An ISFET-based dipstick device for protein detection using the ion-step method

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Abstract: A compact and easy-to-operate device for the detection of proteins in solution is described. The device consists of an ISFET pH-sensor in the middle of a Ag/AgCl electrode, on top of which a microporous composite membrane is deposited. The measurement proceeds by offering an ion step and measuring the transient change of the ISFET potential versus the Ag/AgCl electrode. The potential transient is modulated by adsorption of protein to the membrane by incubation in a protein-containing solution. Theory is developed to describe the ISFET potential transient versus the Ag/AgCl electrode starting from the ISFET and Ag/AgCl potential transients versus an external reference electrode. Conditions are formulated under which the Ag/AgCl electrode potential is sufficiently stable to be used as the reference. Both theory and preliminary measurements show that this condition is met under several characteristic measuring conditions. When the internal reference electrode is used, the ion step can be offered simply by manual transference ('dipping') of the device between vessels of different salt concentration instead of by using a complex flow-through set-up. Measurements show a remarkable absence of noise in the potential signal of the dipstick device. ©1997 Elsevier Science Limited

Keywords: biosensor, ISFET, Ag/AgCl electrode

INTRODUCTION

When a microporous membrane, deposited on an ISFET, is subjected to a sudden increase in the salt concentration of the bathing electrolyte, the ISFET potential shows a transient potential change. This transient is related to the fixed-charge density of the membrane, and can be modulated by adsorption of charged macromol-

ecules to the membrane. This so-called ion-step measuring method is a valuable tool for the ISFET-based detection of charged macromolecules like the protein lysozyme (Schasfoort *et al.*, 1990a; Eijkel *et al.*, (1997a, b)). Prior immobilization of affinity ligands in the membrane causes specific adsorption of one ligand, as was demonstrated for the couple human serum albumin/anti-HSA, the couple heparin/protamin (Schasfoort, 1989; Schasfoort *et al.*, 1990a; van Kerkhof *et al.*, 1993) and for the non-charged molecule of progesterone by using a competition reaction with a charge-labelled derivate

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(Schasfoort *et al.*, 1990b). In the original set-up, the ISFET potential is measured versus a reference electrode placed in the bathing solution, whilst the ion step is offered using a flow-through system (Schasfoort, 1989; Schasfoort *et al.*, 1990a). This set-up is not practicable when the method is to be used for clinical diagnostics, and certainly when it is used in a bed-side situation. The present paper offers a possible solution for this problem by the development of a device with an integrated (pseudo-)reference electrode for the ISFET. Such a device can be removed from solution without disconnecting the electrical contact between both electrodes, so that the ion step can be offered in a dipstick manner.

In this paper, theory is developed to interpret the potential transient of the integrated device in proton-buffered solutions and measurements are performed to check its functioning.

The measurement device and set-up

A cross-sectional representation of the measuring device is shown in Fig. 1. It consists of a chip with a pH-sensitive ISFET in the middle of a pCl-sensitive Ag/AgCl electrode. On top of both sensors a microporous membrane is deposited with a thickness of 8 μm , consisting of polystyrene beads of 112 nm diameter in an agarose matrix. The device is placed in a bathing electrolyte. Measurements are performed by increasing in a step-wise manner the KNO_3 concentration in the electrolyte (the ion step). In the electrolyte the pH and pCl are kept constant. On the ion step a potential transient can be measured in three measuring configurations: measuring the ISFET versus the SCE, the Ag/AgCl electrode versus the SCE or the ISFET versus the Ag/AgCl elec-

trode (the dipstick configuration). Use of an SCE necessitates the use of a flow-through system to offer the ion step. In the dipstick configuration the ion step can also be offered by manual transfer of the device between vessels with a different KNO_3 concentration, since the electrical connection between ISFET and Ag/AgCl electrode is guaranteed by the electrolyte remaining in the membrane pores.

