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Characterization of Silylated ISFETs by Ion-Step Measuring Method

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The ion-step measuring method is used to characterize Ta_2O_5 ion-sensitive field-effect transistors (ISFETs) which are treated with γ-aminopropyltriethoxysilane (APS) to create amine groups on the surface. Different procedures of silylation are used and the stability with respect to hydrolysis is examined. It is shown to be difficult to fabricate reproducible APS layers which are stable with respect to hydrolysis. The sensitivity of the silylated ISFETs to heparin is also examined. In this case positively charged APS is used as an affinity ligand for the negatively charged heparin. The results show a linear relationship between the change in ion-step response and the heparin concentration in PBS. The silylation procedure is also used to 'tune' the ion-step response of the Ta_2O_5 ISFET in such a way that the silylated ISFET does not show a response to an ion step at pH 7. In this way the ion-step response of a porous membrane mounted on top of the gate area of the ISFET can now be specifically determined without an interfering ion-step response of the underlying ISFET.

1. Introduction

In 1990 a new measuring method (the ion-step measuring method) was introduced to detect changes in charge density in a porous membrane deposited on an ISFET.[1] Schasfoort *et al.* have already shown the possibility of determining anti-human serum albumin (aHSA) concentrations in buffer solutions using HSA as an affinity ligand immobilized in a membrane of polystyrene beads. At this moment the ion-step measuring method is under further investigation aiming at the development of a heparin sensor.[2,3] Heparin is an

anticoagulant drug that is intravenously administered to prevent the formation of blood clots (thrombosis). In most cases, heparin treatment must be monitored, usually by means of clotting assays.[4] A simple heparin sensor system would be useful for the monitoring of heparin treatment.

For measuring heparin concentrations, we have used protamine as an affinity ligand. Protamine and heparin form complexes by electrostatic interaction of anionic heparin sites with cationic protamine sites.[5] Under physiological conditions, heparin has high negative charge and protamine has high positive charge, and their electrostatic bond is therefore very stable.

The device initially used in the ion-step measuring method is an ISFET with a porous membrane of polystyrene beads in an agarose gel mounted on its gate, as is schematically shown in Fig. 1. Proteins can be immobilized in the membrane via physical adsorption or covalent bonding to the polystyrene beads. The titratable groups of proteins result in a net charge density in the membrane which is pH dependent. If the concentration of electrolytes in the solution in which the device is immersed is suddenly changed (the ion step) while the pH is kept constant, the ISFET shows a transient response of which the amplitude is a function of the net charge density in the membrane. This is schematically shown in Fig. 1. The response is mainly caused by a release or uptake of protons by the protein molecules in the membrane, which results in a temporary pH change in the membrane, as measured by the pH-sensitive ISFET.[2]

During the experiments with membrane-covered ISFETs, it was observed that a bare ISFET, i.e., without a membrane, also shows a response to an ion step. The mechanism behind this transient ion-step response of a bare ISFET was elucidated, and appears to be a function of the surface charge density of the gate oxide.[6] At the pH where the net surface charge is zero (the point of zero-charge pH_{pzc}), the ion-step response of the ISFET is zero. However, the pH_{pzc} of Ta_2O_5 ISFETs is 3, which indicates that at around pH 7 the surface has a significant negative charge density, and when a membrane is applied, the ion-step response of the ISFET can interfere with the ion-step response of the membrane, which can be clearly observed from the experimental results.

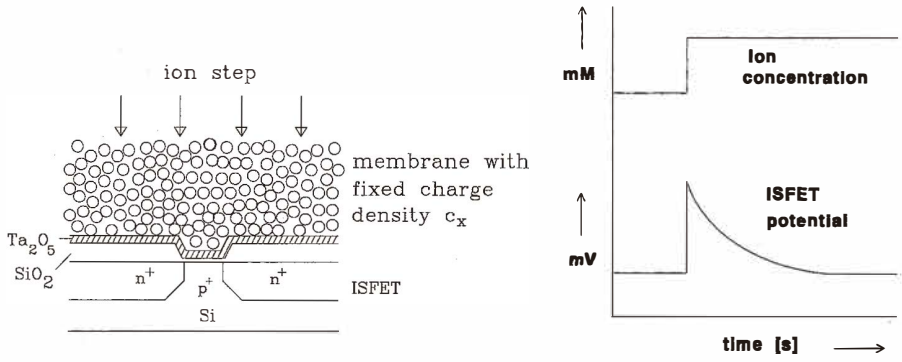


Fig. 1. Schematic presentation of the ion-step measuring method.

