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Short communication

Electrochemical enhancement of glucose oxidase kinetics: Gluconic acid production with anion exchange membrane reactor

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1. Introduction

Enzyme-catalysed reactions provide a means to perform many industrial processes because they enhance chemical reactions specifically and avoid the formation of by-products and the use of toxic organic solvents. Current enzyme applications range from laundry detergent supplements [1, 2] to the destruction of nerve gas agents [3, 4]. Although enzyme specificity is attractive, there are also significant disadvantages to enzymatic catalysis. One of the principal disadvantages being relatively short lifetimes, ranging from a few hours to several days [5, 6]. However, literature has shown that by immobilizing an enzyme on a support matrix, the lifetime of the enzyme is increased since the rigidity of the support matrix helps prevent unfolding [3, 4, 7]. Microfiltration membranes are often a good choice for enzyme attachment. The high surface area in the pores allows for enzyme attachment [8, 9] and reduction of mass transfer limitations.

Ion exchange (IE) membranes, on the other hand, are seldom thought of as good candidates for enzyme attachment. Their dense structure prohibits pore attachment, allowing only surface attachment, which typically yields significantly lower attachment density. For instance, if a membrane has 2 $m^2\ g^{-1}$ internal surface area (standard microfiltration membrane), a 100 μm thickness, and a density of approximately 1 g cm^{-3}, the relative amount of enzyme that could be attached with a monolayer in a pore compared to an IE membrane surface (one side) is about 20. However, given this limitation, IE immobilized-enzymes may be advantageous in certain cases. These cases include enzymes systems where charged ion products could be selectively transported away, thus possibly increasing the reaction efficiency (product-inhibited cases) as well as reducing separation steps.

For these reasons, we have chosen to study a model enzyme system on IE membranes. The enzyme, glucose oxidase (GOd), converts glucose to gluconic acid and hydrogen peroxide. If GOd is immobilized on an anion-exchange membrane (AIE), a current across the membrane can selectively remove gluconic acid from the glucose/enzyme solution. Because this reaction is product-inhibited [10], the removal of the gluconic acid should increase the reaction rate. Thus, the purpose of this study was (i) to achieve the attachment of GOd on an AIE membrane, (ii) to test the stability of enzyme on the AIE membrane compared to that of the solution phase, and (iii) to compare the overall effectiveness of the enzyme immobilized AIE membranes to other bioreactor configurations.

2. Experimental details

Commercially available AIE membranes from Tokayama were used for enzyme immobilization experiments. These membranes included AFX, AMX, and AM-1, which are all strongly charged, dense, anion exchange electrodialysis membranes [11]. Membrane preparation was conducted using gluteraldehyde-amine chemistry as outlined in Table 1 footnotes. This treatment consists of a 0.5% sodium alginate soak for 30 min, 1% PEI (polyethylene imine) soak for 30 min, and a 2% gluteraldehyde soak for 30 min. The membrane surface is then placed in 20–100 mg L^{-1} GOd and 2–10 mg L^{-1} catalase for 180 min. The testing was conducted in 1–10 g L^{-1} glucose solutions. The results of these experiments are measured using HPLC, an enzymatic-visible method to determine GOd consumed, and ionic conductivity measurements. The membrane testing takes place in an electrically assisted H-cell. The cell consists of a 150 mL feed side volume and a 100 mL diluent side volume with a platinum coated titanium oxide cathode and a 'dimension stable electrode' (chlorine resistance) for the anode. The membrane area was 4 cm^2.

Glucose and gluconic acid concentrations were determined with an HPLC column using a modified method of one found in literature [12]. The gluconic acid concentration was linear from 0.1–20 g L^{-1} with a determined error of <4%. The glucose was linear from 0.5–50 g L^{-1} with a determined error of <5%. The enzymatic assay for GOd included additions of sodium acetate buffer, *o*-dianisidine solution, and peroxidase enzyme solution [13]. We found GOd concentration to be linear in the range 10–100 mg L^{-1} with an error of 11%.

Table 1. Effect of membrane processing steps on electrical resistance and enzyme attachment
Resistance determined in 0.5 N gluconic acid

Membrane type	Initial resistance /Ω cm^2	Resistance* /Ω cm^2	Resistance† /Ω cm^2	Resistance‡ /Ω cm^2	Resistance§ /Ω cm^2	Enzyme attachment /mg cm^{-2}
AMX	58.1 ± 0.9	62.8 ± 0.6	63.6 ± 1.2	63.2 ± 1.1	63.7 ± 0.4	4.1×10^{-4}
AM1	49.4 ± 1.0	53.6 ± 1.5	53.1 ± 0.8	53.9 ± 1.8	53.5 ± 0.8	6.1×10^{-4}
AFX	44.5 ± 0.7	BVL	BVL	BVL	45.6 ± 0.5	6.0×10^{-5}

* Functionalization consists of 0.5% sodium alginate soak for 30 min.
† 1% PEI soak for 30 min.
‡ 2% gluteraldehyde soak for 30 min.
§ 20 mg L^{-1} enzyme soak for 2 h.
BVL Binding very low, so detailed resistance study not performed.

