Evaluation of the sensor properties of the pH-static enzyme sensor

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ABSTRACT

The pH-static enzyme sensor consists of a chemical sensor-actuator system covered with a thin enzyme-entrapping membrane. By the electrochemical generation of protons or hydroxyl ions, pH changes induced by the conversion of a substrate by the enzymatic reaction are compensated. The pH inside the membrane remains at a constant level and the control current is linearly related to the substrate concentration and independent of the buffer capacity of the sample. The sensitivity and linearity of the sensor response are evaluated. Depending on the enzyme load of the membrane, the operation of the sensor is either diffusion controlled or determined by the enzyme kinetics.

The practical application of enzyme sensors based on the measurement of pH changes in an enzymatic membrane is limited by the influence of sample pH and buffer capacity on their response. This problem has been recognized since the first publication on ISFET-based enzyme sensors [1] and several workers have tried to describe accurately the complicated response of these electrodes (e.g., [2–4]). The factors that complicate the response are the buffer capacity of the sample, which itself is also a function of pH, the pH-dependent enzyme kinetics and the fact that the products of the enzymatic reaction may be weak protolytes so that the amount of H^+ or OH^- ions produced per mole of converted substrate also depends on pH. For example, the products of urea hydrolysis, 2 mol of ammonia and 1 mol of carbon dioxide per mole of urea, will increase the pH of an acidic sample solution up to ca. pH 9. On the other hand, the pH of more alkaline solutions will

be decreased by the same molecules [5]. As a result of these three factors, the response of ISFET-based enzyme sensors is strongly non-linear and the dynamic range depends on the composition of the sample solution. The general conclusion must be that, at present, an elaborate calibration is required and that the practical value of these sensors is limited [6].

In previous papers [6,7], it was demonstrated that by coulometric control of the pH inside the enzymatic membrane, it is possible to overcome these problems. The pH-static enzyme sensor measures the pH inside the membrane with an ISFET and controls it through the generation of protons or hydroxyl ions at a noble metal electrode, spaced closely around the ISFET gate area. The acidic or alkaline products of the enzyme reaction are thus continuously neutralized. The generating current needed to maintain the pH at a constant level now becomes the output signal of the sensor. It is linearly related to the substrate concentration and independent of the buffer capacity of the sample.

In fact, an electrochemical pH-actuating mechanism, the current-controlled electrolysis of water,

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is used to compensate the biochemical pH actuator, i.e., the enzyme, converting substrate molecules and thus generating pH change. The biochemical actuator is considered first.

The response of an enzyme-modified ISFET is generally pH dependent. However, if the pH is kept at a virtually constant level, the sensitivity may also be considered to be constant. This sensitivity, the change in the steady-state ISFET output voltage V_{out} per change in substrate concentration $[S]$, is given by

$$\partial V_{\text{out}}/\partial [S] = EB/\beta \quad (1)$$

where B (mV pH^{-1}) is the sensitivity of the ISFET, β ($\text{mol l}^{-1} \text{pH}^{-1}$) is the buffer capacity of the sample and E is the enzymatic sensitivity parameter representing the change in equivalents of H^+ or OH^- in the membrane as a function of the substrate concentration. E is determined by the enzyme load of the membrane, the enzyme kinetics, the diffusion constants of the relevant species in the membrane and the ratio in which protons or hydroxyl ions are produced per mole of substrate. For reactions that produce acidic products the value of E is positive and reactions that produce OH^- yield a negative value of E . At constant pH, the value of E can be considered to be constant provided that the substrate concentration $[S]$ is considerably smaller than K_m , the Michaelis-Menten constant of the enzyme, or when the response of the sensor is limited by diffusion of the substrate instead of by the enzyme kinetics.

A similar consideration holds for the electrochemical actuator. For small changes in pH, the

steady-state response of the output voltage on a current I through the generating electrode may also be considered to be linear and is given by

$$\partial V_{\text{out}}/\partial I = AB/\beta \quad (2)$$

where A ($\text{mol l}^{-1} \text{A}^{-1}$) is the sensitivity parameter of the chemical sensor-actuator system and is determined by the area of the generating electrode (and thus the current density) and the diffusion speeds in the membrane.

