

How to Design a Biosensor

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Abstract

Amperometric sensors for continuous glucose monitoring could prevent acute and chronic complications of diabetes, but research is needed to improve accuracy and stability. In designing sensors, interference from non-glucose analytes can be minimized by use of filtration membranes or electron transfer mediators that allow polarization at low potentials. If oxygen is required for the enzymatic reaction with glucose, then the outer permselective membrane must have substantial oxygen permeability. For this reason, during development of permselective membranes, permeability studies (such as performed by Tipnis and colleagues in this issue) can be used to measure transport of glucose and oxygen and optimize membrane structure. Tipnis and colleagues present a novel biosensor based with separate layers for glucose-oxygen permselectivity, enzymatic conversion, and avoidance of interference. They also address sensor stability, in part by comparing sensor function during ascending vs descending glucose levels. By measuring the difference, they were able to minimize this aspect of instability (hysteresis), which assisted them in selecting a promising permselective membrane based on iron and humic acid.

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In this issue of *Journal of Diabetes Science and Technology*, Tipnis and colleagues report a novel multilayer method for constructing an amperometric biosensor. During design of a biosensor, what are the performance goals that should be kept in mind?

One important requirement is specificity for the analyte of interest, which is glucose in the article by Tipnis *et al.* Glucose oxidase has high specificity for β -D-glucose. Nonetheless, as recognized by the authors, the sensor under discussion was polarized at 700 mV, a potential that will oxidize not only hydrogen peroxide, but other

antioxidant compounds, such as ascorbic acid. Because ascorbic acid is so widely taken as a drug, patients cannot be asked to avoid its ingestion. In fact, the intended population (people with diabetes) often use ascorbic acid and other antioxidants to inhibit oxidant pathways that promote the formation of advanced glycosylation end products, which may contribute to diabetic complications.¹ In the Tipnis and colleagues article, a specificity membrane is applied directly over the platinum indicating electrode to avoid the passage of interfering compounds to the indicating electrode.

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The particular specificity membrane is an electropolymerized compound, poly(phenylene diamine) (PPD), originally reported by Malitesta and colleagues.² During electropolymerization, monomers are oxidized at a polarized electrode during which time the molecules bond together to form a polymer. When the resultant polymer is insulating, the growth of the film is largely self-limited. For conducting polymers, the final film thickness is influenced by the duration of the fixed potential or the sweep. According to Malitesta *et al.*,² the polymerized PPD compound effectively prevents ascorbic acid from reaching the underlying electrode. In subsequent reports from the University of Connecticut group, it would be helpful to provide verification that these membranes do, in fact, prevent generation of signals from ascorbic acid and other interferents. It is often difficult to duplicate the findings of other groups and, for this reason, evidence that confirms or refutes the experience of others would be helpful. During such testing, the concentration of ascorbic acid or acetaminophen that should be tested is that which occurs in plasma after the ingestion of large therapeutic amounts of the drugs (but not toxic overdoses).

When such a sensor cannot properly prevent an interferent from becoming oxidized, a "glucose-like" signal typically occurs, and the user may well be misled into believing that his or her glucose is elevated. In addition to the method used in the Tipnis *et al.* report, other compounds such as cellulose acetate, interleaved with Nafion,³ and polysulfone derivatives^{4,5} have been reported to effectively screen out antioxidant interferents. When the analyte signal is mediated by the oxidation of small redox compounds rather than hydrogen peroxide, it may be possible to avoid interfering currents, especially in the presence of interference-reducing films, such as horseradish peroxidase.⁶ An advantage of using mediators is that some of the redox mediators are oxidized at potentials below that which would oxidize acetaminophen, ascorbic acid, or uric acid.