The ionic conductivity was measured (0.5 N gluconic acid) using a Keithley Instruments LCZ meter equipped with four-point measuring probes with <5% experimental error for three different membrane samples.

3. Results and discussion

Enzyme attachment requires a balance between attachment density, stability and reactivity. Because IE membranes are dense, we used a surface treatment technique. Although overall attachment density is expected to be low [14], we felt that the advantages of simultaneous separation would make up for this drawback. Separation requires that functional AIE membranes still maintain their ionic conductivity properties after enzyme attachment. For this reason, we performed a study looking at the change in the ionic conductivity of the membrane as a result of surface modification and subsequent enzyme attachment. As shown in Table 1, each of the immobilized-enzyme membranes showed only slight changes in electric resistance upon enzyme immobilization (~3–10%). It is interesting to note that increase in membrane resistance, AFX<AM–1<AMX, is similar to that seen for NaCl [11]. It should also be noted that catalase attachment, which is present in the mixture at a ratio of 1:10 that of GOd, was not measured and, therefore, changes in enzyme attachment reflect those of GOd only.

Studies performed with AMX and AM–1 membranes (also shown in Table 1) show that most of the change in electric resistance of these membranes takes place due to the sodium alginate soak. This is because the sodium alginate is negatively charged and serves as a neutralizing agent to surface charge on the positively charged AIE membranes. However, we have used this step in the functionalization because it makes it more likely that we can get a PEI coating, a source of primary amines, which is necessary for gluteraldehyde cross-linking of the enzymes [14].

Finally, Table 1 shows an attached enzyme concentration ranging from 0.6×10^{-4} to 6.1×10^{-4} mg cm^{-2}. Given that the molecular weight of this enzyme is about 63 000 Da (dalton) [15], this gives a maximum molar attachment of about 1×10^{-7} mmol cm^{-2}. This activity can be compared to some other enzyme results found in literature. For instance, with BSA (67 500 Da), Koguma et al. [16] found enzyme attachment to range from 9×10^{-8} to 5×10^{-7} mmol cm^{-2} which is consistent with the attachment that we found. Further, Bourdillon et al. [17] found that a single layer of GOd on the membrane had an attachment density of about 2.65×10^{-9} mmol of active enzyme cm^{-2}. Our results differed from those of Bourdillon et al. because their technique measured active enzyme while we measured total enzyme attached. Given that we found the enzyme to be 32% active on the surface, and the amount of active enzyme attached is about 3.2×10^{-8} mmol cm^{-2}, we surmise that the enzyme is probably forming multiple layers on the membrane surface. The loss of activity is attributed to nonspecific, random attachment [8].

Another factor key in the design of bioreactors is enzyme stability. Since the lifetime of polymeric membranes is significantly longer than an enzyme lifetime, it is important to increase enzyme stability in order to realize the full potential of the bioreactor. Figure 1 shows normalized enzyme activity against time for immobilized GOd on AMX and AM–1 along with solution-phase GOd. As shown, the AMX membrane exhibits an enzyme stability of >80% of initial activity for six days and >50% activity for 15 days with the AM–1 membrane losing activity at a much more rapid rate. The most probable cause for this difference is the

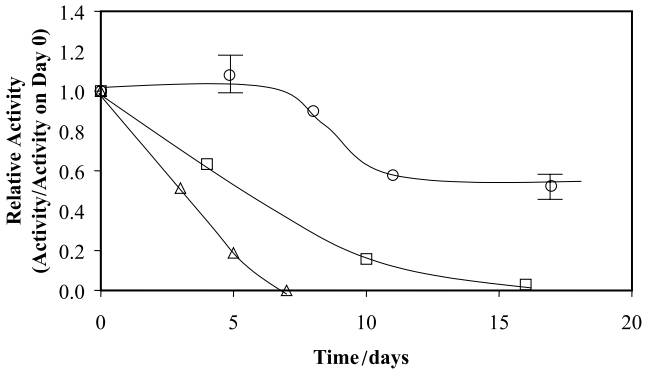

Fig. 1. Stability of membrane attached and solution phase glucose oxidase enzymes. Key: (□) AM–1 immobilized; (○) AMX immobilized; (△) solution. Temperature for storage 4 °C and testing 23 °C.