The generating current is used to compensate the pH changes produced by the enzyme and hence $\partial V_{\text{out,current}} = -\partial V_{\text{out,enzyme}}$. Combination of Eqns. 1 and 2 shows that the sensitivity of the pH-static enzyme sensor is given by

$$\partial I/\partial [S] = -E/A \quad (\text{A mol}^{-1} \text{l}) \quad (3)$$

and is independent of the buffer capacity β . Provided that E remains constant, the response is linearly dependent on the substrate concentration.

Figure 1 shows the control loop that represents the operation of the pH-static enzyme sensor. The pH inside the enzymatic membrane is measured with an ISFET, resulting in an output voltage V_{out} which is compared with V_{set} , the desired output voltage that for instance reflects the original pH of the sample. The error signal ϵ is transformed to a generating current I by the controller. The current I delivers C equivalents of either H^+ or OH^- ions to the membrane, which in turn change the pH of the membrane in a ratio depending on its internal buffer capacity. From Fig. 1 it is again obvious that the effect of the enzyme reaction is no longer dependent on the buffer. The enzymatic reaction products are neutralized at the central

Fig. 1 Block diagram of the control system for the pH-static enzyme sensor

summing point in the figure, before the buffer can assert its influence.

In previous papers [6,7], preliminary results showed the feasibility of the pH-static enzyme sensor. Independently, a similar approach has also been described in a patent [8]. In this paper the construction of the sensor and the control system are described in more detail and the sensor properties are evaluated further. In this study, urease was used as the enzyme, but in principle the method is applicable to all enzyme reactions that produce pH changes on conversion of substrate. The excellent stability and low cost of urease make it an ideal model system for the work described here.

EXPERIMENTAL

Sensor chip

The layout of the sensor chip is shown in Fig. 2. It contains a single ISFET fabricated with NMOS technology. Ta_2O_5 is used as the pH-sensitive gate insulator. The generating electrode is constituted by a thin film of gold or platinum that closely surrounds the gate of the ISFET. The active area of the noble metal electrode measures

1 mm^2 and is defined by a patterned polyimide layer. The die size is chosen as large as $3 \text{ mm} \times 4 \text{ mm}$ to facilitate encapsulation. This chip was designed as a generally applicable device for the study of chemical sensor-actuator systems [9–11].

Encapsulation

The sensor chips are mounted on a printed circuit board carrier and are encapsulated with epoxy resin using a Teflon mould. This mould ensures that the thickness of the epoxy layer on top of the chip is very reproducible and of the order of $200 \mu\text{m}$. In the mould, a Teflon bolt with a diameter of 2 mm is adjusted on the surface of the sensor chip. Thus, a micro pool is formed in the epoxy in which the active area of the sensor is exposed. After curing the epoxy, the sensor is removed from the mould and the surface of the encapsulant over the chip is polished flat which the use of fine-grain sandpaper.

Membrane fabrication

Urease is immobilized in a polyacrylamide membrane [12] which is formed in the epoxy micro pool described above. The membrane contains 10% (w/w) of the polymer and 2.5% of protein. Unless mentioned otherwise, 25 mg ml^{-1} of the

Fig. 2. Layout of the integrated chemical sensor-actuator chip

enzyme are immobilized. Experiments with a lower urease content of the membrane failed initially as the enzyme leaked out very rapidly. The addition of bovine albumin up to a total protein content of 25 mg ml⁻¹ considerably improved the stability. All chemicals for the fabrication of the membrane were supplied by Sigma (St. Louis, MO, U.S.A.).

The procedure is as follows. A 97.5-mg amount of acrylamide, 2.5 mg of *N,N'*-methylene-bisacrylamide and 25 mg of urease (E.C. 3.5.1.5, Sigma Type IX) are dissolved in 1 ml of 33 mM phosphate buffer solution (pH 7) containing 0.1 mg ml⁻¹ of ribovlavin and 2.5 mg ml⁻¹ of ammonium persulphate. The micro pool is filled with a drop of this solution and subsequently closed with a Teflon-coated microscope cover-glass. The monomer solution is then exposed to visible light from a Schott KL 1500 Cold Light Source (Schott Glaswerke, Wiesbaden, F.R.G.) for 30 min, after which polymerization is complete and the cover-glass can be removed. Finally, the protein in the membrane is moderately cross-linked by dipping it into a 0.5% glutardialdehyde solution for 5 min.