As a result of understanding the bare ISFET behavior, we considered the possibility of direct immobilization of protamine as an affinity ligand to the Ta_2O_5 gate oxide surface. In this case, a membrane of polystyrene beads (through which diffusion is relatively slow), would no longer be required. Physical adsorption of protamine to the Ta_2O_5 surface resulted in heparin sensors which were successfully used to determine heparin concentrations in blood plasma.[3] Another possibility which seemed worthwhile to examine, was modification of the negatively charged tantalum oxide surface by functional amine groups, which is the focus of this work.

In this paper, results of experiments with Ta_2O_5 ISFETs treated with γ-aminopropyltriethoxysilane (APS) to create positively charged amine groups on the oxide surface are presented. This silylation procedure to create functional NH_2 groups at the surface might be favorable for several reasons.

- Amine groups are frequently used functional groups for covalent coupling of proteins to surfaces. They can be used for covalent coupling of, for example, protamine as an affinity ligand to bind heparin.
- The point of zero-charge pH_{pzc} of a Ta_2O_5 ISFET ($pH_{pzc} = 3$) might be 'tuned' to higher values by introducing positively charged amine groups. This option would be useful because at pH_{pzc} the ISFET itself does not respond to an ion step and the response of an additional layer of proteins or a membrane can then be measured without an interfering ISFET response.
- If the density of the positively charged amine groups is sufficiently high, then the modified surface could be used directly to bind heparin without using an additional affinity ligand.

In the following, results of different types of ion-step experiments with different objectives are presented. First, ion-step responses are used to characterize silylated Ta_2O_5 ISFETs. Then, the sensitivity of silylated ISFETs to heparin is determined by means of ion-step responses. In this case the amine-functionalized silane is used directly as an affinity ligand for binding heparin molecules from a buffer solution as well as from blood plasma. Next, the silylation procedure is used to shift the point of zero charge of the silylated Ta_2O_5 ISFET to pH 7, as determined by the ion-step response. On top of these silylated ISFETs, different membranes of polystyrene beads are mounted. It will be shown that the measured ion-step response is now fully determined by the membrane, because the ISFET itself does not show an ion-step response in this case.

Before the results are presented, a brief introduction is given on silane coupling agents, focusing on the amine-functionalized silane which was used.

2. Silane Coupling Agents

Organofunctional silanes are hybrid organic-inorganic compounds that are used as coupling agents across organic-inorganic interfaces such as organic polymers and mineral substrates.[7,8] The reactions between the inorganic part of a silane coupling agent and the hydrophilic mineral substrate (*e.g.*, a metal oxide) are equilibrium reactions. Bonds are formed and broken in reversible reactions determined by concentrations and equilibrium

constants. The organic reactions between the organofunctional group and other organic molecules (*e.g.*, proteins) are determined by the thermodynamics and kinetics of different competing reactions. Formation and breakage of these bonds are generally irreversible.

A silane coupling agent may function as a surface modifier or as a primer. When it is used as a surface modifier, it is used only to chemically modify a surface without forming a layer with any mechanical properties. As a primer, however, it must have good mechanical film properties to withstand the mechanical load of the layer to be attached to the primer. A silane layer used for surface modification usually has a thickness of one or more monolayers, whereas a primer layer can have a thickness up to several micrometers.

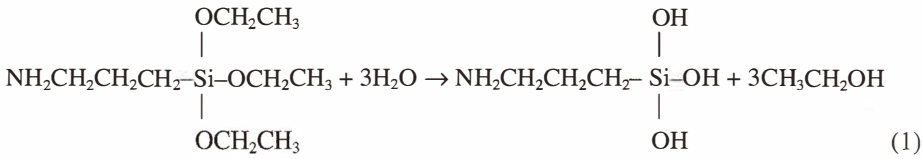
A specific class of silane coupling agents is that of organofunctional alkoxysilanes with the general formula $(OCH_3)_3Si\text{-}RY$. These silanes contain three hydrolyzable ethoxy groups and one organofunctional group RY. The three hydrolyzable groups are intermediates in the formation of silanol (Si-OH) groups for bonding to mineral surfaces. The RY group is chosen for reactivity or compatibility with the specific organic molecules which are to be coupled. Several of these silanes with different organofunctional groups are commercially available. Some examples of the functional group RY are vinyl, chloropropyl, methacrylate, mercapto and primary amine.

In the following, one specific silane and one specific mineral substrate are considered. The silane is APS and the substrate is Ta_2O_5 which is applied as the gate oxide of the ISFETs used for the ion-step experiments.

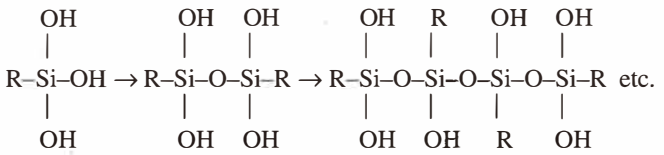
2.1 *APS in aqueous solution*

APS can be applied to a Ta_2O_5 surface from an aqueous solution, an organic solvent or a mixture of an organic solvent and water. First, the reactions of APS with water will be considered. The reaction with the Ta_2O_5 surface and the subsequent formation of an APS layer is considered later.