THEORETICAL SECTION

It will be shown that the Ag/AgCl-measured transient is caused by a rapid change of the average membrane potential, followed by a slightly slower diffusional re-equilibration of the Cl^- ions between electrolyte and membrane. Both phenomena influence the Ag/AgCl potential in opposite directions, causing the transient to return to zero in time. The initial membrane potential change is caused by the increased shielding of the membrane-fixed charge due to the ion step. The diffusional re-equilibration restores electrochemical equilibrium of the Cl^- ions. The ISFET-measured transient basically is caused by identical phenomena now involving proton diffusion. However, its amplitude is larger because it represents the potential change at the pore walls and not the average pore potential change. This phenomenon occurs because the protons are buffered by groups fixed at the pore walls. These groups will release protons into the pore solution to maintain equilibrium on an ion step. The resulting pH change is measured by the ISFET in addition to the average potential change. Because the proton release continues during the diffusional re-equilibration phase, it will also give the ISFET-measured transient a longer duration. (Eijkel *et al.*, (1997a, b)).

Fig. 1. Cross-sectional representation of the ion-step measuring device.

Assumptions

For the description of the processes occurring on an ion step the following assumptions are made:

- (1) The specific adsorption of ions other than protons is negligible.
- (2) The agarose has a net zero charge in the investigated pH range, and shows no proton association. The assumption is justified since the type of agarose used is characterized by a zero endo-osmotic flow in the investigated pH range.

- (3) The bead surface can be represented by an isolated flat polystyrene surface, since the Debye length is negligible in relation to the bead diameter at the salt concentrations used (Schulz *et al.*, 1994).
- (4) The surface charge is smeared-out.
- (5) Pores have dimensions of 10–100 nm, so that the electrical double layer does not extend to the pore centre.
- (6) Between membrane and Ag/AgCl electrode exists an electroneutral electrolytic interlayer with a negligible thickness relative to the membrane.
- (7) The system can be described as one-dimensional in the direction perpendicular to the sensor surface. This is justified since the pore diameter is small enough to assure a constant equilibrium in the plane parallel to the sensor surface. The electrolyte/membrane interface is located at $x = 0$, the ion sensor surfaces at $x = d$ (see Fig. 1).
- (8) Both ion sensors react Nernstian and instantaneously to the concentration of their potential determining ion at $x = d$.

The origins of the measured potential transient

The ion sensor responses in three configurations

The ISFET is connected to an ISFET amplifier of which the output voltage will be denoted as the 'ISFET potential'. In the measuring configuration of ISFET versus SCE, the measured potential can change owing to the generation of the electrical potential difference between $x = 0$ and $x = d$, and to a pH change at the ISFET surface. Defining the potential transient relative to $t = 0$ we obtain

$$\Delta\Phi_{\text{ISF}}(t) = \Delta\Phi(t, d) - \frac{2.3RT}{F} \Delta\text{pH}(t, d) \quad (1)$$

where Φ is the electrical potential (volts), D is the location of the ISFET surface and the other symbols have their usual meaning. The potential transient of Ag/AgCl versus SCE is in an analogous way given by

$$\Delta\Phi_{\text{Ag/AgCl}}(t) = \Delta\Phi(t, d) + \frac{2.3RT}{F} \Delta\text{pCl}(t, d) \quad (2)$$

The ISFET versus Ag/AgCl potential transient in the dipstick configuration is deduced by subtracting Eq. (2) from Eq. (1).

$$\Delta\Phi_{\text{Dip}}(t) = - \frac{2.3RT}{F} (\Delta\text{pH}(t, d) + \Delta\text{pCl}(t, d)) \quad (3)$$

Thus, the term representing the potential change at $x = d$ is absent in the dipstick-measured transient. It will be shown that the transients can be described by four characteristics, namely the amplitude extreme, $\Delta\Phi_{\text{extr}}$, and three time constants. The time constants are, in ascending order of magnitude, those of the offered ion step, τ_{step} , of the diffusion of the (equally mobile) K^+ , Cl^- and NO_3^- ions through the membrane, τ_{Cl} , and of buffer diffusion through the membrane, τ_{Buf} .

