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Detection of protein concentrations using a pH-step titration method[1]

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Abstract

A stimulus–response method based on the application of a pH step is proposed for the detection of protein immobilized in a membrane on top of an ion-sensitive field-effect transistor (ISFET). The ISFET response to a step-wise change in pH, applied at the interface between the membrane and the surrounding solution, depends on the concentration of protein immobilized in the membrane because proton-dissociation reactions of the protein cause a delayed diffusion of protons and hydroxyl ions through the membrane. Our theoretical description shows that the delay in ISFET response is linearly related to the concentration of protein immobilized in the membrane. Experiments performed with lysozyme as a model protein show the feasibility of this detection principle. © 1997 Elsevier Science S.A.

Keywords: Protein; Ion-sensitive field effect transistor (ISFET); pH-step titration

1. Introduction

Biosensors capable of determining protein concentration have been extensively studied throughout the past few decades. Since the invention of the ion-sensitive field-effect transistor (ISFET) [1], research has been focused on the development of ISFET-based biosensors for this purpose. It was shown [2,3] that the static response of an ISFET cannot be modulated by (charged) protein molecules immobilized on top of the ISFET gate, due to the suspension effect counteracting any potential effect. However, proper detection of immobilized protein with an ISFET was shown to be possible by using the ion-step method [4,5]. This stimulus–response method exploits the dependence of the proton-dissociation equilibria of immobilized protein molecules on the electrolyte concentration. The transient ISFET response results from an uptake or release of protons by the protein molecules after a step-wise change in electrolyte concentration [6].

The pH dependence of the proton-dissociation equilibria of immobilized protein molecules can also be exploited by making use of titration. One possible way to add titrant to the protein membrane is by means of coulometric generation of protons or hydroxyl ions, using a noble metal electrode deposited closely around the ISFET gate. This ISFET-based coulometric sensor-actuator device has been used for the measurement of acid or base concentration in solution [7] and, after modification with a porous gold actuator electrode, for the determination of the buffer capacity of protein in solution [8]. More recently we showed that this sensor-actuator device can also be used for the measurement of protein concentration in a membrane on top of the device [9]. A drawback of this method is that the pH at the surface of the actuator electrode cannot be controlled. This sometimes leads to loss of membrane adhesion and may lead to protein damage.

As an alternative method to adding titrant, a known pH step can also be applied at the membrane-solution interface. The ISFET response on the pH step, which is delayed as a result of proton-dissociation reactions of immobilized protein molecules, will be related to the protein concentration.

A theoretical description of this process, in which protons and hydroxyl ions take part in both diffusion

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and chemical reactions, is presented. Furthermore, a qualitative description of the delayed ISFET response is given, using a one-dimensional simulation model. As an example, the influence of lysozyme adsorption on the measured pH-step response has been investigated, using membranes consisting of polystyrene beads in an agarose gel, deposited on top of the ISFETs.

2. Theory

After a step-wise change in pH at the interface between the protein-containing membrane and the surrounding electrolyte solution both protons and hydroxyl ions will start to diffuse through the membrane in opposite directions. (We assume 1 M KCl solutions of pH 4 and 10 without addition of any pH-buffering components). These diffusion processes will be delayed as a result of protein-dissociation reactions of immobilized protein molecules. For protons this delayed diffusion process can be described by a modified form of Fick's second law of diffusion [10].

$$\frac{\partial c_{H+}}{\partial t} = D_{H+}\frac{\partial^2 c_{H+}}{\partial x^2} - \frac{\partial c_{HProt}}{\partial t} \quad (1)$$

where c_{H+} is the concentration of mobile protons in the membrane, D_{H+} is the diffusion coefficient of these protons in the membrane and c_{HProt} is the total concentration of protonated groups in the membrane. When only diffusion in one dimension is taken into account, all concentrations are a function of time t and distance x from the ISFET surface. According to Eq. (1), the change in the proton concentration at a location x in the membrane is a function of both diffusion (first term on the right-hand side) and proton-dissociation reactions of immobilized protein molecules (second term on the right-hand side). This last term can be written as