When in contact with water, APS hydrolyzes irreversibly in a two-step reaction. The three ethoxy groups will hydrolyze very quickly, resulting in the formation of silane triols (reaction (1)). These silane triols have good stability in water at pH 3 to 5, but condense rapidly to siloxanes at pH 7 to 9 according to reaction (2).[7]



(1)



(2)

$R = -CH_2CH_2CH_2NH_2$

If APS is hydrolyzed in excess acetic acid at pH 4, silane triols are immediately formed and solutions with concentrations of up to 50% (by weight) are stable due to the strong hydrophilic nature of the aminopropyl group. When an aqueous solution of APS is prepared at its natural pH (which is very high due to the amine group), siloxane oligomers will be formed immediately, which retain solubility in water at moderate concentrations. It is assumed that the solutions are an equilibrium mixture of low-molecular-weight siloxanes (oligomers) with silanols, stabilized by hydrogen bonds to amine groups, forming cyclic cage structures. However, in very dilute aqueous solutions of APS (<1% by weight), a significant amount of monomeric silanetriol will be present. The proportion of monomeric silanetriol increases rapidly with decreasing concentration of APS for concentrations < 1%.[9] Other APS-class silanes with other organic functional groups are not very stable in aqueous solution because the formation of siloxanes results in an insoluble gel within a few hours.

2.2 *Formation of APS layers on Ta_2O_5 surfaces from aqueous solution*

To bind to a Ta_2O_5 surface, silanol groups of a silane triol or a siloxane oligomer must condense with hydroxyl groups at the oxide surface, as is shown in reaction (3). It is known that neutral silanes (with a neutral functional organic group) condense very slowly on an oxide surface and may require a catalyst for optimum condensation. Amine functional silanes, such as APS, seem to be self-catalytic for surface condensation.

$$\text{Ta–OH} + \text{HO–}\underset{\text{OH}}{\overset{\text{OH}}{\underset{|}{\overset{|}{\text{Si}}}}}\text{–CH}_2\text{CH}_2\text{CH}_2\text{NH}_2 \rightleftarrows \text{Ta–O–}\underset{\text{OH}}{\overset{\text{OH}}{\underset{|}{\overset{|}{\text{Si}}}}}\text{–CH}_2\text{CH}_2\text{CH}_2\text{NH}_2 + \text{H}_2\text{O} \qquad (3)$$

As mentioned previously, the inorganic reaction between APS and a Ta_2O_5 surface (reaction (3)) is an equilibrium reaction and the oxane bonds between APS and Ta_2O_5 can hydrolyze again depending on the availability of water and the equilibrium constant.

Plueddemann has estimated equilibrium constants for hydrolysis of different coupling agents on silica, shown by reaction (4).[10]

$$\text{Si–O–Si–(coupling agent)} + \text{H}_2\text{O} \rightleftarrows \text{Si–OH} + \text{HO–Si–(coupling agent)} \qquad (4)$$

For an organofunctional trialkoxysilane (*e.g.*, APS), the estimated value of the equilibrium constant of reaction (4) is 10^{-4}. Although there is no firm evidence that silane coupling agents have similar interactions with metal hydroxide surfaces (such as Ta_2O_5) as with silica, it seems most probable because almost all metals are found in nature as silicate minerals. Nevertheless, equilibrium constants for hydrolysis of various metal-oxane bonds can be expected to differ from those of silica-oxane bonds, but the factors that determine such constants are unknown.

The stability with respect to hydrolysis of APS-treated glass was studied by Royer and Liberatore under continuous flow conditions over a pH range of 4 to 9.[11] They determined

the concentration of amine remaining on the surface of commercially available porous glass (550 Å pores and a particle size of 125–177 μm) after pumping 1 l of aqueous solution through a packed bed of these particles at a flow rate of 1 ml/min. At pH 4 about 5% of the amine was lost, at pH 6 about 25% and at pH 8 about 65%. It was concluded that retention of the amine function on glass is poor at any pH above 6. It is known that both acids and bases are powerful catalysts for hydrolysis. However, since the hydrolysis reaction is an equilibrium reaction, only the rate of hydrolysis is affected by the pH, not the point of equilibrium.

A good bond between APS and Ta_2O_5 requires that the equilibrium in reaction (3) does not shift too far to the left. Conditions favorable for bonding are therefore a maximum initial formation of Ta-oxane bonds and a minimum equilibrium concentration of water at the interface.