The Ag/AgCl versus SCE transient

The charge fixed at the bead surfaces gives rise to a pore wall potential, Φ^w , with a double layer extending into the membrane pore. When the KNO_3 concentration in the pores rises, the salt ions will increase the screening of the fixed charge at the pore walls and thus decrease the absolute value of the wall potential. In positively charged membranes, a wall potential decrease results and in negatively charged membranes a potential increase. The potential change at the electrolyte/membrane interface will, according to Eq. (2), cause a change of the Ag/AgCl electrode-potential versus the external SCE. The time constant of this change is equal to the time constant of the offered ion step, τ_{step} . The Ag/AgCl electrode will measure the average potential change over the total pore area, which will be denoted $\overline{\Delta\Phi}$. It determines the extreme of the Ag/AgCl-measured transient:

$$\Delta\Phi_{\text{Ag/AgCl,extr}} = \overline{\Delta\Phi} \quad (4)$$

The value of $\overline{\Delta\Phi}$ will depend on the pore geometry. Since this is very complex, its calculation will not be undertaken. When the KNO_3 -concentration rise reaches the interlayer, the potential change at the electrolyte/membrane interface is mirrored at the membrane/interlayer interface. This would cause a return of the potential transient to zero (see Eq. (2)). However, directly following the change of the wall potential, Cl^- ions are redistributed across the pores and between interlayer and pores according to Boltzmann. The resulting extreme change of the pCl in the electroneutral parts of pore and interlayer is determined by the average change of the pore potential, $\overline{\Delta\Phi}$, according to (cf. Overbeek, 1956)

$$\Delta pCl_{extr} = \frac{F}{2.3RT} \overline{\Delta\Phi} \quad (5)$$

Since the change of the interlayer pCl as formulated in Eq. (5) will exactly compensate the effect of the potential change at the membrane/interlayer interface, $\overline{\Delta\Phi}$ will still be measured. From electrically mediated, the transient has become chemically mediated. The transient will return to zero due to the diffusional re-equilibration of the Cl^- ions between membrane and electrolyte solution with as a driving force the pCl difference between membrane and electrolyte which now exists. The time constant of this Cl^- re-equilibration, τ_{Cl} (seconds) is given by (Crank, 1975)

$$\tau_{Cl} = \frac{4d^2}{\pi^2 D_{Cl}} \quad (6)$$

where D_{Cl} (metres squared per second) is the membrane diffusion coefficient of Cl^- ions.

The ISFET versus SCE transient

Proton release or uptake. Following the KNO_3 -concentration rise, the protons redistribute rapidly across the pores according to Boltzmann. This redistribution proceeds differently from that of the Cl^- ions since protons are buffered by chemical binding both to wall-fixed groups and to mobile buffer molecules in the pores. For example, following the decrease of the wall potential in a positively charged membrane, protons will diffuse from the pores to the walls. At the walls they will be taken up by the buffering wall-fixed groups, thus resisting the concentration change at the walls. Analogously, the mobile buffer molecules in the pore solution will release protons and resist the concentration change in the pore solution. If, now, the buffer capacity of the wall-fixed groups is much larger than that of the buffer molecules in the pore solution, as is generally the case in the experimental situation, the increase of the pore (and interlayer) pH reaches an extreme, which is directly proportional to the extreme change of the wall potential (Eijkel *et al.*, 1997a):

$$\Delta pH_{extr} = -\frac{F}{2.3RT} \Delta\Phi_{extr}^w \quad (7)$$

This extreme wall potential change can be calculated, for example, by using a Gouy-Chapman model for the double layer. For an ion step

with a 1:1 salt from concentration $c^{e'}$ to $c^{e''}$ it then equals (Eijkel *et al.*, 1997a)

$$\Delta\Phi_{extr}^w = \frac{2RT}{F} \ln \left(\sqrt{\frac{c^{e''}}{c^{e'}}} \frac{s^w + \sqrt{(s^w)^2 + c^{e'}}}{s^w + \sqrt{(s^w)^2 + c^{e''}}} \right) \quad (8)$$

where $c^{e'}$ and $c^{e''}$ (mol/m³) are the salt concentrations at $t = 0$ and $t = \infty$, s^w is $\sigma^w / \sqrt{8RT\epsilon_0\epsilon_r} = 269\sigma^w$ (mol^{0.5} m^{-1.5}), σ^w (C/m²) the surface-charge density on the pore walls, ϵ_0 (C V⁻¹ m⁻¹) the permittivity of free space and ϵ_r the relative permittivity of water. It follows from Eq. (8) that the change of the wall potential can maximally be ± 59 mV per decade step in KNO_3 ; this value is reached at high values of the surface-charge density σ^w relative to $c^{e'}$.