Another key element of a biosensor is a sufficient sensitivity to the analyte of interest. To the extent that background noise will reduce the signal:noise ratio, a substantial sensitivity is generally considered to be beneficial. Nonetheless, a potential argument against a very high sensitivity is that amperometric sensing reduces the concentration of analyte in the immediate vicinity. If there is rapid diffusion of glucose into an enzyme layer that breaks down glucose, the concentration of glucose in the ambient fluid will decline. A "perfect" biosensor will not perturb the concentration of analyte in the ambient tissue or fluid. At any rate, sensor sensitivity must be sufficient to overcome noise; in the article by Tipnis *et al.*, sensitivity was quite high, in the microampere range. During

prolonged studies of stability over time, the analyte in the test fluid should be tested before and after such studies by an independent method. (One common reason for a decline in the concentration of an analyte such as glucose, which could masquerade as sensor instability during long-term *in vitro* studies, is bacterial degradation.)

Many workers in the field believe that the response to the analyte of interest should be linear or nearly linear over the range of interest. A high degree of linearity allows use of a simple linear calibration method and ensures that changes in the upper concentration regions will be detectable. Even with the best outer membrane in their study (humic acid interleaved with iron), the sensor described in the article by Tipnis *et al.* begins to rise less steeply above 5 mM. Fortunately, complete leveling off is not seen even at levels of 20 mM. So even though the response is clearly not linear in this report, the fact that a plateau is not observed is quite favorable. The authors acknowledge this nonlinearity and discuss possible explanations. Although there may be several potential mechanisms, one likely explanation is an oxygen deficit. If the ratio of glucose to molecular oxygen in the enzyme layer is greater than 1, then incremental addition of glucose to the ambient test liquid will not result in a proportionate rise in the signal.

The situation in a living mammal, under typical circumstances, demands more of the sensor than the situation on the benchtop. Consider that three different studies all found that a typical oxygen concentration in the subcutaneous space of mammals is approximately 35–70 torr.^{7–9} As arithmetic conversion to the same units is carried out, it becomes obvious that mammals (especially those with diabetes) have orders of magnitude more glucose in their subcutaneous tissue than oxygen. Therefore, a sensor that may be linear *in vitro* (during which the sample liquid is directly exposed to air) may be nonlinear due to an oxygen debt *in vivo*, especially under hypoxic conditions, such as cardiac disease, pulmonary disease, or high altitude.

One effective way to address this issue during sensor development is to measure the oxygen effect directly. One releases small amounts of oxygen, mixed in different proportions with nitrogen or argon, into the sample liquid and monitors the dissolved oxygen tension with a commercial oxygen meter. One can keep the analyte of interest (e.g., glucose) fixed at physiologic values and vary the oxygen tension over a large range. As the oxygen tension is reduced, there will be a point at which the glucose signal declines. To the degree that such an oxygen tension is in a range seen in normal humans or in common disease states, a change in oxygen tension *in vivo* after calibration has taken

place will lead to sensing error. For this reason, it is ideal to use a membrane that has a high degree of hydrophobicity to enhance oxygen entry. The degree of hydrophilicity must be sufficiently low to serve as a partial barrier to glucose. Too great a reduction in hydrophilicity will lead to very low sensitivity.

Gough and colleagues¹⁰ have successfully employed a different method of avoiding error due to changes in oxygen. In their device, glucose is measured by comparing the change in oxygen (as glucose changes in the opposite direction) at an enzyme electrode with the concentration of oxygen at a nonenzyme electrode. Using such a device, if oxygen changes *in vivo* after calibration, the glucose signal will remain stable, since the oxygen signals both at the enzyme and at the nonenzyme electrode will change in parallel.

