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ISFET Based Enzyme Sensors

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ABSTRACT

This paper reviews the results that have been reported on ISFET based enzyme sensors. The most important improvement that results from the application of ISFETs instead of glass membrane electrodes is in the method of fabrication. Problems with regard to the pH dependence of the response and the dynamic range as well as the influence of the sample buffer capacity have not been solved. As a possible solution we introduce a coulometric system that compensates for the analyte buffer capacity. If the pH in the immobilized enzyme layer is thus controlled, the resulting pH-static enzyme sensor has an output that is independent of the sample pH and buffer capacity and has an expanded linear range.

Key words: ISFET, enzyme, urea, IC-technology, buffer dependence, coulometry, pH-static enzyme sensor.

INTRODUCTION

Potentiometrically operating enzyme sensors suffer from a number of problems, partly due to the properties of immobilized enzymes and partly resulting from the use of the particular ion sensitive electrode. Focussing on enzyme electrodes in which the conversion of a substrate causes a change in the local pH, these problems are: pH-dependence of response, detection limit and dynamic range; response dependency on the analyte buffer capacity; dependence on immobilization technology; and individual membrane casting and control of membrane thickness.

During the three-day workshop on the theory, design and biomedical application of solid-state chemical sensors (held at Case Western Reserve University in Cleveland, Ohio, on March 28–30, 1977), Professor Guilbault made a request to the audience, saying that he would welcome as much help as possible in the area of enzyme electrodes from workers in the field of ion-sensitive field effect transistors (Guilbault, 1978). He probably expected that at least some of the problems could be solved by the application of ISFETs.

It will be investigated in this paper whether this expectation is realistic or not. First of all, therefore, we have to list the properties of ISFETs and conventional electrodes in order to be able to compare the performances and operational mechanism.

THE GLASS ELECTRODE

pH-Glass electrodes (Bates, 1973) are based on the properties of a glass membrane which is contacted on both sides by an electrolyte solution. In practice, the glass membrane is formed at the end of a glass tube which is filled with a reference solution. On both sides of the membrane a pH-dependent potential is formed, giving rise to a *trans* membrane potential which is measured by a reversible electrode, such as an Ag/AgCl electrode, in the inner solution and a reference electrode, e.g. a saturated calomel electrode, in the analyte. For proper operation, the glass membrane has to be in contact with an aqueous solution for a certain conditioning period in order to hydrate. This period is of the order of 24 h. The substrate of the glass membrane is relatively dry, although a certain degree of conduction is necessary for the actual measurement of the surface potentials. This gives the electrode a rather high resistance, resulting in a high sensitivity to electrical interference which necessitates a shielded cable.

The response time of a glass electrode is governed by a rather slow diffusion process of ions in the hydrated glass layer which means that fast pH changes cannot be measured. Time constants in the order of 1–3 s are typical for standard electrodes. With miniaturization of the electrode this value increases considerably.

The sensitivity of a glass membrane electrode is Nernstian (59 mV dec^{-1} at room temperature) over a pH range from pH 1 to 12. The temperature behaviour is such that an athermal point is realized for pH 7 and 0 mV output. Due to this thermally insensitive reference point, compensation for the temperature sensitivity can now easily be made by the use of a temperature sensor, the output of which controls the amplification of the electrometer amplifier. The stability of glass membrane electrodes and the

adhering reference electrodes is very good and typical drift rates are less than 1 mV week^{-1}. Again, also the drift increases with miniaturization of the electrode.

THE ISFET

The ISFET (Sibbald, 1986) belongs to the class of Chemically Sensitive Electronic Devices. This means that the device is in the first place a transistor and can be operated as such. The so-called threshold voltage of the transistor is, however, a function of the pH of the analyte which contains a reference electrode contacted to the common of the specially developed ISFET amplifier (Bergveld, 1981). The operational mechanism of the ISFET originates from the pH sensitivity of the inorganic gate oxide, such as SiO_2, Al_2O_3, Si_3N_4 or Ta_2O_5. This mechanism is purely a surface phenomenon which can be explained by the site dissociation model (Bousse *et al.*, 1983). Surface hydroxyl groups react with the analyte in an acidic or a basic manner, resulting in a corresponding surface charge and potential. Depending on the specific properties of the gate oxide, this surface potential is in the order of 25 mV dec^{-1} (SiO_2) to 55 mV dec^{-1} (Ta_2O_5). In general, the operation does not need any conditioning period. The response is fully determined by the kinetics of the surface reactions and response times in the order of milliseconds are typical. The ISFET incorporates an impedance transformation, which makes the device connection leads less sensitive to electrical interference.

The temperature sensitivity of the ISFET is mainly determined by the solid-state part of the sensor, i.e. the FET. The best procedure to compensate for this temperature sensitivity is the use of a differential pair of ISFETs or an ISFET and a MOSFET, as usual in electronic systems. Because an ISFET is an open transistor, the device is also sensitive to light, but this can also be largely compensated for by the application of a differential pair.

The drift of an ISFET is still a subject of research, although in the main it is thought to be caused by the polarization of the applied inorganic gate oxide. Drift rates of the order of 0·1–1 mV h^{-1} are typical (Arnoux *et al.*, 1987). For short-term use, compensation for this drift can be made by the use of a programmable drift correction.

It should be mentioned that ISFETs are essentially chips which are of a planar construction, are very small and can be manufactured with integrated circuit (IC) technology, which means that mass production of identical chip sensors can easily be performed. In contrast with the glass electrode, the electrical connections to the ISFET chip have to be made within the liquid

surroundings. This implies very stringent requirements with respect to the packaging and is probably the most important reason for the limited commercial success of ISFETs to date.