The procedure results in membranes with an accurately reproducible thickness of 200 μ m. Because the polymerization takes place in a closed cavity, the enzyme load and the diffusion constants of ions and molecules inside the membrane are also well controlled. Figure 3 shows a cross-section of the complete sensor.

Between measurements, the sensors are either stored dry or in a phosphate buffer solution at 4°C. Dry-stored membranes sometimes rupture, mostly at those places where air bubbles are entrapped accidentally in the membrane. However,

those membranes which remain intact can be kept refrigerated for at least 3 months without losing their original sensitivity.

Interface electronics

Figure 4 shows the general measurement set-up that is used for the pH-static enzyme sensor. The system uses two pH-sensitive ISFETs with an integrated actuator electrode, one for the actual enzyme sensor and a second as a reference that measures the background pH of the sample solution. The second chip is identical with the first, except that its membrane does not contain the enzyme. Both ISFETs measure with respect to a common (quasi-) reference electrode which is actually the platinum electrode on the reference ISFET. The ISFETs are operated with a constant source-drain voltage and a constant drain current using source and drain follower circuits [13]. Their differential signal reflects the substrate-dependent pH change induced in the membrane of the first ISFET.

To ensure that the current source is completely electrically isolated from the pH-measuring circuit, it is battery powered. The current source consists of two parallel sources, one for positive and the other for negative currents. The control of the output current is obtained via opto-couplers by pulse-width modulation of constant current sources. At high current densities at the generating electrode, gas bubbles (either hydrogen or oxygen, depending on the direction of the current) may be formed and therefore the amplitude of the current sources, and hence the maximum output current, is limited to 2 μ A.

Controller

The controller itself is realized on an Apple IIe computer. The differential output voltage of the ISFETs is measured, after a 100 $\times$ preamplification, with a 12-bit analogue-to-digital converter. An input range of 10 V is used so that the ISFET signal can be measured with a resolution of 25 μ V or ca. 0.0005 pH unit. Offset compensation is done manually before the actual measurements start.

The controller operates at a sample interval of 2 Hz. The clock that controls the output pulse

Fig. 3. Cross-section of the pH-static enzyme sensor. The height of the epoxy encapsulant determines the thickness of the polyacrylamide membrane

Fig. 4 Measurement set-up for the pH-static sensor

width of the current source runs at 100 kHz so that, with a maximum output current of $2 \mu\text{A}$, the current can be regulated in 40-pA increments.

The control program is written in Applesoft BASIC in connection with some machine language routines for input/output and timing. The control algorithm was derived from a study of the so-called open-loop behaviour of the system. Equation 2 gives the steady-state change in output voltage of the chemical sensor-actuator system. The dynamic behaviour can be examined when a step-wise current ∂I is applied to the generating electrode at $t = 0$. It appears that the pH change in the membrane, as measured by the ISFET, can be described in terms of a delay time T_D and a time constant τ according to

$$V_{\text{out},t} = V_{\text{out},0} + (AB/\beta) \{1 - \exp[-(t - T_D)/\tau]\} \partial I \quad (4)$$

for $t > T_D$, where $V_{\text{out},t}$ is the output voltage at time t and $V_{\text{out},0}$ is the initial output voltage. The delay time T_D is due to the time needed for ions to diffuse from the generating electrode to the

ISFET gate. The control algorithm contains both a differentiating action to compensate for τ and an integrating action to compensate for T_D and to minimize the static error. Because the open-loop transfer of the system is buffer dependent, the maximum amplification of the controller that ensures a stable operation is a function of β . Therefore, the system is able to determine β and to adapt the amplification to the actual sample solution automatically. A detailed description of the control system is given elsewhere [14].

Measurement set-up

Measurements are carried out in 15 ml of buffer to which small aliquots of concentrated urea solution are added. The solution is continuously stirred using a magnetic stirrer. Because of the relatively thick membrane, the steady-state response of the sensor is independent of stirring. However, stirring ensures that the added urea is homogeneously distributed in the solution very rapidly so that the measured response time can be entirely attributed to the sensor itself. All buffer solutions used contain 0.1 M potassium nitrate as supporting electro-

lyte. Measurements were carried out at room temperature without further thermostating.

