Letters

Enzyme-Linked Biosensors: Michaelis–Menten Kinetics Need Not Apply

Enzyme-linked biosensors have become fairly easy to prepare and use. Two recent articles published in this *Journal* featured student fabrication and use of a tyrosinase-linked phenol sensor *(1)* and a glucose oxidase-linked glucose sensor *(2)*. We have implemented the phenol biosensor project in our upper-division biochemistry laboratory, with good results. Students enjoy this project because they make their own biosensor and then use it to assay concentrations of a biologically relevant solute.

Biosensors are typically used by adding aliquots sequentially to a stirred cell and measuring the electrochemical response. Similar to all solute-specific electrodes, biosensors have a finite range of linear response: Below the linear range, electrode response is small (or even zero), and above the linear range, the response saturates. At the low concentration end, electrode response for the first one or two aliquot additions is often smaller than subsequent additions (Figure 1). For tyrosinase-linked electrodes, we have found that this effect disappears in subsequent runs; we conclude that a conditioning process takes place at the electrode surface during the first titration. We therefore instruct our students to do a preliminary standard titration with a freshly prepared electrode, followed by two more standard titration runs. Electrode response for the final two standard titrations should be reproducible, even for the first aliquot.

At the high concentration end, it is interesting to ask whether electrode response declines because of enzyme–substrate saturation or because of electron transfer or diffusion limits at the electrode surface. If diffusion is sufficiently fast, then enzyme catalysis is rate-determining: Current measurements should then obey Michaelis–Menten kinetics and saturate hyperbolically, and Lineweaver–Burke plots (1/current vs 1/concentration) should be linear. Although this behavior is assumed by a number of authors *(1, 3)*, we have consistently found this NOT to be the case for tyrosinase–phenol biosensors. The fit to the equation[1] for hyperbolic decline

$$i_{\mathrm{corr}} = \frac{i_{\infty}}{1 + \dfrac{C_{1/2}}{C}}$$

is fairly poor (Figure 2) and the double-reciprocal plot is nonlinear over the entire range of phenol additions (Figure 3). In fact, linear regression of the double-reciprocal plot yields a positive slope and a negative y intercept (Figure 4 in the supporting information[2] of ref 1 or the attached Figure 3), which gives nonsensical negative values for K_m and V_{max}.

Electrode saturation is in fact nonhyperbolic. The full calibration curve (Figure 4) comprises two linear portions: an initial linear decline in current up to about −20 μA (slope = −0.64 μA/nmol), followed by electrode saturation at about −24 μA. Saturation occurs at analyte concentrations about 2–3 times higher than the highest concentration in the linear-response range. In contrast, hyperbolic saturation is typically observed at concentrations ≥100 times higher than the high concentration end of a pseudolinear response.

Furthermore, there is a good reason why electrode saturation is nonhyperbolic, namely, saturation is almost certainly not due to the kinetics of enzyme catalysis. Within the biosensor's linear-response region, current is diffusion-limited[3] and all of the substrate is catalytically converted to product at the electrode surface. Because diffusion rate increases essentially linearly with bulk concentration, electrode response is linear. In this concentration range, current is influenced only by bulk substrate concentration and not by the kinetic properties of the enzyme. At concentrations above the diffusion-limited regime, the enzyme is not capable of converting all of the substrate to product. Substrate concentration at the electrode surface is influenced by a complex series of three-dimensional transport processes. As even more substrate is added, substrate concentration at the electrode surface approaches that in the bulk solution, and the system becomes more truly kinetically limited. An important point noted by others[3] is that "enzyme saturation may occur at substrate concentrations below where there is truly kinetic limitation, so it is conceivable that there is never kinetic limitation except in the thoroughly uninteresting case where the enzyme is saturated." The diffusion-limited linear region, transitional region, and enzyme-saturation region can all be clearly seen in Figure 4, and there appears to be very little scope within these ranges for a truly kinetically limited enzymatic process. The bottom line is that electrochemical theory suggests that Michaelis–Menten kinetic theory should not apply to the response of the tyrosinase-linked phenol biosensor.

This behavior is explored by Kamin and Wilson *(4)*, who use an enzyme-modified rotating electrode. They show (Figure 3 in ref 4) that only when the electrode rotates at speeds ≥900 rpm does the amperometric reaction saturate hyperbolically (giving a linear Lineweaver–Burke plot); at these high speeds, diffusion to and away from the electrode is fast, and enzyme catalysis is rate-limiting. At rotation speeds ≤400 rpm, the Lineweaver–Burke