GLASS MEMBRANE ELECTRODE COMPARED WITH ISFET

Now that we have summarized the essential features of glass membrane electrodes and ISFETs, we can easily deduce the advantages and drawbacks of both types of pH sensors from Table 1. From this table it can be concluded that ISFETs, in comparison with glass membrane electrodes, have the

TABLE 1

	Glass electrode	*ISFET*
Appearance	Bulb type surface	Flat surface
Dimensions	$>100\ mm^2$	$<1\ mm^2$
Impedance	Very high	Very low
Sensitivity	$59\ mV\ dec^{-1}$	$<55\ mV\ dec^{-1}$
Drift	$<1\ mV\ week^{-1}$	$0{\cdot}1\text{–}1\ mV\ h^{-1}$
Multi sensor	Individual types	Integrated
Production	Individual	Large scale
Time constant	Seconds	Milliseconds
Conditioning	24 hours	Not required
Encapsulation	Not problematic	Critical

advantages of: planar construction, small dimensions, low impedance, fast response, large scale production, ease of multi sensor (differential) realization and immediate use after dry storage. A drawback is the larger drift rate and the necessary stringent encapsulation of the chip edges and bonding leads. In the next section we will investigate which of the advantages over pH glass membrane electrodes can be useful with respect to the application in enzyme electrodes.

TYPICAL ISFET PROPERTIES, ADVANTAGEOUSLY APPLICABLE IN ENZYME ELECTRODES

It will be obvious that membrane casting onto flat surfaces gives a better control of membrane thickness and improved sealing possibilities than with the use of curvilinear surfaces from bulb type electrodes. Furthermore, the deposition of membrane material is no longer individual per sensor, but in

the ISFET processing the membranes can be deposited on a whole wafer containing hundreds of ISFETs. This can be done by means of spinning as usual with photoresist layers in the ISFET processing. The layer, from which the thickness can be controlled by the viscosity of the membrane material and the spinning speed, can further be patterned by the application of photolithography, a technology which is also compatible with the usual ISFET production process. In this way very small quantities of enzymes are necessary per sensor, which is advantageous, especially for expensive enzymes.

In order to compensate for the pH of the analyte, it is very easy to carry out a differential measurement between an enzyme modified ISFET and a bare ISFET, because two ISFETs can easily be integrated in one chip without increasing the price.

The advantages mentioned above look very promising and we will now summarize the literature concerning ISFET based enzyme electrodes in order to be able to discuss the real significance of the relevant ISFET features.

REPORTED RESULTS ON ISFET-BASED ENZYME SENSORS

Although Janata & Moss (1976) had already suggested in 1976 the possibility of designing an enzyme-modified ISFET, the first preliminary results were not published until 1980. In that year Caras & Janata (1980) described a penicillin-responsive device, which comprised a dual pH sensitive ISFET, one ISFET having a membrane on the gate of cross-linked albumin-penicillinase and the other having a membrane of only cross-linked albumin. The device was called an ENFET. When penicillin was present in the analyte, the penicillinase present in the active gate membrane catalyzed the hydrolysis of penicillin to penicilloic acid. The released protons caused a local decrease in the pH near the ISFET gate, resulting in an ISFET output signal. The second ISFET remained unaffected, because its membrane did not carry the enzyme penicillinase, so this ISFET can be operated as a reference device (REFET). A schematic drawing of their measurement set-up can be seen in Fig. 1.

The advantages of the differential-mode measurement were reported to be the relative insensitivity towards thermal effects and variations in the analyte pH. It was also reported that the sensors need only a minute amount of enzyme and that the attachment can be so good that the membrane does not need frequent replacement as with conventional enzyme sensors. Response times of approximately 25 s and lifetimes of two months with intermittent usage were reported.

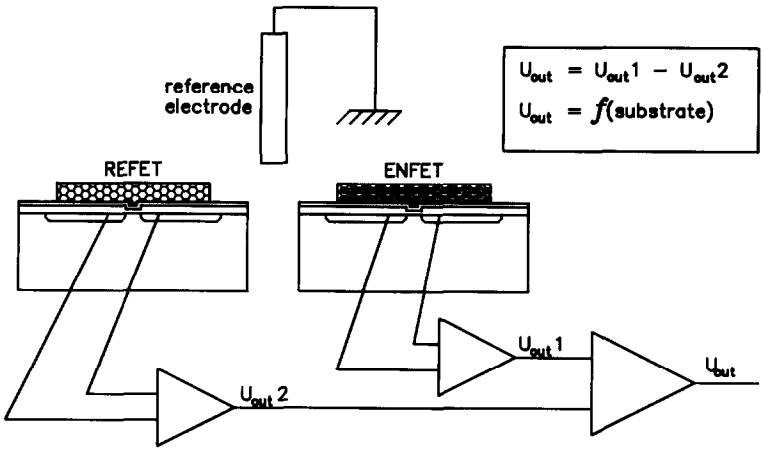

Fig. 1. Experimental set-up for ENFETs using one ISFET with an enzyme loaded membrane as the sensor and one ISFET with an unloaded membrane as a reference.

It was mentioned, however, that the buffer capacity of the analyte had a profound influence on the sensor sensitivity and its linearity as well as on the concentration range. A decrease in analyte buffer capacity suppressed the internal membrane pH less, enabling greater device sensitivity, whereas an increase in buffer capacity caused the reverse. A buffer concentration of 5 mM phosphate at pH 7 resulted in a linear response to penicillin in the range of 0·2–6 mM at a sensitivity of 16 mV $mmol^{-1}$ penicillin. A buffer concentration of 20 mM caused a decrease in sensitivity to 3·1 mV $mmol^{-1}$ but increased the linear range to 25 mM. The upper limit of the response curve increases with higher buffer capacity. In the case of a 20 mM buffer this upper limit was found to be 70 mM, corresponding to an internal membrane pH at the gate interface of approximately 4·7. The pH dependence of penicillinase activity is bell shaped with a maximum pH 7. The authors calculated that this result would correspond to a reduction of the penicillinase activity at pH 4·7 of at least 72%. It was also noted that the device response was sensitive to solution stirring, which is not surprising because substrate and product concentration in the membrane are of course complex functions of distance and diffusion rates.

It can be concluded that the first reported results of ENFETs show that these devices have indeed some advantages, especially with respect to the dimensions, the membrane fixation and the dual sensor design for easy differential measurements, but that various problems already known from conventional enzyme electrodes, such as buffer dependence of sensor response and pH dependence of enzyme activity, are not solved. This is of course not surprising, because the ISFET application does not influence these typical enzyme electrode related properties.