$$\frac{\partial c_{HProt}}{\partial t} = \frac{\partial c_{HProt}}{\partial \mathrm{pH}}\frac{\partial \mathrm{pH}}{\partial c_{H+}}\frac{\partial c_{H+}}{\partial t} = \beta_{Prot}\frac{1}{2.3c_{H+}}\frac{\partial c_{H+}}{\partial t} \quad (2)$$

where β_{Prot} is the buffer capacity of the immobilized protein and $2.3c_{H+}$ that of the supporting electrolyte. For β_{Prot} we can write:

$$\beta_{Prot} = c_{Prot} f(c_{H+}) \quad (3)$$

where c_{Prot} represents the immobilized protein concentration and f is a complex function which is independent of this protein concentration, but dependent on c_{H+} and on reaction constants of protein groups. If we assume that $\beta_{Prot} \gg 2.3c_{H+}$, then a combination of Eqs. (1)–(3) gives:

$$\frac{\partial c_{H+}}{\partial t} = \frac{1}{c_{Prot} f'(c_{H+})}\frac{D_{H+}}{}\frac{\partial^2 c_{H+}}{\partial x^2} \quad (4)$$

Eq. (4) shows that the diffusion of protons in the membrane is delayed by a factor that depends linearly on c_{Prot}. Diffusion of hydroxyl ions through the membrane can be described in a similar way. Thus, the time needed to obtain a certain change, ΔpH at the ISFET surface always depends linearly on c_{Prot}.

3. Experimental

ISFETs with a Ta_2O_5 gate insulator, having a sensitivity of -58.5 mV pH^{-1} were fabricated in the MESA clean room following the usual processing steps. After mounting the chips on a piece of printed circuit board, they were encapsulated using hysol epoxy, leaving a circular area around the gate with a diameter of 1.7 mm and a depth of 150 μm uncovered. The membranes, consisting of polystyrene beads ($\varnothing = 0.1$ μm) in an agarose gel with a thickness of 8–16 μm, were casted in this area [4]. Lysozyme (from chicken egg white, Sigma, St. Louis MO) was adsorbed in the membrane by incubation in solutions containing lysozyme (3E-7–3E-4 M) in 100 mM KNO_3, pH 7.4. Except for the first series of experiments the protein solution was stirred during the incubation, in order to reduce protein-concentration gradients in the solution. After each incubation step the devices were rinsed for 1 min, before mounting them in the wall-jet cell of a flow-through system in which a pH-step was applied in 0.1 s [5]. All experiments were performed in 1 M KNO_3, using HNO_3 and KOH to adjust the pH to 4 and 10, respectively. After mounting the devices in the wall-jet cell, they were rinsed with the pH 4 solution for 2 min, before the pH step was applied. The ISFET response, measured as the output voltage V_s of a source-drain follower, was recorded with a Nicolet 310 digital oscilloscope.

4. Results and discussion

4.1. Simulation results

A one-dimensional simulation model based on the Nernst–Planck and Poisson equations [6], in which all the acid–base reactions occurring in the membrane are taken into account, has been used to give a qualitative description of the pH-step titration process. In these simulations, a pH step is applied at a 2 μm thick stagnant layer which is assumed to be present in front of an 8 μm thick membrane. Diffusion coefficients in the membrane are assumed to be 4/10 of those in water (this value is based on experience with ion-step experiments). Lysozyme, used as a model protein, is assumed to contain 11 carboxylic groups ($pK_a = 4.4$), two imidazole groups ($pK_a = 6.0$) and nine amino groups ($pK_a = 10.4$) per molecule. Concentration profiles of all species involved in the titration process at any moment in time

as well as the pH at the ISFET surface as a function of time can be calculated.