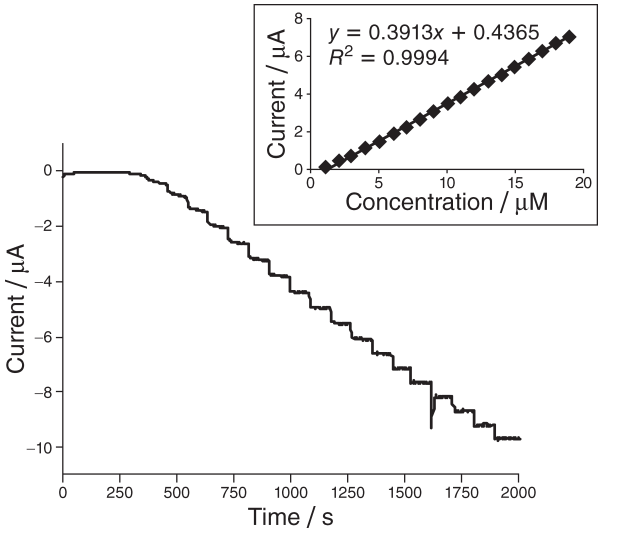

Figure 1. Steady-state current–time response for increasing phenol concentration at the tyrosinase-linked electrodes (Figure 4 from ref 1. Reprinted with permission from ref 1. Copyright 2007 American Chemical Society and Division of Chemical Education, Inc.).

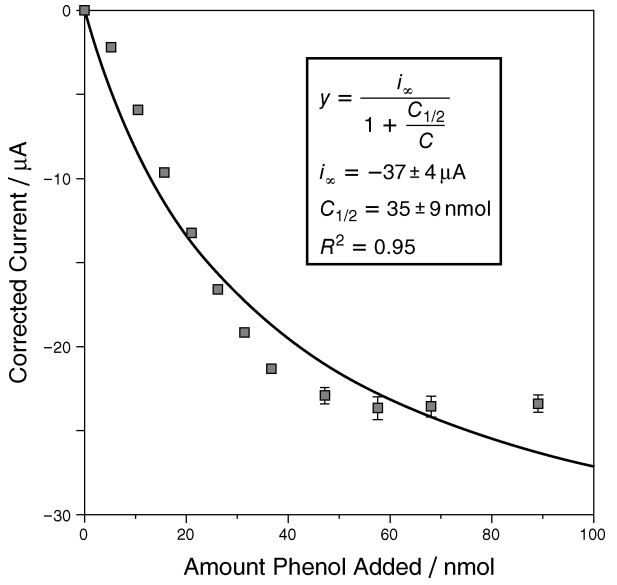

Figure 2. Current versus phenol concentration fitted with hyperbolic equation shown in the legend.

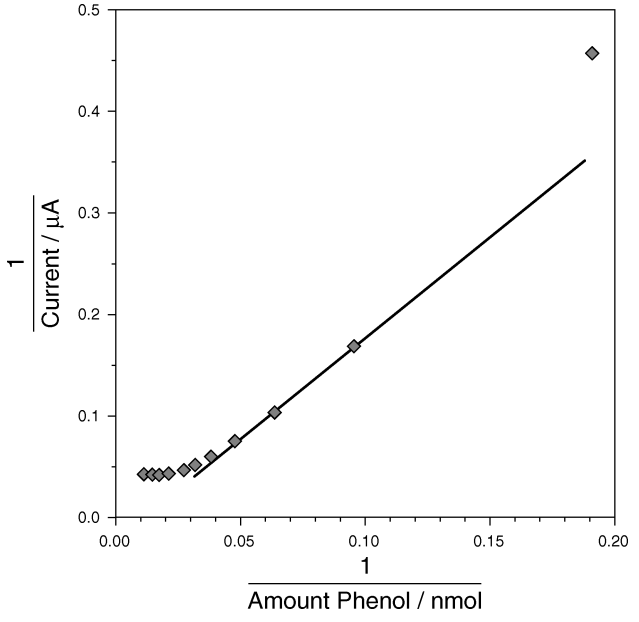

Figure 3. Double reciprocal plot of current versus the phenol concentration.

plot is nonlinear, because diffusion becomes rate-limiting. Kamin and Wilson's calibration curve (current vs concentration) at 100 rpm is qualitatively similar to those observed for our stationary biosensor electrodes: an initial linear range, followed by saturation at a concentration about 3 times higher than the maximum linear-response concentration. It makes sense that if diffusion is rate-limiting for an electrode rotating at 400 rpm or less, it will certainly be rate-limiting for stationary electrodes such as those described in this *Journal (1, 2)*.