The proceedings of the first international meeting on chemical sensors, held in September 1983 in Fukuoka, Japan, contain two contributions on ENFETs. The experiments of Miyahara *et al.* (1983) confirm the results mentioned above. They describe a urea and an acetylcholine ENFET, measurement being made with respect to a reference FET on which glycine was immobilized instead of urease and acetylcholinesterase, respectively. It was reported that although the pH response of the enzyme modified ISFETs did not change due to the formation of an organic membrane, the response of the devices to a step change of pH was much slower than that of the bare ISFETs. It was further found that the temperature and ambient pH changes in the solutions were automatically compensated by the differential-mode measurement, if the temperature and pH characteristics of ENFET and REFET were the same. The sensitivity of the ENFETs was also found to be affected by the concentration of the phosphate buffer, which suppresses the locally produced pH change with increasing concentration.

Hanazato & Shiono (1983) report the results of a glucose oxidase ENFET, measured with respect to a bare ISFET. This means that the time constants of the two devices are unequal, resulting in a temporary differential signal after a pH change in the sample solution which was not observed by the aforenamed authors. On the other hand, this paper explicitly describes the use of the differential measurement through the application of a platinum pseudo-reference electrode. This is an important advantage because this electrode is much easier to fabricate and miniaturize than the Ag/AgCl/saturated KCl reference used by others.

More papers on ENFETs appear in the open literature in 1985, concerning the theoretical basis as well as technology and application. Both Caras and co-workers (Caras & Janata, 1985*a*,*b*; Caras *et al.*, 1985*a*,*b*) and Eddowes and co-workers (Eddowes, 1985; Eddowes *et al.*, 1985*a*,*b*) independently published extensive theoretical models. The main factors, as indicated in Fig. 2, on which the device response depends are the relative rates of diffusional mass transport of all the species involved, the enzyme kinetics (which are pH dependent) and the buffer capacity and bulk pH of the sample solution. This was confirmed by the corresponding experimental verification on glucose oxidase modified ENFETs and penicillinase-modified ENFETs. Very recently, Eddowes (1987) made another contribution to this series of theoretical papers in which he presents an analytical solution for the response in the presence of a buffer. It can be concluded from these extensive studies that it is the enzymatically controlled proton generating process which creates the practical problems of ambient interference, and not the actual sensor part of the device, i.e. the ISFET. Partial solutions can be found by the differential measuring approach, but in practical applications a rigid control over the sample pH and buffer capacity is required.

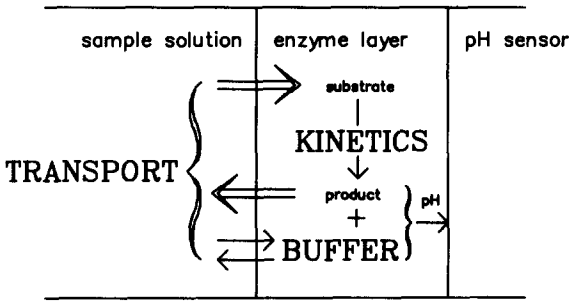

Fig. 2. Diagram to illustrate that the response of pH-based enzyme sensors is governed by the transport rates of the chemical species, the enzyme kinetics and the buffer capacity of the sample.

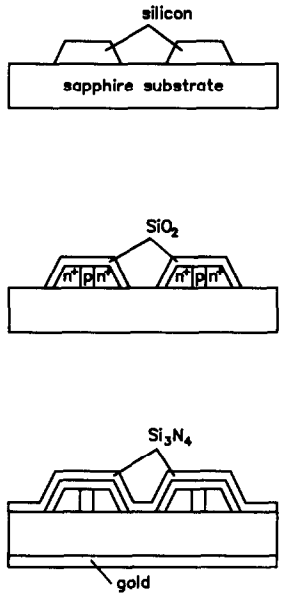

Fig. 3. Diagram to show that in the Silicon on Sapphire technology (SOS), transistors are fabricated in small silicon islets, deposited on an insulating Al_2O_3 substrate.

For the fabrication of the ISFET part of their enzyme sensors, Miyahara and co-workers (Miyahara *et al.*, 1985; Moriizumi & Miyahara, 1985) and Kimura and co-workers (Kimura *et al.*, 1985, 1986; Kuriyama *et al.*, 1985) use silicon on sapphire (SOS) technology. Figure 3 shows that here ISFETs are formed in silicon islets, deposited on an insulating Al_2O_3 substrate. This means that on the wafer all ISFETs are electrically insulated. Furthermore, the chips are surrounded by sapphire and surface insulation layers of SiO_2 or Si_3N_4, so that the edges are insulated from measurement solutions too.

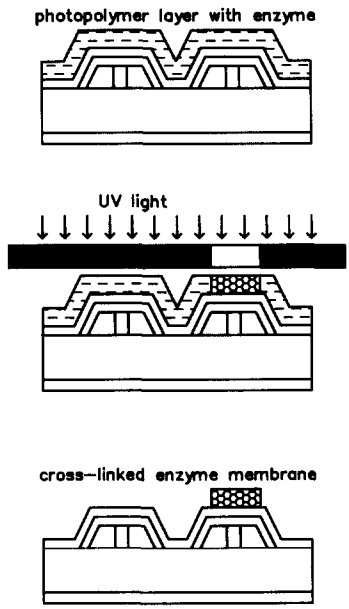

Fig. 4. Diagram to show the use of photo-sensitive PVA for the selective deposition of enzyme membranes over the ISFET gate areas.

Accordingly, when many ISFETs are formed on the wafer, and the individual sensors are taken by cutting the wafer, there is no need for insulating the cut edge. Thus, sensor encapsulation is made easier because only the bonding wires have to be insulated carefully. As the SOS ENFET is differentially measured against an inactivated SOS ENFET, a metallic pseudo-reference electrode can be used. Therefore, Kimura uses a layer of gold deposited on the backside of the insulating sapphire substrate.