difference in surface charge and attachment density. Since the AMX membrane has less surface charge than the AM–1 membrane, enzyme interactions (deactivation, unfolding) could be less [18]. Further, because of this charge, localized aggregation of the enzyme could take place resulting in the increased loss of activity. However, both of the enzyme immobilized membranes still showed an increase in stability over homogeneous solution as expected. Given the significant increase in stability of the AMX over the AM–1 membrane, the AMX membrane was used for the remainder of the enzyme studies.

We used the best results of enzyme attachment to study the effects of simultaneous separation on the rate of reaction. Given the enzyme system in question, we determined three likely processing scenarios (Figure 2). The only difference in these three reactors (for the purpose of this study) is the amount of product being transported. In case A, there is no product transport away and thus product inhibition is expected to be high. In case B, the amount of gluconic acid transported to the surface of the membrane is controlled by the diffusion rate as opposed to the reaction rate as in case C. Because the reaction rate is much higher than the rate of diffusion to the surface, we expect the current utilization for the electrically-assisted membrane would be less than the membrane immobilized electrically-assisted case. The results of experiments with these three reactors can be seen in Figure 3. As shown, the amount of gluconic acid formed increases in the order C > B > A (note in the membrane functionalized-electrically assisted case, only active enzyme was considered). At 150 minutes, we have shown a 4-fold increase in product formation with membrane immobilized (C) as opposed to the homogeneous solution (A). Both of the membrane separation techniques (C and B) were expected to have improved performance over the solution phase bioreactor. However, at equal current density, the functionalized membrane should have better transport rates across the membrane. This is because the concentration of gluconate relative to hydroxide (competing ions) at the membrane surface will be higher due to the surface reaction (immobilized enzyme).

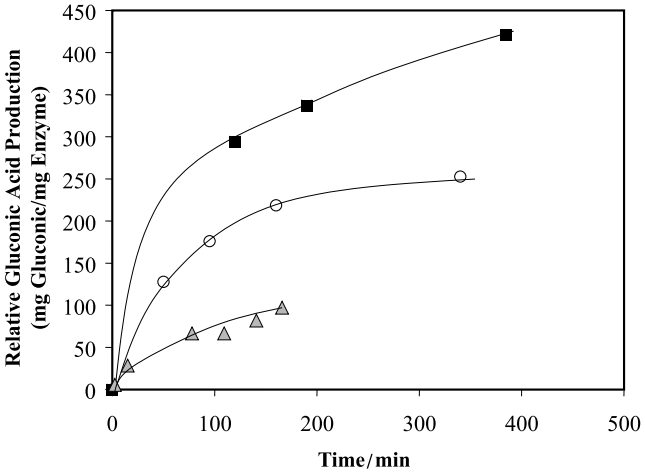

Fig. 3. Rate of gluconic acid production with different bioreactor configurations. Parameters: 1 g L^{-1} glucose feed; current density 2 mA cm^{-2}; pH 4.5–5.5. Key: (■) membrane immobilized, electrically assisted; (○) solution phase, electrically assisted; (▲) solution phase, diffusion controlled.

Considering the observed increase in gluconic acid transport, we would expect the immobilized enzyme to have a higher degree of separation at identical currents due to the decrease in the transport boundary layer with enzyme attachment. Thus, Figure 4 shows the relative product (gluconic acid) concentration of both feed and product sides with enzyme-attached and free-solution electrically assisted enzymes. As shown, with the immobilized enzyme (C), gluconic acid in the product compared to the feed is 9:1 at 400 min compared to about 1.5:1 for the free solution enzyme (B). This is the expected result since the enzyme at the surface should have much better current utilization for separation (decrease in diffusion boundary layer). Further, we have shown that, at these rates of current utilization, there was very little glucose transport.

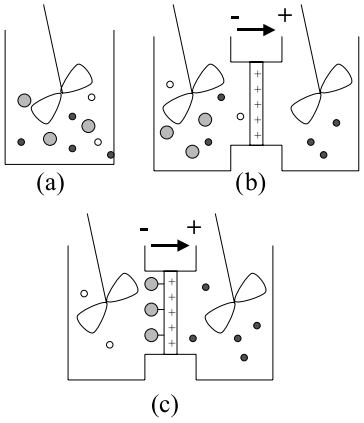

Fig. 2. Possible bioreactor configurations including (a) solution phase, (b) electrically assisted, and (c) membrane functionalized-electrically assisted. Key: (◍) enzyme; (●) product; (○) substrate.