Before the described pH change has reached the interlayer, the ISFET versus SCE transient will be determined by the change of the average pore potential, and be identical to the Ag/AgCl versus SCE transient. After this moment, the interlayer pH change will determine the ISFET potential. Substitution of Eq. (7) into Eq. (1) shows that its extreme change equals the extreme of the change of the wall potential:

$$\Delta\Phi_{ISF,extr} = \Delta\Phi_{extr}^w \quad (9)$$

Thus, the ISFET-measured transient has a larger amplitude than the Ag/AgCl-measured amplitude formulated in Eq. (4).

Proton re-equilibration. The proton re-equilibration phase has been described in the absence of mobile buffer (Eijkel *et al.*, 1997a). Here equations are derived for the case where mobile buffer is present. It is assumed that protons are transported only by mobile buffer molecules, with a negligible contribution of the proton and hydroxyl ion fluxes. Fig. 2 shows an arbitrary moment during the re-equilibration phase. In the electrolyte Buf^- and $BufH$ concentrations are equal since in all experiments $pH = pK_{a,Buf}$.

Assuming one type of protonatable wall-fixed group A and a mobile buffer Buf , the relevant protonation reactions are

Equations (10) and (11) can be combined to the net membrane reaction

Fig. 2. Cross-sectional representation of the measuring device during the phase of proton re-equilibration between electrolyte and membrane. The lines indicate the concentration profiles of the mobile species Buf^- and BufH and the arrows the direction of diffusion. The profiles result from simultaneous diffusion and reaction with fixed AH groups.

In good approximation the diffusion coefficients of the protonated and non-protonated buffer molecules will be equal, so that the sum of the BufH and Buf^- concentrations in the membrane remains constant during the proton re-equilibration and no net transport of buffer into the membrane takes place. In this case the Buf^- concentration will change owing to Buf^- diffusion and to reaction with AH, and the following modified form of Fick's second law of diffusion can be applied (cf. Crank, 1975, p. 326):

$$\frac{\partial c_{\text{Buf}}(t,x)}{\partial t} = D_{\text{Buf}} \frac{\partial^2 c_{\text{Buf}}(t,x)}{\partial x^2} - \frac{\partial c_A(t,x)}{\partial t} \quad (13)$$

where D_{buf} (m^2/s) is the membrane diffusion coefficient of the buffer molecules. It can be derived that

$$\begin{aligned} \frac{\partial c_A(t,x)}{\partial t} &= \frac{\partial c_A(t,x)}{\partial \text{pH}(t,x)} \frac{\partial \text{pH}(t,x)}{\partial c_{\text{Buf}}(t,x)} \frac{\partial c_{\text{Buf}}(t,x)}{\partial t} \\ &= \frac{\beta_A(t,x)}{\beta_{\text{Buf}}(t,x)} \frac{\partial c_{\text{Buf}}(t,x)}{\partial t} \end{aligned} \quad (14)$$

where β is the buffer capacity in moles per cubic metre. Substituting Eq. (14) into Eq. (13) and rearranging gives

$$\frac{\partial c_{\text{Buf}}(t,x)}{\partial t} = \frac{D_{\text{Buf}}}{K(t,x) + 1} \frac{\partial^2 c_{\text{Buf}}(t,x)}{\partial x^2} \quad (15)$$

with

$$K(t,x) = \frac{\beta_A(t,x)}{\beta_{\text{Buf}}(t,x)} \quad (16)$$

The dimensionless term $K(t,x)$ represents the retardation of the diffusion-generated concen-

tration change of Buf^- in the pore solution due to the reaction with the wall-fixed AH groups. Equation (16) shows that K depends on the ratio of the proton buffer capacities of the A^- groups and the Buf^- molecules. K will be approximately constant in the pH range traversed during the ion step in the case that the wall buffer capacity and the mobile buffer capacity are determined by the same functional group (e.g. a carboxyl group). Assuming a constant K , the time constant for buffer diffusion, τ_{Buf} (seconds), will equal (Crank, 1975)

$$\tau_{\text{Buf}} = \frac{4(K+1) \cdot d^2}{\pi^2 D_{\text{Buf}}} \quad (17)$$

The Buf^- concentration at $x = d$ determines $\text{pH}(t,d)$, which in turn determines the ISFET-measured transient versus the SCE. The time constant for the decay phase of this transient will thus be determined by τ_{Buf} .