Both research groups mentioned above with regard to SOS technology also introduce IC-technology compatible methods for the deposition of enzyme membranes on ISFETs. This is an important advantage over the previously shown individual membrane casting techniques because only then can the full potential of microfabricated sensors be exploited. Moriizumi & Miyahara (1985) used photosensitive polyvinyl alcohol (PVA) for selectively depositing membranes that contain urease. The polymer films are applied by spin coating and then patterned photolithographically as illustrated in Fig. 4. The sensitivity of the sensors fabricated in this way is lower than that of those with individually cast membranes because the membranes are thinner. The lifetime of the sensors was reported to be 20 days. Kuriyama *et al.* (1985) describe a different approach that is shown in Fig. 5. They deposit an acetylcellulose membrane over an entire wafer by spin coating and successively immobilize urease in this membrane using

glutardialdehyde. Finally, the enzyme is locally inactivated by ultraviolet light through the use of a photomask. An attractive feature of this technique may be that ENFET/REFET pairs that are created this way behave identically in every respect, electrically as well as chemically, except for the substrate sensitivity of the ENFET.

A lift-off method for selectively depositing membranes over the pH-sensitive gate area of ISFETs, using membrane materials that are not

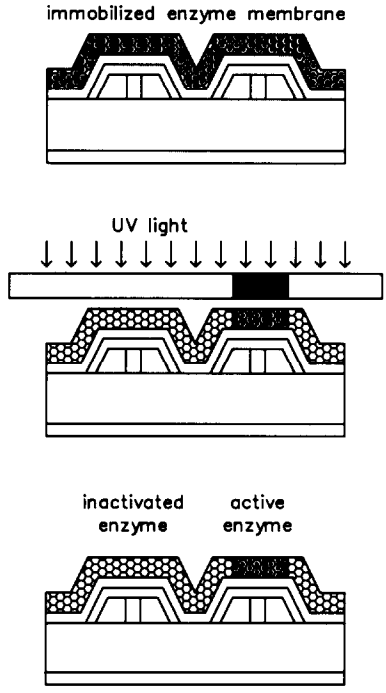

Fig. 5. Diagram to show that through the local inactivation of immobilized enzymes, ENFETs and reference-FETs are fabricated on the same chip without the need for patterning the membrane.

photosensitive themselves, was described by Kuriyama *et al.* (1986). In Fig. 6 it can be seen that first a layer of photoresist is deposited over the entire wafer and selectively removed from the gate areas. After silanization of the surface, a solution containing albumin, the enzyme and an amount of glutardialdehyde for cross-linking is spin coated on the wafer. After the cross-linking is complete, the enzyme membrane other than that over the gate areas is removed by dissolving the underlying photoresist in acetone.

A further advantage of the use of ISFETs for enzyme sensors could be the construction of multi-sensors. The possibility of fabricating several transistors on one chip surely surpasses the glass electrode as a detector. In

the literature, a number of multi-enzyme FETs are reported; however, up to now these devices are always fabricated by individual casting of the different membranes.

Kimura and co-workers (Kimura *et al.*, 1985, 1986; Kuriyama *et al.*, 1985) used membranes of enzyme cross-linked with albumin, deposited in micro-pools over the ISFET gates. A sensor that simultaneously measures glucose and urea is described and the cross-talk between the different sensors located at a distance of 300 μm is investigated (Kimura *et al.*, 1986).

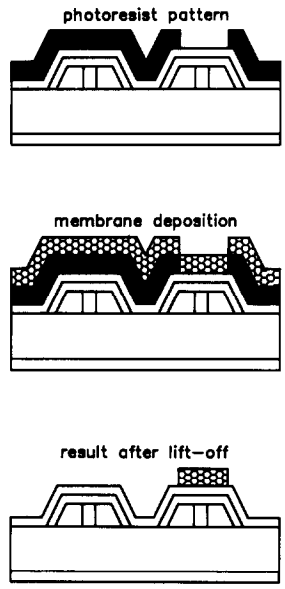

Fig. 6. Diagram to show that the lift-off technique can be used to pattern membrane materials that are not photo-sensitive themselves.

Another construction of a multi-enzyme sensor is described by Hanazato *et al.* (1986*a*) for the case of a combined urea, glucose, pH and pseudo-reference electrode. Here separate chips are used, assembled together on an epoxy laminate. The particular enzyme solutions were dropped over the gate areas and spun out to relatively thin layers without mixing. The experimental results are again similar to those already described above.

A fabrication method for multi-enzyme sensors that overcomes the problem of individual manual membrane casting is proposed by Kuriyama *et al.* (1986). On the wafer, micropools are formed around the gate areas using a photo-polymer. In these micropools, minute drops of enzyme solution are injected by means of an ink jet nozzle. The wafer is mounted on a directed

X–Y stage so that mass production should be possible. Unfortunately, no experimental details are available yet. Recently, it was reported that the lift-off technique can also be applied for the fabrication of multi-enzyme sensors (Murakami *et al.*, 1987). A disadvantage of this method is that some loss of enzyme activity occurs in the process.

Anzai *et al.* (1985) again describes a urea sensor based on an ISFET. They found, in agreement with the results mentioned above, that the sensor shows a higher response in a slightly acidic buffer solution (pH 6) than in buffer solutions of pH 7·1 and 8, which can be explained in terms of the activity of the immobilized urease, the catalytic ability of which is a function of pH. Having a maximum activity at pH 7–7·5, it is reasonable to start at pH 6, because then the urea reaction shifts the pH value of the membrane interior in the alkaline direction due to the consumption of H^+. Thus it is preferable to employ the sensor in a slightly acidic buffer, to obtain the maximum performance of the sensor. Again it was observed that even under these optimal conditions, increasing the buffer concentration, e.g. from 0·5 to 20 mM, decreases the sensitivity to 20% of its maximum.

Another urea ENFET is reported by Karube *et al.* (1986). It is shown that the pH sensitivity of the original ISFET is not affected by the 1 μm thick urease-containing membrane nor by the organic composition or method of attaching, which can be chemical or physical. The response time appears to be strongly influenced by the thickness of the membrane as would be expected. The authors have compared the properties of the free and the immobilized enzyme and they conclude that, while for the free urease the pH for optimal activity is 6·5–7·5, the highest activity for the immobilized enzyme is found at pH 6.

The investigations described up to now deal mainly with urea and glucose sensors. The positive and negative experiences are, however, not caused by the enzymes chosen for these feasibility studies. Gotoh *et al.* (1986) report similar experiences with an ATP modified ISFET. Their sensor system consisted of two ISFETs, one having a cross-linked poly vinyl butyral resin loaded with H^+-ATPase and the other having only the cross-linked poly vinyl butyral resin membrane. Both devices were measured differentially. The response to ATP typically showed a rapid initial increase in the differential output, followed by a gradual decay. This is explained by the fact that the rapid generation of hydrogen ions measured by the ISFET is followed by a period where this pH change causes a decrease in enzyme activity. This is the well-known pH dependency of the enzymes.