The purpose of our communication is to warn instructors that just because a biosensor may be linked to an enzyme, one should not a priori expect the electrode response to obey Michaelis–Menten kinetics. Wilson and others *(1, 4, 5)* point out that, even in cases where electrodes do show hyperbolic

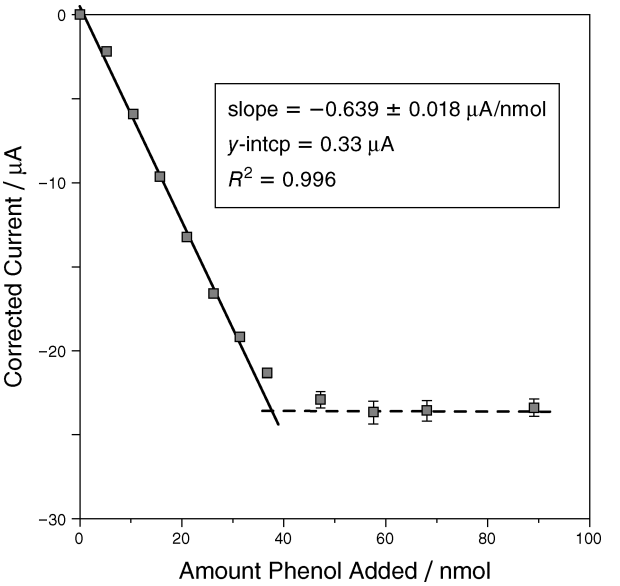

Figure 4. Current versus phenol concentration fitted with linear equation shown in the legend.

saturation, the resulting Michaelis–Menten parameters obtained are "effective", not necessarily the same as those that characterize enzyme behavior in solution. In many cases for stationary electrodes, we would expect to find nonhyperbolic saturation and derived Michaelis–Menten parameters are liable to be useless (e.g., negative). Applying Michaelis–Menten kinetic theory, including Lineweaver–Burke plots, to an electrode system that is not kinetically controlled is inadvisable.

Notes

1. Here C is the concentration (or amount) of substrate or analyte after aliquot addition, i_{corr} is the current measured after each aliquot addition, corrected for baseline current in the absence of substrate ($i - i_0$), i_∞ is the saturating current in the presence of very high substrate concentration, and $C_{1/2}$ is the concentration of the substrate necessary to give half-maximal electrode response. For a Michaelis–Menten enzyme, $C_{1/2} = K_m$ (the Michaelis–Menten constant) $\approx K_d$, the equilibrium constant for the dissociation of the noncovalent enzyme–substrate complex. A double-reciprocal plot of $1/i_{corr}$ versus $1/C$ gives a straight line with y intercept of $1/i_\infty$ and slope of $C_{1/2}/i_\infty$.
2. It is worth noting that, in calculating analyte concentration, Njagi et al. *(1)* neglect the increase in total volume with each aliquot addition. The resulting error grows from +0.1% for the first aliquot to +1.9% for the last (19th) aliquot. Although this error is small, it is simple to correct, and students should be encouraged to account for dilution effects during titrations.
3. We wish to thank reviewer 2 for his or her contributions to our discussion in this paragraph.

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Response to Enzyme-Linked Biosensors: Michaelis–Menten Kinetics Need Not Apply

We thank Dr. Silverstein for adopting our experiment *(1)* in the classroom and for his insightful remarks. The goal of our article *(1)* was to provide an experiment to expose students to the development stages of a modern biosensor as a bioanalytical instrument. The experiment was *not* intended as a study of enzyme kinetics, although we agree with the Silverstein's point in this letter that this aspect requires special and careful consideration.

Dr. Silverstein makes the point that the response of an electrode with an immobilized enzyme does not follow Michaelis–Menten kinetics. We agree that a simple Michaelis–Menten Menten model is not entirely applicable for immobilized enzymes and that this model should be used with caution. There are in fact multiple factors that affect the kinetics of immobilized enzymes, and thus, a much more complex model should be adopted. Nevertheless, the assumption that the electrode saturation is only due to diffusion is simplistic. In addition to diffusion, the process is dependent on the rate of electron transfer, possible adsorption of the reaction product, conformational changes of the protein, or film thickness *(2–5)*. The process is also dependent on the immobilization matrix, the local environment, the electrode, and materials used. Thus, for a true kinetic model, all these parameters should be considered and it is difficult to assess the contribution of these individual factors. Calculation of "apparent" Michaelis parameters of immobilized enzymes using a Michaelis–Menten approach is commonly reported in literature *(2–4)* including for tyrosinase–phenol biosensors *(6–12)*. In our experience, we have found that, depending on the immobilization method and experimental conditions, the response of the enzyme electrode can vary largely and this is also evident from the available literature data *(6–12)*. The study by Kamin and Wilson *(13)* cited by the Dr. Silverstein refers to glucose oxidase as the immobilized enzyme and H_2O_2 as the reaction product, which is different from that of the tyrosinase biosensor described in our article. The phenol–tyrosinase system is special because the reaction product, *o*-quinone, is very reactive and can strongly adsorb and inactivate the electrode surface, especially at high substrate concentrations. This can account for the anomalous behavior observed at the high concentration end. We have suggested in our article that students can compare the "kinetics" of the immobilized enzyme extracted from electrochemical data with the kinetics of the enzyme in free solution. They will discover that immobilized enzymes behave differently than soluble enzymes and can describe the kinetic limitations of the two models. This is a useful educational instrument to introduce students to this nontraditional, but important area of immobilized biocatalysts while constructing a practical biosensor.

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