Note well: to obtain the same dimensions for the concentration of groups Buf and A, the wall concentration of groups A, c_A^w , is multiplied by the amount of pore volume per wall area to obtain the concentration per pore volume, c_A :

$$c_A = c_A^w \frac{3p}{(1-p)r} \quad (18)$$

where p is the polystyrene bead volume fraction in the membrane and r (metres) the bead radius.

The ISFET versus Ag/AgCl transient

According to Eq. (3) this extreme will be determined by the change of the pCl and pH at $x = d$. If $\Delta \text{pCl} \approx 0$ at the moment that ΔpH is still

maximal (see next section), then the transient extreme is given by Eq. (9), and its time constant by Eq. (17).

Conditions for the use of the Ag/AgCl electrode as a reference electrode

The main point of interest is whether the Ag/AgCl-measured potential transient can be considered constant on the time scale of the ISFET transient. This is true if the pCl change on an ion step is much smaller than the pH change, or if the time constant for chloride diffusion is much smaller than for proton diffusion. It follows from Eqs (5) and (7) that the former condition is fulfilled if

$$\Delta\Phi_{\text{extr}}^w \gg \overline{\Delta\Phi} \quad (19)$$

which will depend on the charge density, the pore size and the pore geometry. The latter condition is fulfilled if (combining Eqs (6) and (17) and assuming that $K \gg 1$ in Eq. (17))

$$\frac{\beta_A}{\beta_{\text{Buf}}} \gg \frac{D_{\text{Buf}}}{D_{\text{Cl}}} \quad (20)$$

In the experiments buffer solutions will be used with a β_{Buf} of about 0.25 mM ($c_{\text{Buf}} = 0.5$ mM and pH equal to the pK_a of the buffer). Assuming that D_{Cl} is about twice D_{Buf} , the buffer capacity of the wall-fixed groups must be about five times larger than that of the mobile buffer (i.e. larger than 1.5 mM) to satisfy Eq. (20).

MATERIALS AND METHODS

The sensor part of the measuring device is shown schematically in Fig. 3. ISFET chips with a $15 \times 550 \mu\text{m}$ Ta_2O_5 gate have a 20 nm titanium film and subsequently a $1 \mu\text{m}$ layer of silver deposited around the gate. Using a polyimide mask a square surface of 1 mm^2 of Ag is left uncovered. The chips are mounted on a piece of printed circuit board and encapsulated with Hy-Sol epoxy. Around the gate a circular area with a diameter of 1.7 mm and a depth of $150 \mu\text{m}$ is left uncovered. Subsequently, approximately 120 nm of Ag is coulometrically chloridized ($4 \mu\text{A}/\text{mm}^2$ for 300 s in 0.1 M HCl) (Ives & Janz, 1961). The pCl sensitivity of the Ag/AgCl layers before membrane deposition is 59 mV/pCl. The layers show small transients (amplitude 0–5 mV) of short dur-

ation (1/e fall time 0.1 s) to an ion step from 10 mM KCl to 10 mM KCl + 40 mM KNO_3 . The ISFETs show a pH sensitivity close to Nernstian (– 58 to – 59 mV/pH) and give without a membrane short-lasting (1/e fall time 0.2 s) transient responses to an ion step (amplitude 10–30 mV), whose transient is pH-dependent (van Kerkhof *et al.*, 1994).

In the circular area a polystyrene beads/agarose membrane is cast (Schasfoort *et al.*, 1990a). The casting solution is prepared by 1:1 mixing of a 2.5% solution of polystyrene beads of 112 nm diameter (Polysciences, Warrington, PA, USA) and a 0.25% solution of agarose (Biorad, Hercules, CA, USA; Zero Mr) in demineralized water. After careful mixing at 50°C $3 \mu\text{l}$ of this solution is deposited in the circular area. Subsequently, the device is cooled at 4°C for 12 h to allow slow evaporation of the water. Membranes have a thickness of $8 \mu\text{m}$ when measured in dry conditions with a surface profiler (Sloan Dektak). Finally, the device is heated at 55°C for 1 h. For one device lysozyme (from hen egg white, Sigma, St Louis, USA) is adsorbed in the membrane by incubation for 72 h in a 20 mg/ml lysozyme solution in PBS.