As an example the ISFET response to a pH step from 4 to 10 applied outside an 8 μm thick membrane containing 3 mM lysozyme in equilibrium with a 1 M KNO_3 electrolyte solution is simulated. Fig. 1 shows the calculated concentration profiles of protons and hydroxyl ions at three consecutive moments in time during the pH-step titration. At t_1, the concentration of protons, c_{H+}, at the interface between membrane and stagnant layer (at $x = 8$ μm) has already decreased to

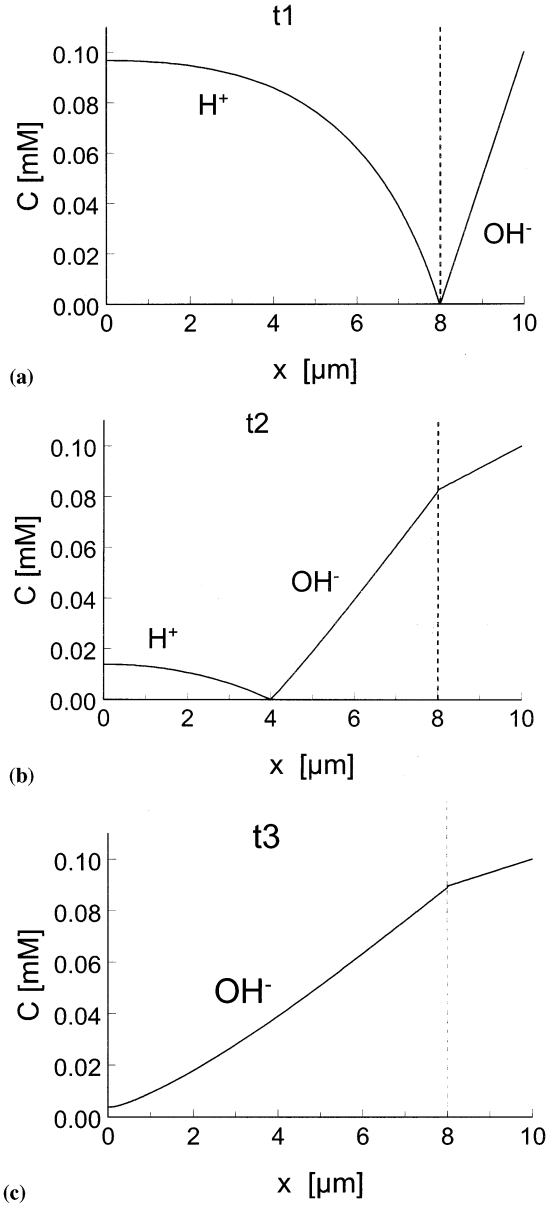

Fig. 1. Calculated concentration profiles of mobile protons and hydroxyl ions in the membrane and the stagnant layer at three consecutive moments in time after a simulated pH step from pH 4 to 10. The vertical dashed lines indicate the interface between the membrane (0–8 μm) and the stagnant layer (8–10 μm)

1E-7M (pH 7), which is approximately zero on a linear scale. This reaction plane where pH 7 moves through the membrane towards the ISFET surface, reaching $x = 4$ μm at t_2. At t_3, the reaction plane has reached the ISFET surface ($x = 0$), thus, the concentration of hydroxyl ions has become larger than the proton concentration. It should be noted that not only mobile protons react with hydroxyl ions but also protons that dissociate from acidic and basic lysozyme groups. These dissociation reactions strongly delay the titration process. Where in the case of a bare ISFET the situation at t_3 would be reached in ≈ 50 ms, in the case of a membrane with 3 mM of lysozyme it would take more than 4 s.

Fig. 2 shows the concentration profiles of proton-dissociating groups for the same moments in time. Each graph shows the profiles of the three different types of proton-dissociating groups considered. At t_1 all the amino groups ($pK_a = 10.4$) and almost all the imidazole groups ($pK_a = 6.0$) are still in their undissociated form, since the pH in the membrane ranges from almost 4 at the ISFET surface to 7 at the interface between membrane and stagnant layer. For a lysozyme concentration of 3 mM, this is equivalent to 6 mM of protonated imidazole groups (t_1: curve 2) and to 27 mM of protonated amino groups (t_1: curve 3). Only part of the acidic carboxylic groups ($pK_a = 4.4$) lost their protons (t_1: curve 1). Note that the dissociation equilibrium of these carboxylic groups follows the local pH exactly, resulting in a concentration profile ranging from 23.6 mM of protonated carboxylic groups at the ISFET surface, which is the equilibrium concentration at pH 4 to approximately 0 mM at the interface between the membrane and the stagnant layer.