Another type of ENFET is described by Hanazato and co-workers (Hanazato *et al.*, 1986*b*; Nakako *et al.*, 1986) for neutral lipid determination. Here, photosensitive poly vinyl pyrrolidone (PVP) is used for the membrane and the authors claim that the composition of this material is much

easier to optimize than that of the previously used PVA. Again, two ISFETs are used, one having an immobilized lipase membrane and the other unmodified. It is shown that the higher the lipase content, the bigger the differential output voltage of the system. Unfortunately an increase of the lipase content made the membrane mechanically very weak. The optimum was found to be a mixture of 15 mg lipase, 10 mg BSA and 200 μl of photopolymer solution for a practical membrane. Using lipase B instead of lipase A (B having about a 50 times greater activity per milligram than A) increased the sensitivity four to five times. Also, in this case, dependence on the buffer capacity was observed

COMMENTS ON REPORTED RESULTS

This paper started with an inventory of the problems that are related to the fabrication and application of pH sensor based enzyme electrodes. When the reported ENFET results, as reviewed above, are analyzed, it can be concluded that so far the most important improvements obtained by the use of ISFETs are in the methods of fabrication. ENFETs have the potential of being mass produced and in the light of the number of papers that arise from industrial research laboratories, it is to be expected that these devices will become commercially available in the not too distant future.

To date, ENFETs have been completely analogous to sensors based on pH glass electrodes, albeit fabricated in a more sophisticated manner. Problems with regard to the pH and buffer dependence of the response have not been solved. However, a number of ENFET papers have made meaningful contributions to the theoretical understanding of these devices. It is important to recognize that enzyme sensors actually consist of two distinct functional parts as was shown in Fig. 2. First, there is the immobilized enzyme membrane which provides selective recognition of the substrate. In this selector part of the biosensor, protolytes are generated through the catalytic conversion of substrate and thus a pH change is created in the membrane. The second part of the biosensor is the transducer part, i.e. the FET, that converts the selectively induced pH change into an electrical signal. Through this bipartition it is very easy to see that the adjective 'Nernstian', that is used by some authors to describe the response of enzyme sensors, has no meaning. The term Nernstian should be reserved to describe the potential that arises from the thermodynamic equilibrium condition that exists in an electrochemical cell. The response from enzyme electrodes may in some cases produce a change of 1 pH dec^{-1} of substrate concentration but this is only so under very specific conditions.

The most popular substrates that have been measured with ENFETs are

glucose and urea. In Tables 2 and 3, the experimental results of several authors are summarized. The results for glucose-FETs as given in Table 2 are difficult to compare because of the differences in sample pH and buffer capacity. For instance, Hanazato and co-workers (Hanazato & Shiono, 1983; Hanazato *et al.*, 1986*a*) used a 20 mM phosphate buffer at pH 5, but at this pH the solution has a very low buffer capacity. Most other authors use a pH around 7 although the optimal pH range for the enzyme glucose oxidase (GOD) is around pH 5. It can be seen, however, that especially at physiological pH values the sensitivity of these sensors is limited to some millivolts per decade. Considering the relatively high drift rate of ISFETs (1 mV h^{-1}) it can be concluded that these devices need frequent calibration and thus are, apart from the problem of the pH and buffer dependence of the response, not serious candidates for in-vivo measurement of glucose. Also, for in-vitro applications, these sensors have serious competition from amperometrically operating devices (Turner & Pickup, 1985).

The results for urea sensitive FETs as given in Table 3 are more comparable because of the greater uniformity in sample pH and buffer capacity. The sensitivity in this case is of the order of some tens of millivolts per decade. In general, the response is faster than for the comparable glucose-FETs. Because of the quite acceptable experimental results and the lack of an amperometric alternative, it may be expected that the prospects for the practical use of urea-FETs are fairly good.

The response of ENFETs depends on the pH and buffer capacity of the sample and thus for a practical application, these variables have to be rigidly controlled. So far, this problem has not been brought any nearer to a solution by the application of an ISFET instead of a pH glass electrode, and thus the general applicability of ENFETs is still limited. Further improvement in the operation of enzyme sensors has to be found in the use of a compensation method for the buffer capacity and by stabilizing the operation of the enzyme in the membrane. In the next section we will present a possible solution to this problem.

COMPENSATION METHODS FOR THE BUFFER CAPACITY OF THE SAMPLE

The sensitivity of pH based enzyme sensors is strongly dependent on the buffer capacity and pH of the sample solution. Furthermore, the response is non-linear for a number of reasons. First, the buffer capacity of a solution is pH dependent. A simple buffer system consisting of one acid/conjugated base pair is most effective when its pH is equal to the pK_a value of the acid, i.e. when the concentration of acid and base are equal. It can be stated that

for each pH unit deviation from this optimum value, the buffer capacity decreases approximately tenfold. Of course more effective buffer solutions can be composed using mixtures of buffering components, but in general the buffer capacity will be sample dependent.

Secondly, products that are formed in the enzymatic reactions are locally changing the pH in the membrane. However, these products are not in general free protons or hydroxyl ions but weak acids and bases, the dissociation of which depends on the pH of the solution. In the case of a glucose sensor, gluconic acid is formed. This acid has a pK_a of 3·77 so when measurements are performed above pH 5, it may be considered as completely dissociated and the response is rather straightforward. In the case of urea, the products are bicarbonate, ammonium ions and hydroxyl ions and the influence on pH is more complicated. Also, at low buffer concentrations of the sample solution, the products of the enzymatic reaction may contribute considerably to the overall buffering capacity of the solution in the membrane and thus influence the response.