RESULTS

Time response

Figure 5 shows a typical output registration from the pH-static enzyme sensor. Curve A represents the control current and curve B shows the ISFET output voltage. First, the system temporarily opens the control loop and applies a current pulse of 10-s duration and measures the slope of the induced pH change. From this slope, the buffer capacity of the solution is estimated, the amplification of the controller is adapted accordingly and the control action is resumed. Then, urea is injected into the solution at the moments indicated by the arrows to the concentrations shown. Finally, the solution is replaced with fresh buffer solution that contains no urea. The response to a stepwise increase in substrate concentration is complete within 3 min and it is linear with concentration. As can be seen, the pH is controlled within 1.5 mV or 0.03 pH unit.

Buffer influence

The response of the pH-static enzyme sensor is independent of the buffer capacity of the sample solution. Figure 6 compares the output of a "classical" ISFET-based enzyme sensor with that

Fig. 5. Response of the sensor to stepwise additions of urea

Fig. 6 Response of a "normal" ISFET-based enzyme sensor (closed symbols) compared with that of the pH-static sensor (open symbols) in sample solutions of different buffer capacities. ■, □, 2 mM, ●, ○, 10 mM, ▲, △, 50 mM phosphate buffer at pH 7

of the pH-static sensor. The lines with solid symbols show the measured pH change as a function of the urea concentration at pH 7 in phosphate buffers of 50, 10 and 2 mM. The lines with open symbols show the control current used by a pH-static sensor as measured simultaneously with the first three curves. As can be seen, the sensitivity and dynamic range of the "normal" enzyme sensor depend strongly on the buffer capacity. In a weak buffer, the response saturates quickly as the pH in the membrane approaches 9. In a strong buffer, the maximum concentration that can be measured is much larger but the sensitivity is reduced. Because the pH-static sensor operates at a constant pH, the enzyme activity and the influence of the reaction products on the pH remain constant.

Linearity

The results with the pH-static sensor as presented in Fig. 6 shows that the response is fairly linear over the concentration range used. As can be seen, the sensitivity of the sensor is of the order of $700 \text{ nA } 1 \text{ mmol}^{-1}$. Because the output of the current source is limited to $2 \mu\text{A}$, the maximum concentration that can be measured is ca. 3 mM urea. The dynamic range of the sensor can be expanded by lowering the sensitivity of the sensor, i.e. the value of the sensitivity parameter E as given in Eqn. 1. The most obvious way to decrease the value of E is to lower the enzyme load of the

Fig. 7 Lineweaver-Burke plot for the response of a pH-static sensor with an enzyme load of 0.5 mg ml^{-1} in the membrane.

membrane. However, as can be concluded from the almost linear relationship between urea concentration and controller current in Fig. 6, the responses of the sensor must be determined, at least partially, by diffusion of the substrate. When the enzyme concentration in the membrane is lowered, this may become the limiting factor and give rise to a non-linear response. In that case, the response can be described with Michaelis-Menten kinetics and is the sensor output given by

$$I = I_{\max} [S] / \{ K_m + [S] \} \quad (5)$$

where $I_{\max}$ is the controller current when the maximum reaction velocity of the enzyme in the membrane is reached. Only when $[S] \ll K_m$ will the sensor output be approximately linearly related to the substrate concentration. On the other hand, when for larger concentrations the inverse of the response is plotted against the inverse of the substrate concentration, a linear relationship is found, the so-called Lineweaver-Burke plot. From this plot $I_{\max}$ and K_m can easily be determined.

In Fig. 7 a Lineweaver-Burke plot is given from the response of a pH-static sensor where the enzyme concentration in the membrane is reduced to 0.5 mg ml^{-1} . From the perfectly linear fit it can be concluded that the enzyme kinetics are in this case indeed the response-determining factor. The value of K_m as found from this plot is 6.85 mM , which is in reasonable agreement with the literature value of 10 mM for the free enzyme [15].

pH dependence

As shown in the previous section, the response of the pH-static sensor can be limited by diffusion on the one hand and by the enzyme kinetics on the other. Between these two there will be a gradual transfer from diffusion-limited to kinetically controlled response as the enzyme load of the membrane decreases. In the case of kinetic limitation, the response of the sensor should depend on the pH of the sample solution and show a maximum sensitivity at the optimum pH for the enzyme. Figure 8 gives the response of the pH-static sensor at various pH values of the sample solution, the sensitivity at pH 7 being normalized to a value of 1. It can be seen that the maximum sensitivity is found between pH 6.5 and 7. This value is lower than that reported elsewhere for urease in a phosphate buffer [12]. However, when the sensitivity is corrected for the amount of OH^- ions liberated at the different pH values [5], the optimum pH for the enzyme is between 7 and 7.5. On the other hand, the influence of the immobilization procedure on the enzyme parameters is not known.