At t_2 only a small part of the originally undissociated carboxylic groups is still in possession of its proton (t_2: curve 1). Also approximately half of the imidazole groups and a small part of the amino groups have dissociated their proton (t_2: curves 2 and 3, respectively).

Finally, at t_3 all the carboxylic groups and almost all the imidazole groups have dissociated their proton. Since the highest pH in the membrane at t_3 is about 9.9 (at $x = 8$ μm), only a small part of the amino groups has become deprotonated.

At t_3 ≈ 33 mM of protons dissociated from protein groups have reacted with hydroxyl ions, compared to 0.1 mM of free protons, indicating that the protein completely determines the titration process. This is in agreement with the condition necessary for Eq. (3) that the buffer capacity of the protein β_{Prot} has to be much larger than the buffer capacity of the electrolyte solution.

Fig. 3a shows the ISFET response at a pH-step from pH 4 to 10 as a function of time for different concentrations of lysozyme present in the membrane. The dashed

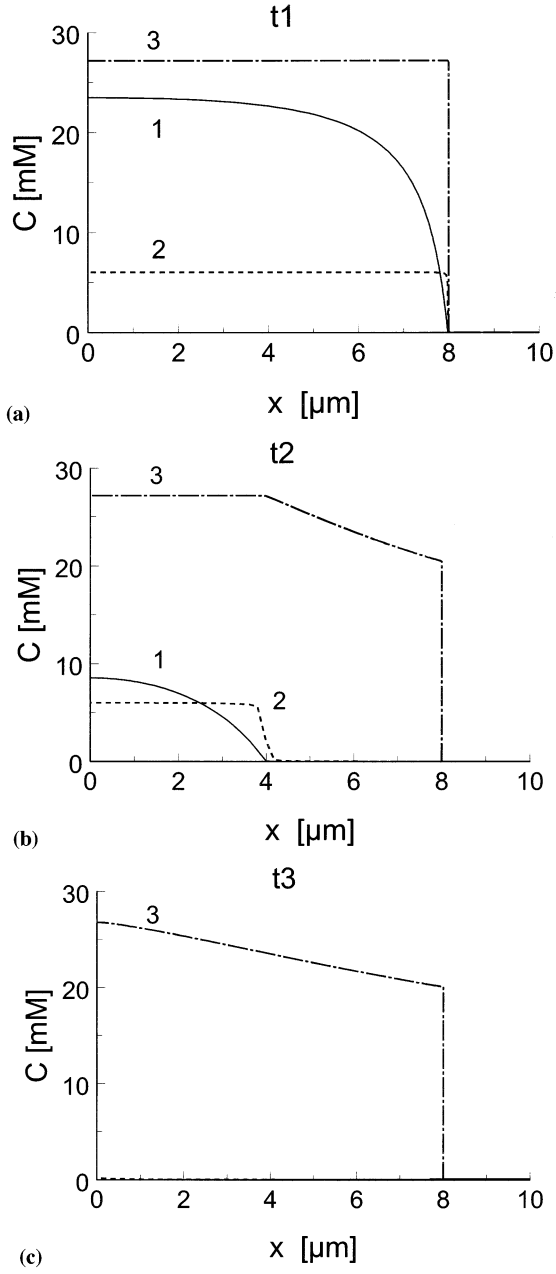

Fig. 2. Calculated concentration profiles of the three types of immobile proton-dissociating groups in the membrane at three consecutive moments in time (see Fig. 1) after a simulated pH step from pH 4 to 10. Concentrations of protonated carboxylic (1) imidazole (2) and amino groups (3).

line indicates the ISFET response corresponding to pH 7. Clearly, an increasing in the concentration of lysozyme immobilized in the membrane corresponds to a stronger delay in ISFET response. Fig. 3b, curve 1 shows the linear relation between the time needed to reach pH 7 at the ISFET surface, defined as t_{pH7}, and the concentration of immobilized lysozyme, c_{Prot}, that can be derived from these responses. This linear relation is in agreement with Eq. (4).