Experiments were performed after an equilibration time of a few hours in a 10 mM KCl solution. In the non-dipstick experiments, the device was mounted in the flow-through system shown in Fig. 4(A). The solutions were transported by creating a nitrogen pressure in the two bottles containing the solutions. Transport proceeds through PTFE tubing towards a valve that can switch between both solutions. A section of tubing with an internal diameter of 0.304 mm and a length of 30 mm leads from the valve to a wall-jet cell where the measuring device is mounted. A tube section with a length of 1.55 cm and a diameter of 1 mm connects the wall-jet cell with the SCE. All ion steps were offered by increasing the KNO_3 concentration in the electrolyte from 0 to 40 mM while keeping the electrolyte pH constant and the Cl^- concentration at 10 mM. The time constant of the concentration rise of KNO_3 at $x = 0$ is about 0.1 s. In the dipstick experiments illustrated by Fig. 4(B) the device is manually transferred from the electrolyte with low salt concentration to the one with high salt concentration. After transference the solution is continuously stirred by hand, using the device. The electrolyte is buffered with 0.5 mM HEPES buffer at pH 7.6 or with 0.5 mM acetic acid at

Fig. 3. Top view of the measuring device.

pH 4-8. All measurements were performed at room temperature (about 21°C). The chemicals used were of analytical grade and were used without further purification. The water was demineralized.

The potential of the Ag/AgCl electrode versus the SCE placed downstream is measured using a

unity gain amplifier with an input impedance of 1 TΩ. A potential proportional to the surface potential of the Ta₂O₅ ISFETs is obtained by the use of a source-drain follower set-up, grounding the solution either with the SCE or with the Ag/AgCl electrode (Bergveld & Sibbald, 1988). All potentials were recorded on a Nicolet 310 Memory Scope.

Fig. 4. (A) The flow-through system used for the non-dipstick measurements. (B) The dipstick measurement procedure.

RESULTS

A device with a bare polystyrene beads/agarose membrane

To a device with a bare membrane ion steps were applied using the flow-through system. Fig. 5 shows the transient potentials measured in the three-electrode configurations. The signal of the Ag/AgCl electrode versus the SCE (1) has been filtered by using a 20 Hz low-pass software filter, the other signals are not filtered.

All transients have a positive sign, indicating negatively charged pore walls. This is as expected for polystyrene beads, carrying sulphate groups

Fig. 5. Potential transients measured in three configurations, using the flow-through system. The device has a bare 8 μm membrane and the ion step is from 10 mM KCl to 10 mM KCl + 40 mM KNO_3 at $\text{pH}^e = 7.6$ (buffer 0.5 mM HEPES).

($\text{pK}_a = 1.7$) and carboxyl groups ($\text{pK}_a \approx 4.5$). Transient (1) is determined by the change of the pCl and of the potential at the interface of membrane and Ag/AgCl electrode, as formulated in Eq. (2). The positive transient indicates an initial increase of the mean pore potential followed by a re-equilibrating decrease of the pCl in the membrane. The re-equilibration phase can be described using a $\tau_{\text{Cl}} = 1$ s, which implies a membrane Cl^- diffusion coefficient of $2.6 \times 10^{-11} \text{ m}^2/\text{s}$, about 0.013 times the value in the electrolyte.

Transient (3) is determined by the change of the pH and of the electrical potential at the interface of membrane and ISFET, as is formulated in Eq. (1). According to theory, its amplitude is directly proportional to the change of the pore wall potential (see Eq. (9)), and its duration is determined by the relative magnitude of the proton buffer capacities of the pore walls and the pore solution.

Since transient (3) has a much larger amplitude than transient (1), the change of the wall potential is much larger than that of the mean pore potential, which is as expected. Its amplitude of +22 mV, according to Eq. (8), indicates a surface-charge density σ^w of about $-1.1 \mu\text{C}/\text{cm}^2$. This is an acceptable value, as literature values range between -1 and $-20 \mu\text{C}/\text{cm}^2$ (see e.g. Norde, 1986). The surface charge density can be converted to a fixed charge concentration as in Eq. (18). Assuming a polystyrene volume fraction p of 0.7, an average fixed charge concentration of -14 mM is obtained.