A third, and perhaps even more important, cause for the non-linear response of enzyme electrodes are the pH dependent enzyme kinetics. It is well known that each enzyme has a specific optimum pH value at which its activity is highest. For instance, glucose oxidase has its optimum at pH 5·1 and the maximum activity for urease is found around pH 7–7·5. It must be noted that this optimum pH may change as a result of immobilization (Karube *et al.*, 1986). As a consequence of this pH dependent activity, the dynamic range of enzyme electrodes is limited, as was already described by Caras & Janata (1980) in their first ENFET paper. In low capacity buffers, the sensitivity is high but the upper limit of the linear range is restricted. For buffers with a high capacity the opposite is true. For practical use of enzyme electrodes it is thus required that pH and buffer capacity of the solutions used for calibration and of the sample solutions are equal.

As a potential solution for the problem of buffer dependency we have investigated the possibilities of a coulometric pH control system. This system consists of an ISFET with an integrated noble–metal electrode around its pH-sensitive gate. This electrode can either be used as an anode or cathode to produce hydroxyl ions or protons through the electrolysis of water. Through the generation of these ions, pH changes are created in the vicinity of the ISFET, changes that can be used to determine the buffer capacity. Previously, the present authors have shown the practical value of such a system for rapid acid–base titrations (Van der Schoot & Bergveld, 1985) and in a new type of carbon dioxide sensor with an excellent long-term stability (Van der Schoot & Bergveld, 1986). The application of this technique for the improvement of enzyme electrodes has also been proposed by Chandler & Eddowes (1986).

TABLE 2
Glucose Sensitive FETs

Author	Hanazato (1983)	Miyahara (1985)	Kimura (1985, 1986)	Caras *et al.* (1985*b*)	Eddowes *et al.* (1985*a*)	Hanazato *et al.* (1986*a*)	Hanazato *et al.* (1986*a*)	Kuriyama *et al.* (1986)	Hanazato *et al.* (1986*b*)
Membrane	PVA	PVA	Albumin	Polyacrylamide	poly-HEMA	PVA	PVA	Albumin	PVP
Buffer	Phosphate 20 mM, pH 5	Phosphate 10 mM, pH 7	HEPES 10 mM, pH 7·5	Phosphate 0·2–1 mM, pH 7·2	Phosphate 1–10 mM, pH 7·2	Phosphate 20 mM, pH 6	Phosphate 20mM, pH 5·1	—	Acetate 10 mM, pH 5·5
Dynamic range (mgl^{-1})	–300	100–10 000	50–500	18–180	—	50–1 000	—	100–1 000	–900
Sensitivity ($mV\ dec^{-1}$)	30	8	2·5	50–25	—	—	10 mV at 200 mgl^{-1}	15	10 mV at 540 $mg\ l^{-1}$
Response time 90%	2 min	10 min	—	2 min	—	—	1 min	30 s	1 min
Lifetime	—	Some days	>30 days	2 weeks	—	1 week	—	—	—

TABLE 3
Urea Sensitive FETs

Author	Miyahara *et al.* (1983)	Miyahara *et al.* (1985)	Moriizumi & Miyahara (1985)	Kimura (1985, 1986)	Anzai (1985)	Kuriyama *et al.* (1985)	Hanazato *et al.* (1986*a*)	Kuriyama *et al.* (1986)	Karube *et al.* (1986)
Membrane	Acetyl-cellulose	PVA	PVA	Albumin	Albumin	Acetyl-cellulose	PVA	Albumin	Directly coupled
Buffer	Phosphate 10 mM, pH 7	Phosphate 10 mM, pH 7	—	HEPES 10 mM, pH 7·5	Phosphate 1–20 mM, pH 6	Phosphate 10 mM, pH 7	Phosphate 20 mM, pH 6	HEPES 20 mM, pH 7·5	Tris-HCl 5 mM, pH 6·5
Dynamic range ($mg\ l^{-1}$)	50–10 000	100–10 000	—	10–1 000	30–1 200	10–2 500	50–1 000	5–1 000	1 000–10 000
Sensitivity	25	18	—	50	10–50	10	20	15	<10
Response time	1 min	1 min	—	1 min	Some min	10 s	1–2 min	5 s	<1 min
Lifetime	27 days	Some days	20 days	>30 days	—	—	1 week	—	>3 weeks

Experimental

To investigate the possibilities of the coulometric buffer compensation system we have made use of urea-sensitive ISFETs. The ISFETs are *n*-channel devices fabricated with standard metal oxide semiconductor (MOS) technology. Tantalum oxide, used as the pH-sensitive gate insulator, is formed by oxidation of a tantalum film that is applied onto the wafer by evaporation. The ISFETs are mounted on a carrier and encapsulated with epoxy resin. The enzymatic membrane consists of a cross-linked mixture of albumin and urease. First, the surface of the ISFET is silanized using γ-aminopropyltriethoxysilane (γ-APTES). Twenty five milligrammes of albumin (Bovine albumin, Sigma A-7030) and 25 mg urease (EC 3.5.1.5, Sigma Type IX, U-4002) are dissolved in 0·5 ml 25mM phosphate buffer at pH 7. To this solution, 0·2 ml glutardialdehyde (2% in water) is added and a drop of this mixture is applied to the gate area of the ISFET. After some hours the cross-linking reaction is complete and the ENFETs are ready for use. The membrane thickness is estimated to be of the order of some hundreds of micrometres: however, this thickness is not very reproducible because of the manual casting procedure. The diameter of the membrane is approximately 0·8 mm.

Of course, these ENFETs can be operated in the usual way and their characteristics are very similar to those described in the previously reviewed papers. Figure 7 shows the response of the sensor after repeated additions of urea to the measuring solution. After each addition the solution is stirred, which causes a temporary drop in the response. Ninety per cent of response

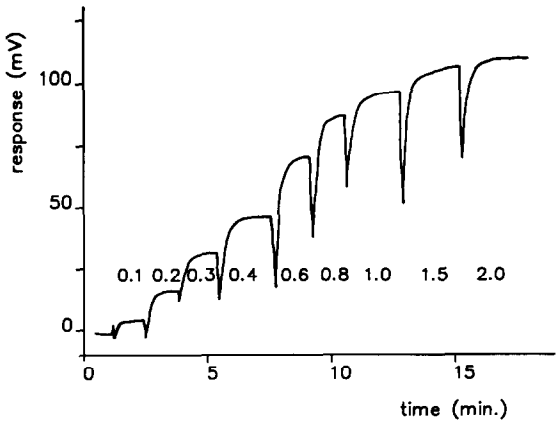

Fig. 7. Time response of urease/albumin ENFETs. The sensor output is sensitive to the stirring of the solution after each addition of urea. The figures at the curve indicate the urea concentration in mmol litre^{-1}.

times are of the order of 10–20 s. The response of these sensors is, of course, also dependent on the buffer capacity of the sample solution as is clearly demonstrated in Fig. 8.