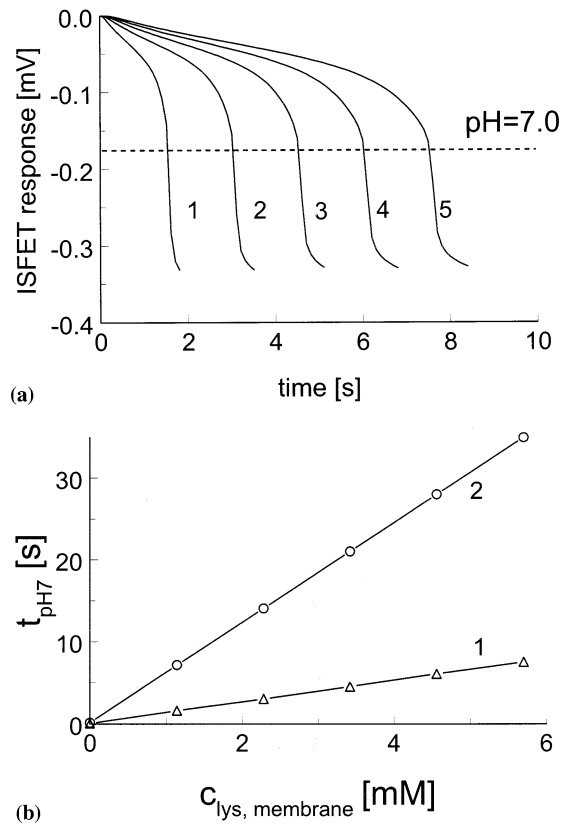

Fig. 3. (a) Simulated ISFET response to a pH step from pH 4 to 10 as a function of time for membranes containing 1.14 (1), 2.28 (2), 3.42 (3), 4.56 (4) and 5.7 mM (5) of lysozyme. The horizontal dashed line represents the ISFET response at pH 7.0; (b) Relation between t_{pH7} and the concentration of lysozyme immobilized in the membrane for a pH step from pH 4 to 10 (1) and 4 to 9 (2), found by simulations.

The magnitude of the pH step also influences the ISFET response. A step from pH 4 to 9 instead of 10 will result in a larger value for t_{pH7}, since less base is added per unit time. Fig. 4 shows the ISFET response

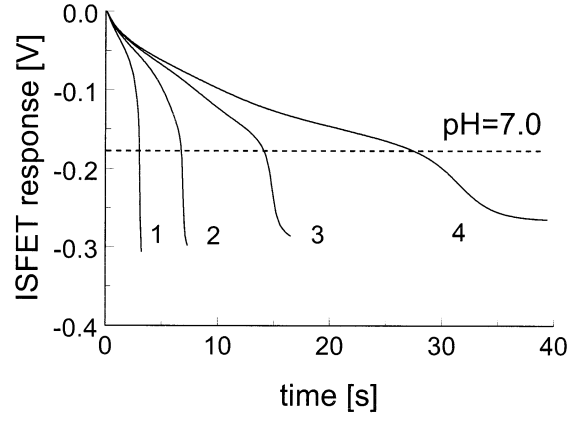

Fig. 4. The dependence of the ISFET response on the magnitude of the pH step found by simulations, for a membrane with a fixed concentration of lysozyme. A pH step is applied from pH 4.0 to 10.0 (1), 9.5 (2), 9.0 (3) and 8.5 (4).

as a function of time for a device with a fixed concentration of lysozyme in the membrane, for different magnitudes of the pH step. Clearly the delay in ISFET response strongly increases when the step magnitude decreases. Curves 1 to 4 are the result of pH steps from pH 4.0 to 10.0, 9.5, 9.0 and 8.5, respectively. Simulated pH steps from 4 to 9 for different concentrations of immobilized protein result in t_{pH7} values that are approximately 4.5 times larger than those where a pH step from 4 to 10 is used (Fig. 3b, curve 2).

The simulation results presented here clearly show the dependence of the ISFET response on the concentration of protein immobilized in a membrane on top of the device. Since the number and type of proton-dissociating groups determine the protein buffer capacity ($\delta c_{HProt}/\delta_{pH}$) and since the ISFET response directly reflects the change in pH as a function of time, in principle the shape of an ISFET response also contains information on the type of protein that is immobilized. This aspect has not been investigated yet.