In sample solutions of a different pH it is possible to change the pH inside the membrane to the optimum of about 7 by means of a coulometric "offset current". In that way, the sensitivity of the pH-static sensor can be adjusted to the maximum value. This procedure is illustrated in Fig. 9. Lines A and B give the response of the sensor in buffers of pH 7 and 6, respectively and C gives the

Fig. 8. Normalized response (sensitivity at pH 7 equal to 1) as a function of pH.

Fig. 9. Response of the pH-static sensor at pH 7 (A); pH 6 (B) and with the inner membrane pH shifted from 6 to 7 (C) The dashed line is identical with C, shifted 420 nA upwards.

response in a pH 6 buffer with the desired output voltage of the system set to a value corresponding to pH 7. It can be seen that at zero urea concentration a generating current of -420 nA is required in order to produce a flux of OH^- ions that brings the pH to the desired value. When urea is added to the solution, the enzymatic reaction also produces hydroxyl ions so that the generating current will be reduced. At higher substrate concentrations the polarity of the generating current even inverts to compensate for the excess of OH^- produced by the enzyme. The dashed line in Fig. 9 is identical with line C, shifted upwards with the value of the offset current at zero urea concentration. As can be seen, the response of the sensor in this last experiment is not exactly linear, for low urea concentrations the sensitivity being higher than the original pH 7 curve. For higher urea concentrations the mutual correspondence between the measurements is better. Possibly the deviation in sensitivity is due to the pH gradient that exists in the membrane during the last experiment. While the pH at the ISFET gate is controlled at 7, the pH at the outside of the membrane is of course still equal to 6. From Fig. 8 it can be seen that the optimum sensitivity is below pH 7 and this may be the reason for the initially higher response.

DISCUSSION

In addition to the sensor properties described under Results, there are a number of points that need further consideration.

Current efficiency

For proper operation, the efficiency of the coulometric control current must be constant. As described above, measurements with the pH-static urea sensor are performed in phosphate buffer solutions containing potassium nitrate as supporting electrolyte. In these solutions the efficiency of the H^+ generating current is probably close to 100%. Chloride ions, on the other hand, present a serious interference. Not only is the amount of anodically generated protons drastically reduced but also the enzyme is denatured by the chlorine radicals or Cl_2 molecules produced. The requirement that chloride must be absent from the sample solution is, of course, a serious limitation to the practical applicability of the pH-static sensor. A possible solution to this problem might be found in the use of a mediating redox system or in the use of alternative electrode materials. Also, for the application of acid-producing enzymes the efficiency problem might be less serious as hydroxyl ions are produced which interfere less. So far this has not been tested, however.

Drift

When the pH-static sensor is in operation, the pH is continuously measured and, of course, when the output voltage of the ISFET drifts, this will result in a compensating drift of the controller current. From Eqn. 2 it can be seen that the magnitude of this compensating current will be directly proportional to the buffer capacity β of the sample solution. Using the experimentally determined values for A and B , it can be calculated that an ISFET drift of 1 mV h^{-1} will result in a drift of 30 nA h^{-1} in the control current of the pH-static sensor working in 5 mM phosphate buffer at pH 7. At a sensitivity of $700 \text{ nA l mmol}^{-1}$ urea, this corresponds to a baseline drift of approximately $40 \text{ } \mu\text{M h}^{-1}$. Of course, the baseline can easily be reset by placing the sensor in a blank solution and restarting the control action.

Sensor construction

The design of the pH-static sensor is not ideal. Because of its construction, the pH is only controlled optimally at the gate of the ISFET. Inside the membrane pH gradients will exist because the sensor, the immobilized enzyme and the electrochemical actuator cannot be physically located at the same point. A possible improvement is to separate the membrane into two layers. A thin enzyme-loaded membrane directly on the chip can be covered with a thicker blank membrane that merely acts as a diffusion barrier to make the sensor independent of convections in the sample solution. The total thickness of this composite membrane should still be limited to ca. 200 μm to ensure a reasonable response time for the sensor. Possibly the non-linearities as they occur in the measurements in Fig. 9 can be eliminated in this way.

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