and covalent immobilization of GO_x. Of these two techniques, matrix entrapment (physical immobilization) afforded the higher loading capacity and higher specific activity of the immobilized enzyme. Microspheres containing physically entrapped enzyme were packed into

a plug-flow bioreactor and a detailed investigation into the enzyme kinetics under conditions of flow was performed. Flow rate/concentration studies revealed a $K_{m(app)}$ value of 13.2 mM, that is similar to solution-borne GO_x (10 mM). This indicates that the substrate has almost solution-like access to the immobilized enzyme within the microsphere and that the hydrogel presented no significant diffusional barrier to enzyme-substrate reaction. The pH studies indicated that two functional groups, imidazolium and sulphydryl, of histidine and cysteine amino acid residues, respectively, may be involved at the enzyme's active site for the oxidation of glucose. This suggests that the hydrogel microsphere matrix influences the mechanism of action of GO_x. Temperature studies revealed two distinct regions; a high (>35 °C) temperature region represented by lower activation energy and a low temperature region represented by high activation energy. Thus, by selecting operating conditions of sufficiently high flow rates and an operating temperature within the range of 20–35 °C (to minimize diffusional limitations), we have successfully designed a flow injection (FI) analysis system incorporating the present bioreactor for the amperometric quantitation of glucose in human serum [19].

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References

[1] O.R. Zarborsky, in: R.C. Weast (Ed.), Immobilized Enzymes, CRC Press, Ohio, 1973, pp. 1–3.
[2] R. Axen, in: M. Salmona, C. Soronio, S. Garattini (Eds.), Insolubilized Enzymes, Raven, New York, 1974, p. 9.
[3] E. Katzir-Katchalski, in: H.H. Weetall, G.P. Royer (Eds.), Enzyme Engineering, Plenum Press, New York, 1980, p. 12.
[4] G.G. Guilbault, Handbook of Enzymatic Analysis, Marcel Dekker, New York, 1977.
[5] L.B. Wingard Jr., E. Katchalski-Katzir, L. Golstein, Analytical Applications of Immobilized Enzymes and Cells in Applied Biochemistry and Bioengineering, Vol. 3, Academic Press, New York, 1981.
[6] P.W. Carr, L.D. Bowers, Immobilized Enzymes in Analytical and Clinical Chemistry, Wiley/Interscience, New York, 1980.
[7] G.G. Guilbault, Analytical Uses of Immobilized Enzymes, Marcel Dekker, New York, 1984.
[8] T.M.S. Chang, Immobilized Enzymes, Antigens, Antibodies and Peptides, Marcel dekker, New York, 1975.
[9] Z. Yang, A.J. Mesiano, S. Venkatasubramanian, S.H. Gross, J.M. Harris, A.J. Russell, J. Am. Chem. Soc. 117 (1995) 4843.
[10] B. Cambou, A.M. Klibanov, J. Am. Chem. Soc. 106 (1984) 2687.
[11] S. Brahim, D. Narinesingh, A. Guiseppi-Elie, Biosens. Bioelectron. 17 (2001) 53.
[12] A. Guiseppi-Elie, G.G. Wallace, T. Matsue, in: T. Skotheim, R. Elsenbaumer, J.R. Reynolds (Eds.), Handbook of Conductive Polymers, 2nd Edition, Marcel Dekker, New York, 1995.
[13] K. Mosbach, B. Mattiasson, in: B.L. Horecker, E.R. Stadtman (Eds.), Current Topics in Cellular Regulation, Vol. 14, Academic Press, New York, 1978.
[14] M. Yoshida, I. Kaetsu, J. Appl. Polym. Sci. 26 (1981) 687.
[15] M. Kumakura, I. Kaetsu, J. Appl. Biochem. 4 (1982) 441.
[16] M.Y. Arica, V. Hasirci, Biomaterials 14 (1993) 809.
[17] C.L. Bell, N.A. Peppas, Adv. Polym. Sci. 122 (1994) 125.
[18] M.D. Lilly, W.E. Hornby, E.M. Crook, Biochem. J. 100 (1966) 718.
[19] A. Guiseppi-Elie, N.F. Sheppard Jr., S. Brahim, D. Narinesingh, Biotechnol. Bioeng. 75 (2001) 475.
[20] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.
[21] U. Mankasingh, D. Narinesingh, T.T. Ngo, Anal. Lett. 33 (2000) 2407.
[22] B.E.P. Swoboda, V. Massey, J. Bio. Chem. 240 (1965) 2209.
[23] J. Kost, T.A. Horbett, B.D. Ratner, M. Singh, J. Biomed. Mater. Res. 19 (1985) 1117.
[24] Y. Arica, V.N. Hasirci, Biomaterials 8 (1987) 489.
[25] N.F. Sheppard Jr., M.J. Lesho, Mat. Res. Soc. Symp. Proc. 331 (1994) 193.
[26] M.V. Pishko, A.C. Michael, A. Heller, Anal. Chem. 63 (1991) 2268.
[27] C.E. Hall, D. Datta, E.A.H. Hall, Anal. Chim. Acta 323 (1996) 87.
[28] F.R. Shu, G.S. Wilson, Anal. Chem. 48 (1976) 1679.
[29] I.H. Segel, Enzyme Kinetics, Wiley/Interscience, New York, 1975, p. 894.
[30] D.E. Koshland Jr., in: F.F. Nord (Ed.), Advances in Enzymology, Vol. 22, Interscience, New York, 1960, p. 57.
[31] N.F. Sheppard Jr., M.J. Lesho, A. Guiseppi-Elie, Biosens. Bioelectron. 11 (1996) 967.
[32] K.J. Laidler, P.S. Bunting, in: D.L. Purich (Ed.), Methods in Enzymology, Vol. 64, Academic Press, New York, 1980, p. 227.
[33] T.T. Ngo, K.J. Laidler, Biochim. Biophys. Acta 525 (1978) 93.
[34] P.V. Sundaram, A. Weedale, K.J. Laidler, Can. J. Chem. 48 (1970) 1498.
[35] S.B. Adeloju, G.G. Wallace, Analyst 121 (1996) 699.
[36] D. Narinesingh, V.A. Stoute, G. Davis, T.T. Ngo, J. Mol. Cat. 45 (1988) 285.