Note that, since in practical experiments the concentration of immobilized protein after an incubation step is related to the protein concentration in the incubation solution through a complex adsorption process, the measured relationship between t_{pH7} and protein concentration is not expected to be linear in that case.

4.2. Experimental results

Fig. 5a shows typical ISFET responses to a step from pH 4 to 10, as a function of the cumulative incubation time in a 3×10^{-5} M lysozyme solution. A time shift, resulting in a clear dependence of t_{pH7} (intersection with dashed line) on the incubation time in a lysozyme solution is found. Since $t_{pH7} \approx 50$ ms for an ISFET without a membrane, the value $t_{pH7} = 1.85$ s found before incubation (curve 1) indicates that the bare membrane already contains a certain amount of buffering components. This is probably due to the presence of carboxylic groups on the polystyrene beads. Since a measured value for t_{pH7} can be regarded as the sum of the time needed for the titration of the bare membrane plus the time needed for the titration of the immobile protein, we define a Δt_{pH7} as the shift in t_{pH7} as a result of incubation in a protein solution. Fig. 5b shows Δt_{pH7} as a function of the cumulative incubation time for incubation in 3E-6, 3E-5 and 3E-4 M lysozyme solutions.

After this first series of experiments we incubated six devices in six different lysozyme solutions for 30 min. The results shown in Fig. 6, curve 1 show a clear dependence of Δt_{pH7} on the lysozyme concentration in the sample solution. We stirred during the 30 min incubation process in order to reduce protein-concentration gradients in the solution.

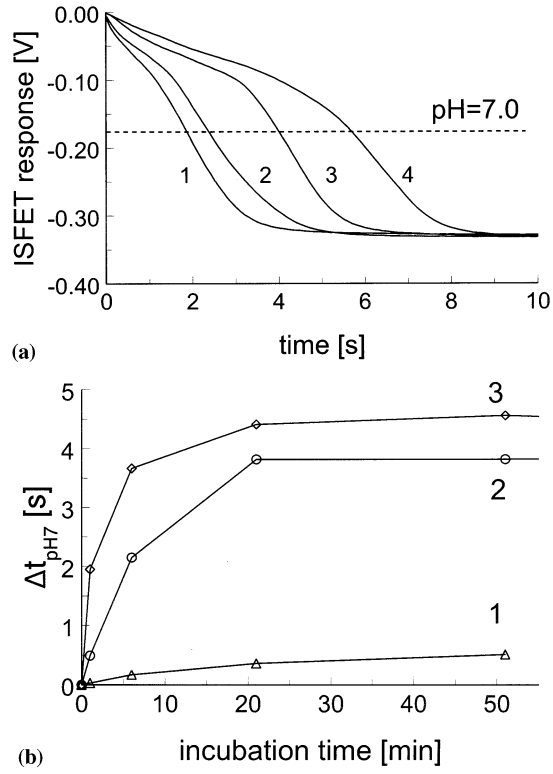

Fig. 5. (a) Measured ISFET response to a pH step from pH 4 to 10: before incubation (1), after 1 min (2), 6 min (3), and 21 min (4) of incubation in a 0.5 mg ml^{-1} lysozyme solution. The dashed line indicates the ISFET response at pH 7; (b) Δt_{pH7} as a function of the cumulative incubation time in a 3E-6 (1), 3E-5 (2) and 3E-4 M (3) lysozyme solution.

The measured effect of the pH-step magnitude on the delay in ISFET response is shown in Fig. 7 for one device with a fixed concentration of lysozyme (3E-6 M) in the membrane. A decrease in step magnitude clearly induces an increase in the response time. The measured values for Δt_{pH7} after a pH step from 4 to 9 are much larger than those measured after a pH step from 4 to 10

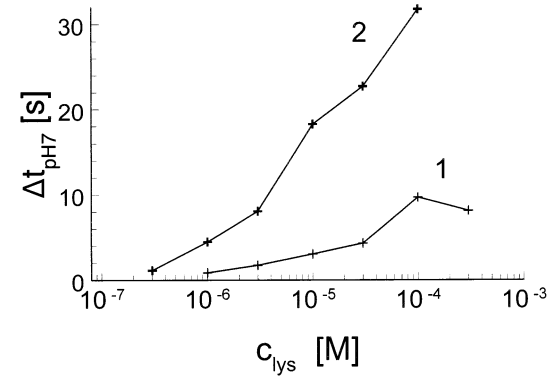

Fig. 6. Measured relation between Δt_{pH7} and the concentration of lysozyme in the incubation solution for a pH step from pH 4 to 10 (1) and 4 to 9 (2), in both cases after an incubation time of 30 min.