In principle, there is a great similarity between an enzyme-FET and an ISFET with a pH-actuator electrode. In both cases, local pH changes are created, either through an enzymatic reaction or through the conversion of electric charge into an equivalent amount of ions. The electroanalytical technique that uses the equivalence between an electrical charge (in Coulombs) and an amount of ions is called coulometry. The analogy between both systems is illustrated in Fig. 9a and b. It can be seen that, just like the enzyme sensor, the response of the coulometric system is also dependent on the buffer capacity of the sample solution.

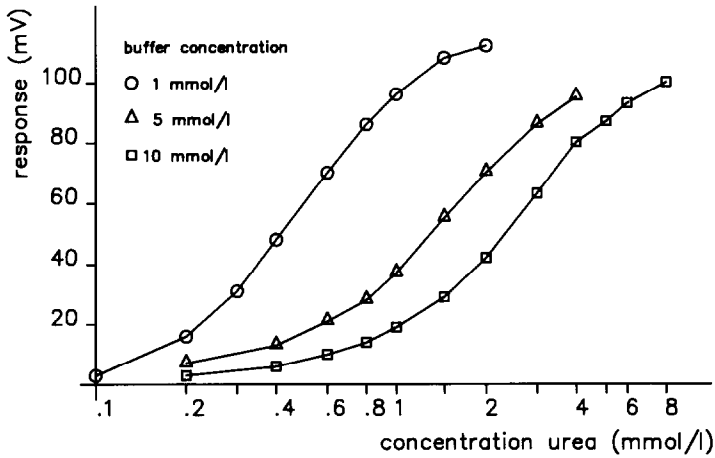

Fig. 8. Graph showing that the sensitivity and dynamic range of the urea-ENFET depend on the buffer capacity of the sample.

To create an enzyme electrode that is independent of the buffer capacity of the sample solution, we have investigated a control system as shown in Fig. 10. When a substrate is offered to the enzyme electrode, this results in a pH change in its membrane which is measured by the ISFET. The operation of the system is such that an identical pH change is generated at the gate of the coulometric reference ISFET which is thus tracking the ENFET. The magnitude of the current that is needed for this pH change should now be a measure for the enzyme activity and thus for the substrate concentration. The 'controller' that was used for these preliminary experiments actually consisted of the human experimenter who manually adjusted the potentiometer that controls the magnitude of the generating current. When the time constants of the system are determined this manual control can easily

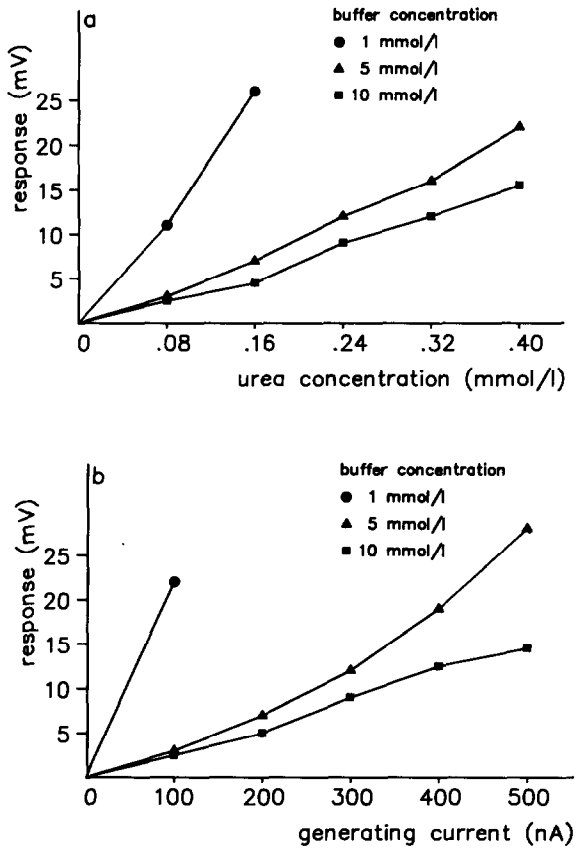

Fig. 9. A comparison between (a) enzymatically and (b) coulometrically induced pH changes in dependence of the buffer capacity of the solution.

be automated as was demonstrated earlier (Bergveld *et al.*, 1983). In Fig. 11 the required current is given as a function of substrate concentration for different buffer solutions. It appears that for relatively high buffer capacities and a low substrate concentration an excellent agreement between generating current and substrate concentration can be observed, independent of the sample solution. However, with higher substrate concentrations as well as with a lower buffer capacity (1 mmol) this method is not of much use. The deviations between the enzymatically and coulometrically induced effects that occur at higher pH changes can be explained as follows. In the first place. the activity of the enzyme is pH dependent whereas the coulometric method is not. Thus, at higher pH changes, the enzyme is inhibiting its own activity, resulting in a non-linear response. The second cause for a levelling off of the response is that the

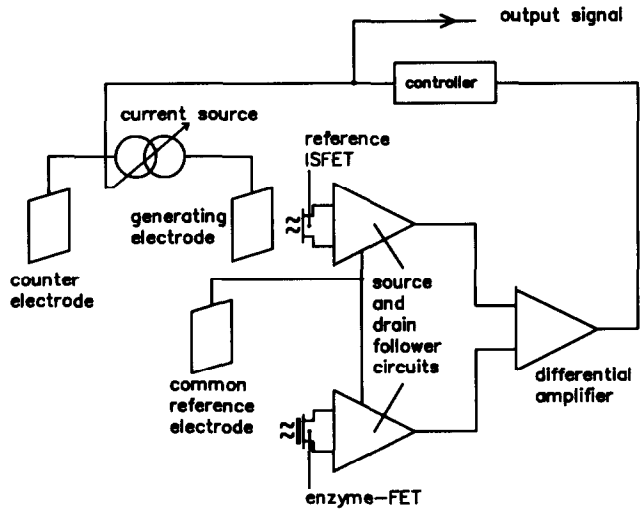

Fig. 10. Coulometric control system in which the pH at the reference-ISFET is kept equal to that at the ENFET.