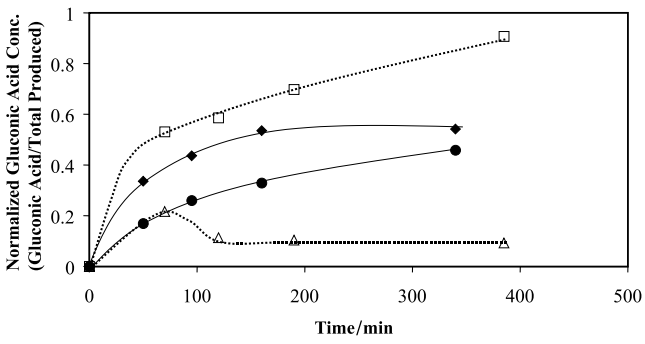

Fig. 4. Relative gluconic acid in feed and permeate with membrane immobilized and solution phase glucose oxidase bioreactors. Parameters: 1 g L^{-1} glucose feed; current density 2 mA cm^{-2}; pH 4.5–5.5. Key: (●) solution phase feed; (♦) solution phase permeate; (△) immobilized feed; (□) immobilized permeate.

4. Conclusions

The following points can now be made:

(i) Glucose oxidase has been immobilized on anion exchange membranes with a <10% loss in membrane conductivity.
(ii) The membrane immobilized enzyme had a >50% activity for as long as 15 days.
(iii) A four-fold increase in the rate of product formation is possible using membrane immobilized enzymes with simultaneous separation as opposed to solution phase enzymatic catalysis.

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References

1. K. Horikoshi, *Microbiol. Molecular Biol. Rev*. **63** (1999) 735.
2. C. Hemachander and R. Puvanakrishnan, *Process Biochem*. **35** (2000) 809.
3. K.E. LeJeune, A.J. Mesiano, S.B. Bower, J.K. Grimsley, J.R. Wild and A.J. Russell, *Biotech. Bioeng*. **54** (1997) 105.
4. K.E. LeJeune, J.R. Wild and A.J. Russell, *Nature* **395** (1998) 27.
5. J. Torrent, J.P. Connelly, M.G. Coll, M. Ribo, R. Lang and M. Vilanova, *Biochem*. **38** (1999) 15952.
6. A.I. Ruiz, A.J. Malave, C. Felby and K. Griebenow, *Biotech. Lett*. **22** (2000) 229.
7. M.C. Millot, B. Sebille and C. Mangin, *J. Chromatog. A* **776** (1997) 37.
8. S. Ganapathi-Desai, D.A. Butterfield and D. Bhattacharyya, *Biotech. Prog*. **14** (1998) 865.
9. M.M. El-Masry, A. De Maio, S. Di Martino, N. Diano, U. Bencivenga, S. Rossi, V. Grano, P. Canciglia, M. Portaccio, F.S. Gaeta and D.G. Mita, *J. Molecular Catal. B: Enzymatic* **9** (2000) 219.
10. F.R. Duke, M. Weibel, D.S. Page, V.G. Bulgrin and J. Luthy, *J. Am. Chem. Soc*. **91** (1969) 3904.
11. Tokyama Product Literature (1995).
12. O.K. Choi, C.G. Kim, Y.D. Kim, H. Kim and J.S. Jo, *J. Chromatog. A* **745** (1996) 249.
13. H.U. Bergmeyer, K. Gawehn and M. Grassl, *in* H.U. Bergmeyer (Ed.), 'Methods of Enzymatic Analysis' (Academic Press, New York, 2nd edn, 1974) pp. 457–458.
14. M.S. Mohy Eldin, M. Portaccio, N. Diano, S. Rossi, U. Bencivenga, A. D'Uva, P. Canciglia, F.S. Gaeta and D.G. Mita, *J. Molecular Catal. B*: *Enzymatic* **7** (1999) 251.
15. G. Wolfhart, S. Witt, J. Hendle, D. Schomburg, H.M. Kalisz and H.J. Hecht, *Acta Crystallogr*. **55** (1999) 969.
16. I. Koguma, K. Sugita, K. Saito and T. Sugo, *Biotech. Prog*. **16** (2000) 456.
17. C. Bourdillon, C. Demaille, J. Moiroux and J.M. Saveant, *J. Phys. Chem. B*. **103** (1999) 8532.
18. P.S. Kushwaha and P.C. Mishra, *Int. J. Quantum Chem*. **76** (2000) 700.