The re-equilibration phase of transient (3) can be described using a τ_{Buf} of about 2.7 s. When a membrane buffer diffusion coefficient is assumed of $0.016 \times 10^{-9} \text{ m}^2/\text{s}$ (as for Cl^- ions 0.013 times its value in the electrolyte) and for β_{Buf} 0.24 mM is used (the buffer capacity of a 0.5 mM buffer solution at a pH that during the ion step lies between its pK_a and its $\text{pK}_a + 0.2$ pH units), the application of Eqs (16) and (17) results in a membrane buffer capacity β_A of 0.06 mM. This value appears to be low in comparison with the averaged fixed charge concentration of $\sim 14 \text{ mM}$, but indeed in this bare membrane such a small buffer capacity is expected, since only carboxyl ($\text{pK}_a \approx 4.5$) and sulphate groups ($\text{pK}_a \approx 0.7$) will be present on the polystyrene surface, which do not buffer at $\text{pH}^e = 7.6$.

Transient (2) (ISFET versus Ag/AgCl) is theoretically determined by the difference in the change of the pCl and pH at the interface of membrane and ion sensors, as described by Eq. (3). In accordance with theory the transient can be obtained by subtracting both measured transients (1) and (3). Since all contributions of the change of the electrical potential difference between $x = 0$ and $x = d$ are absent in (2), the large remaining transient proves that no diffusion potential is generated by the diffusion of KNO_3 through the membrane. The amplitude of transient (2) is slightly smaller than that of transient (3) (ISFET versus SCE), which is caused by the slightly positive potential of the Ag/AgCl electrode at the peak moment. This is mainly the result of the slightly different pCl in both ion-step solutions, as indicated by the small offset of the Ag/AgCl transient after the ion step.

It can be concluded that the Ag/AgCl electrode in this example can function as a reference electrode for the ISFET, mainly because Eq. (19) is satisfied. Thus, the device can be used in the dipstick configuration, which will be demonstrated in the following section. Inspection of Fig. 5 further shows that signal (2) contains much less noise than both other signals. This can be explained by the fact that the measuring electrodes of the integrated device are located at a distance of the order of $10 \mu\text{m}$ (about half a gate-width), whilst in both other signals a tube section with a length of 1 cm and a diameter of 1 mm connects the wall-jet cell with the SCE, offering a large electrical resistance. The amplitudes of transient (3) were reproducible within 0.5 mV.

Offering the ion step by manual dipping in solutions with different salt concentrations. When the device is used in the dipstick configuration (ISFET versus Ag/AgCl), an ion step can be offered by manual transference of the device. A typical measurement with this method is shown in Fig. 6. At the moment indicated by the arrow the device is lifted from the low-concentration solution, and at $t = 0$ it is immersed in the high-concentration solution while stirring the solution with the device itself. The figure shows that the electrical connection between both electrodes remains intact after removing it from solution. It can further be seen that the transient amplitude is a little smaller and the duration somewhat longer than for the one measured in the flow-through system. The probable explanation is that the manual stirring is less effective than the stirring in the flow-through apparatus, causing both τ_{step} and τ_{Buf} to increase. The potential offset remaining at $t > 15 \text{ s}$ could not be explained as yet. Subsequent measurements with one device were reproducible within 1 mV. Above a mild rate of stirring this reproducibility was constant.

A device with adsorbed lysozyme

Fig. 7 shows the transients in the three different measuring configurations after adsorption of lysozyme to a device membrane. Measurements took place at pH 4.8 to ensure a positive membrane charge, which is confirmed by the negative sign of the transient. Adsorption of lysozyme confers a positive charge to the membrane, since one lysozyme molecule has a net positive charge of

Fig. 6. Potential transient measured using the dipstick procedure. All parameters are as in the caption of Fig. 5. The device is removed from the first solution at the moment indicated by the arrow.