Another highly desirable attribute of a biosensor is stability over time. One method of quantifying stability is to sequentially carry out more than one calibration curve. Tipnis and colleagues should be congratulated in this regard. For all the sensors that they tested in an ascending direction, they sequentially carried out tests in descending glucose concentrations and measured the difference between the two test conditions. They termed this difference hysteresis, a phenomenon wherein two physical quantities have a relationship that depends on the history. The sensor whose outer membrane used a humic acid derivative had much less hysteresis than sensors built with other materials. There are several reasons why a second calibration curve may respond differently than the initial curve. One such example is a sensor that has not been allowed to fully equilibrate after polarization, a phenomenon probably related in part to the formation of oxides on the surface of the indicating electrode. The authors are familiar with this possibility and cite the work of Hall and colleagues¹¹ in this regard. Unpublished data from the authors suggest that insufficient equilibration is unlikely to be the primary cause of the hysteresis that was observed (personal communication). Other potential causes of declining sensitivity over time include inactivation of enzyme (or loss of enzyme) and loss of integrity of the reference electrode, although such a reference electrode change usually takes many days. If one knows how much AgCl is initially present on the reference electrode, the sensitivity of the sensor, and the analyte level to which it is exposed, one can calculate the transfer of charge over time and thus determine the "life" of the AgCl layer. To some extent, depending on the electrolytes present in the test fluid, the sensor may continue to measure the analyte even after the AgCl is depleted, but stability will likely be compromised.

How can one distinguish among the different causes of signal loss over time? One useful technique is to purposely test sensors in analytes that do not require enzymatic conversion to generate a signal. Thus, one can compare the signal generated from oxidation of acetaminophen to that of glucose, which of course requires enzymatic conversion. For such a comparison, it would be helpful to omit membranes such as the specificity membrane that would otherwise prevent the acetaminophen from reaching the indicating electrode. If the acetaminophen-induced signal is stable but the enzyme-requiring signal declines over time, then enzyme activity may well be declining (e.g., due to denaturation or to frank loss of enzyme into the surrounding medium). If both signals decline equally, passivation of the indicating electrode or loss of reference electrode integrity may be the source of signal loss.

One can examine the reference electrode in several ways. For example, one can measure the potential of the reference electrode under evaluation as compared to a freshly chloridized or standard reference electrode, as described by Moussy and Harrison.¹² Alternatively, one can compare the sensitivity of a sensor tested over an analyte range (using the reference electrode in question) to the same testing protocol in which the same indicating electrode is tested with a fresh or standard reference electrode. It should be noted that Ag/AgCl reference electrodes can quickly lose AgCl *in vivo* if the electrode is not coated properly with certain polymers.¹² As in the case of the oxygen requirement, the *in vivo* situation can be very different than that of the *in vitro* situation.

One can also examine the permeability of permselective membranes by use of a permeability cell. This can be technically challenging because even a small leak can create artifact. Again, I congratulate Tipnis and colleagues for performing careful measurements and calculations of membrane permeability and diffusion. They found that the humic acid/iron-layered membrane showed favorable transport properties for glucose. They also were able to quantify the transport of hydrogen peroxide. The authors discuss the implications of a topic that heretofore has received little attention: the outward diffusion of hydrogen peroxide, which (being a small molecule) flows easily through the outer membrane into the surrounding medium. My only methodologic quibble in the permeability studies is that it might have been even more convincing to measure glucose and other analytes by independent methods (e.g., using a YSI benchtop device) than by the same electrochemical enzymatic method that was used for other aspects of the study.

By measuring transport through specific membranes and by isolating specific components of a biosensor, it is possible to specifically examine each component of the sensor. Although carrying out all of these measures of stability can be time-consuming, they are important validation procedures if human testing is contemplated. Instability of sensors is considered to be common (especially among clinicians), and rigorous testing by workers in the field will help mitigate against misadventures during which biosensors might be used for clinical decision making. Labeling of currently available glucose sensors caution against using sensor data for clinical decision making, but patients may not always fully understand or always follow this caveat.

The article by Tipnis and colleagues, using humic acid derivatives interleaved with iron, presents valuable new data regarding an outer permselective-sensing membrane. As the authors prepare for animal studies, short-term and long-term stability can be further addressed. The authors should be congratulated for an interesting contribution to the field of biosensing technology.

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