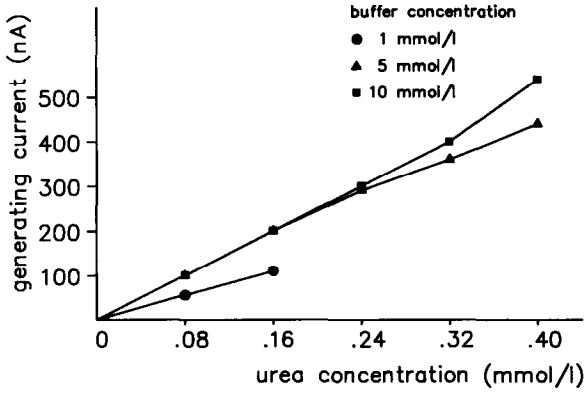

Fig. 11. Generating current as a function of urea concentration for the system depicted in Fig. 10. Only for small pH changes the system is insensitive to the buffer capacity.

products of the enzymatic reaction have buffering properties themselves. In the case of a urea-sensitive electrode, ammonia and bicarbonate are formed in the enzyme layer. This locally increases the buffer capacity of the solution in the membrane and thus depresses the response. In conclusion, it can be said that, although in some cases this control system renders the operation of the enzyme electrode buffer independent, the method is not generally applicable. Therefore we have improved this method by the integration of the enzyme layer and the coulometric pH-actuator on the same device.

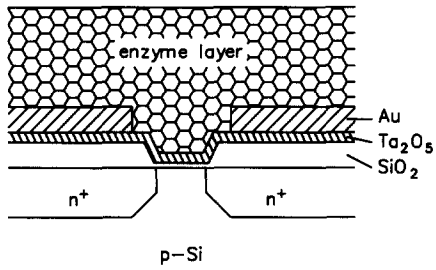

Fig. 12. Schematic cross section of the pH-static enzyme sensor. The enzyme membrane is deposited over the entire structure.

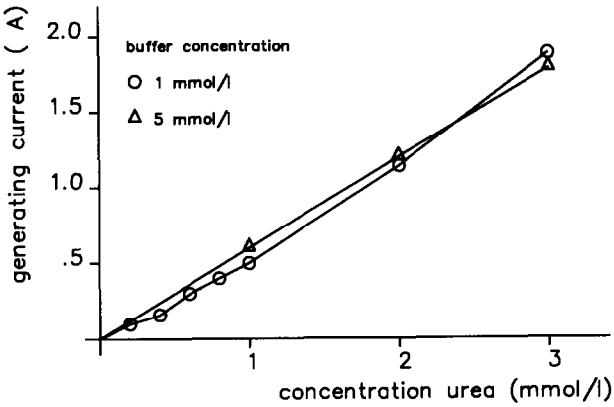

Fig. 13. Graph showing that the output of the pH-static enzyme sensor is independent of the sample buffer capacity and the dynamic range is expanded.

Figure 12 shows a cross-section of an ENFET with an integrated actuator electrode. In this configuration the coulometric system can be used to control the pH inside the immobilized enzyme layer. The sensor is operated in a system similar to that of Fig. 10, only in this case the ENFET and the reference-FET have changed places. The reference-FET is continuously measuring the pH in the bulk of the sample solution. When a substrate is offered and the pH in the enzyme layer is increasing, this is compensated by the generation of H^+ ions at the actuator electrode. Thus, the pH in the enzyme layer is kept equal to that in the bulk of the sample solution. The enzyme operates at a constant pH and we propose to call this sensor a 'pH-static enzyme sensor'. Because the feedback now takes place directly in the enzyme layer, changes in the buffer capacity by the products of the enzymatic reactions are automatically compensated for. Figure 13 shows the relationship between generating current and substrate concentration for

two different buffer solutions. It can be seen that the response is now largely buffer-independent. A second important advantage of this system is that because the enzyme operates at a constant pH, it has a constant activity. The response of this system is linear with concentration as is demonstrated in Fig. 13. From Fig. 8 it can be seen that for a 'normal' ENFET at low buffer capacity the output levels off at substrate concentrations above 1 $mmol^{-1}$. For the pH-static sensor, however, the upper limit of the dynamic range is not restricted by a decreasing activity of the enzyme, simply because it is kept at its optimal pH.

Discussion and conclusions

The results described above for the pH-static enzyme sensor show that it is a practical system. However, the construction that we have used for these first experiments is not yet optimal. The enzyme membrane is attached over the entire sensor–actuator structure. During the coulometric generation of H^+ ions the pH is of course not uniform across the entire membrane but a gradient exists throughout the enzyme layer. Only at the gate area of the ISFET, where the pH is measured, is the pH equal to that of the bulk of the sample solution. At the surface of the generating electrode, the pH is necessarily much lower. As a result of this, deformations occur in the albumin membrane and the response time of the sensor increases as compared to the 'normal' ENFET. After the sensor has been used for some time in the pH-static mode, the response time stabilizes and is in the order of several minutes for 90% response. The construction of the sensor should thus be optimized by choosing a different geometry or other membrane materials.

The integration of an enzymatic membrane and a pH-actuator offers the unique possibility of controlling the internal membrane pH. Of course, the method is not limited to keeping the membrane pH equal to that of the sample solution. As we have seen in a previous section, each enzyme has its own optimum pH range. If the sample pH deviates from this optimum pH, by this coulometric method it should also be possible to shift the pH in the membrane to a more favourable value. In that case, the reference-ISFET also has to be provided with an actuator electrode and should, coulometrically, be brought to the same pH. The difference in generating currents between reference- and enzyme-FET is again directly related to the enzyme activity. In this way, it should be possible to decrease the lower detection limit of the electrode and to improve the stability of the control system.

In conclusion, it can be said that the first results with the pH-static enzyme sensor are very promising. Through the application of a direct feedback, the sensor becomes independent of the buffer capacity of the sample solution,

the response is linear and the dynamic range can be expanded. The general applicability of pH-based enzyme sensors will surely be enlarged by this new method of measurement.

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