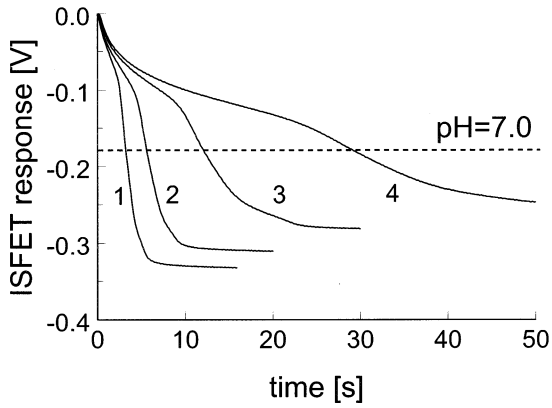

Fig. 7. The measured dependence of the ISFET response on the magnitude of the pH step, for a membrane with a fixed concentration of lysozyme. A pH step is applied from pH 4.0 to 10.0 (1), 9.5 (2), 9.0 (3) and 8.5 (4).

(Fig. 6, curve 2), which is in agreement with simulation results. We did, however, not find a constant ratio between the two curves, which is probably due to differences in thickness and/or density between the individual membranes.

5. Conclusions

After immobilization in a membrane on top of an ISFET, protein molecules can be detected by means of a pH-step titration. The experimental results show that without any optimization of membrane composition measurement of lysozyme in concentrations ranging from 3E-7 to 3E-4 M is possible with the pH-step titration method. Simulation results clearly show a linear relation between the delay in ISFET response, represented by the time needed to reach pH 7 at the ISFET surface, and the concentration of protein immobilized in the membrane. Since the concentration of immobilized protein after an incubation step is related to the protein concentration in the incubation solution through a complex adsorption process, the measured relation between t_{pH7} and protein concentration in the sample solution is not linear. However a clear dependence of t_{pH7} on protein concentration is found, using lysozyme as a model protein. Qualitatively the experimental results are in good agreement with simulations, proving that in principle it is possible to measure protein concentrations using a pH-step stimulus–response method.

In order to lower the detection limit and to improve the device-to-device reproducibility other membrane materials may have to be investigated. Especially for selective protein detection, requiring specific receptor molecules deposited in the membrane, more hydrophillic membrane materials are probably necessary, since the hydrophobic polystyrene beads are known to easily adsorb proteins aspecifically.

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Biographies

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Jan C.T. Eijkel was born in Amsterdam, the Netherlands, on September 3, 1955. He received his M.Sc. degree in pharmacy from the University of Amsterdam in 1979 and his pharmacist degree in 1980. While working as a pharmacist he received his B.A. in theology in 1985 at the University of Utrecht. In 1989, he started working at the Biosensor Technology group of the MESA Research Institute of the University of Twente, where he obtained his Ph.D. degree in 1995. The subject of his dissertation was the potentiometric detection of proteins.

Piet Bergveld was born in Oosterwolde, The Netherlands, on January 26, 1940. He received his M.Sc. degree in electrical engineering from the University of Eindhoven, the Netherlands, in 1965 and his Ph.D. degree from the University of Twente, the Netherlands, in 1973. The subject of his dissertation was the development of ISFETs and related devices and the actual invention of the ISFET. It has also been investigated by many international research groups of Universities as well as industry. Since 1965 he has been a member of the Biomedical Engineering Division of the Faculty of Electrical Engineering (University of Twente) since 1965, and in 1984 was appointed as Full Professor in Biosensor Technology. He is one of the project leaders in the MESA Research Institute. His research subjects concern the further development of ISFETs and biosensors based on ISFET technology as well as physical sensors for biomedical and environmental applications, resulting up to now in more than 250 papers. He was appointed research dean of the Faculty of Electrical Engineering in 1994.