Fig. 7. Potential transients measured in three configurations, using the flow-through system. The device has an $8\ \mu\text{m}$ membrane with adsorbed lysozyme and the ion step is from $10\ \text{mM KCl}$ to $10\ \text{mM KCl} + 40\ \text{mM KNO}_3$ at $\text{pH}^e = 4.8$ (buffer $0.5\ \text{mM}$ acetic acid).

about $+11$ at this pH. The amplitude value of about $-22.5\ \text{mV}$ indicates a surface charge of about $1.14\ \mu\text{C}/\text{cm}^2$, or $15.5\ \text{mM}$. The transient measured by the device with the integrated reference electrode (2) has a slightly smaller amplitude than the one of the ISFET versus the SCE, just as in the previous subsection. It can be seen from the figure that this is caused by a slightly negative potential of the Ag/AgCl electrode at the peak moment.

The re-equilibration phase of transient (1) can be described by using $\tau_{\text{Cl}} = 0.5\ \text{s}$. This implies a membrane Cl^- diffusion coefficient of $5.2 \times 10^{-11}\ \text{m}^2/\text{s}$, about 0.026 times the value in the electrolyte. Transient (3) indicates a value for τ_{Buf} of 4 s. When a membrane buffer diffusion coefficient is assumed of $0.031 \times 10^{-9}\ \text{m}^2/\text{s}$ (as for Cl^- ions 0.026 times the value in the electrolyte) and β_{Buf} is taken as $0.24\ \text{mM}$, application of Eqs (16) and (17) gives a membrane buffer capacity β_A of $0.9\ \text{mM}$. This value is much larger than that of the bare membrane at $\text{pH}^e = 7.6$, which can be explained by the buffer capacity at $\text{pH}^e = 4.8$ of carboxyl groups, both the ones originally present on the polystyrene beads and the carboxyl side-chain groups of adsorbed lysozyme molecules. The other features of the figure have already been discussed in the first subsection of the Results section.

It can be concluded from the above considerations that Eqs (19) and (20) are sufficiently satisfied. Therefore, the dipstick configuration can also be used in this case as will be shown in the next subsection.

Offering the ion step by manual dipping in solutions with different salt concentration

Fig. 8 shows the transient obtained when the dipstick method is used. On removal from the low-concentration solution the device potential is seen to drift in a negative direction, which can be explained by evaporation of water causing a salt concentration rise. In this case the transient amplitude is slightly larger than the one obtained in the flow-through system (see Fig. 7), which can possibly be explained by this negative drift. As in the measurement on the bare membrane, the transient duration is about 1.5 times that measured in the flow-through system, and a potential offset remains at $t > 15\ \text{s}$. Subsequent measurements with one device again were reproducible within $1\ \text{mV}$.

DISCUSSION AND CONCLUSION

The ion-step measurements presented in this paper illustrate three different methods to retrieve a potential transient from a device with an ISFET and an integrated Ag/AgCl electrode. It is shown that the method of measuring the potential of the device ISFET versus the integrated Ag/AgCl electrode has several advantages over the other methods. In the first place the amount of noise is very much reduced as compared to the measurement of the ISFET potential versus an external SCE. In the second place the ion step can be offered simply by transferring the measuring device from one salt solution to another, without the use of a large and complex flow-

Fig. 8. Potential transient measured using the dipstick procedure. All parameters are as in the caption of Fig. 7. The device is removed from the first solution at the moment indicated by the arrow.

through apparatus. The latter measuring method is shown to result in transient amplitudes approximately equal to the ones measured by the ISFET versus an external SCE. This equality was shown to result from the fact that the Ag/AgCl-measured transient potentials are only small and of short duration as compared to the ISFET-measured transients. A theoretical basis is provided for the observed phenomena.

A disadvantage of the dipstick procedure is its smaller reproducibility compared with the use of the flow-through procedure (1 mV versus 0.5 mV). It will depend on the application of the measurement if the obtained reproducibility is sufficient.

Measurements clearly show the effect of the adsorption of lysozyme, expressing itself in a decrease of amplitude and an increase of the membrane buffer capacity. A quantitative analysis of these parameters resulting in a membrane proton titration curve is given in another paper (Eijkel *et al.*, 1997a). The dipstick experiments presented are clearly of a preliminary nature, and some problems must be solved before it can be used as a standard ion-step procedure. These problems, which are currently being investigated, are the drift after removal from the first solution, the possibly related problem of the offset that remains after immersion in the second solution, and the problem of decreased reproducibility relative to the flow-through experiments.

Devices as presented here are intended for single use. Future investigations can include regeneration of the device, for example by incubation in a high concentration electrolyte.

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