Since a silane triol has three silanol groups, it is able to condense with the Ta_2O_5 surface as well as with adjacent molecules. Moreover, aqueous APS solutions already contain low-molecular-weight siloxanes which create a multilayer structure after condensation at the oxide surface. Additional cross-linking in this layer can improve the resistance to water which is favorable for good bonding, according to reaction (3). Further cross-linking can be achieved by drying the layer at elevated temperatures to ensure complete condensation in the multilayer itself, as well as at the Ta_2O_5 surface. The polysiloxane layer fuctions as a protection layer against water, which prevents hydrolysis at the Ta_2O_5 surface. Therefore, multilayer APS coverage is more stable than monolayer APS coverage.

To achieve stable multilayer coverage, aqueous silane baths and silane baths containing a mixture of organic solvent and water are generally stored for at least 2 – 3 hours after preparation to ensure the formation of oligomers. After contact with Ta_2O_5, these oligomers will form a polysiloxane layer which can be stabilized by baking the layer. A drawback of baking APS layers at elevated temperatures is the possibility of damage to the aminopropyl group. To avoid damage by peroxide formation, the treated surface can be heated in an inert atmosphere; however this has not yet been demonstrated.

The resulting thickness of an APS film on a Ta_2O_5 surface depends on the availability of water, the pH (catalyst), and age of the silane solution. Tutas *et al.* observed that the thickness of an APS film deposited on glass was the same after one minute of contact as after one hour of contact with the aqueous silane bath.[12] However, it is not clear whether the degree of cross linking, and therefore the mechanical stability of the layer, changes during contact with the silane bath. Normally, surfaces are placed in contact with the silane bath for 2–3 hours.

2.3 *Formation of APS layer on oxide surfaces from organic solution*

When APS is applied to an oxide surface from organic solvents, it is necessary for the APS to migrate to the oxide surface, react with the surface moisture (physically and chemically adsorbed water) and condense on the surface. Because no siloxane oligomers are formed, silylation from a dilute solution of APS in an anhydrous organic solvent will probably lead to thinner layers (2–3 monolayers) than silylation from an aqueous solution.

3. Materials and Methods

3.1 *Materials*

APS was purchased from Sigma, heparin from Organon Teknika (Thromboliquine®, 5 ml ampoules containing 25000 units) and agarose (low IEE, zero M_r) from Biorad. A 2.5% suspension of 0.12 μm negatively charged polystyrene beads (sulphate groups) in water was purchased from Polysciences, and a 4% suspension of 1.03 μm positively charged polystyrene beads (amidine groups) was purchased from Interfacial Dynamics Corp. All salt and buffer solutions were of analytical grade. Normal blood plasma was a generous gift from the laboratory of the Medisch Spectrum Twente hospital.

3.2 *Measurement setup*

The measurement setup consists of a computer controlled flow-through system. The flow is controlled by the effective pressure (0.1 bar) of nitrogen in the two bottles containing the solutions, resulting in a constant flowrate of 1.3 ml/min. The ISFET is mounted in a wall-jet cell in which the liquid flow is perpendicular to the ISFET surface and a saturated calomel electrode is used to define the potential of the solution. Figure 2 shows a cross section of the wall-jet cell. Two bottles containing the two ion-step solutions (10 and 100 mM KCl) are connected via valves to the wall-jet cell in such a manner that the electrolyte concentration at the ISFET surface can be increased with a rise time (to 90% of the final value) of 200 ms. The 10 mM KCl solution is buffered with 0.5 mM HEPES, and the 100 mM KCl solution with 0.2 mM HEPES. In some cases the solutions were both

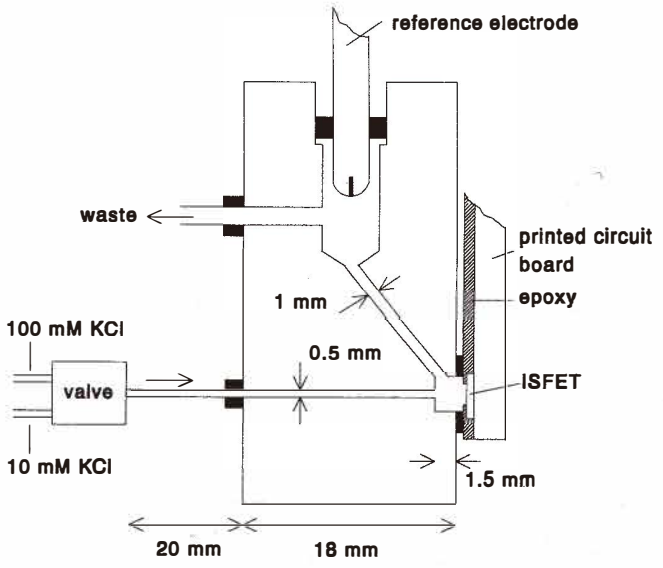


Fig. 2. Cross section of the wall-jet measurement cell.

buffered with 0.1 mM phosphate buffer.

The ISFETs are connected to a source-drain follower, the output of which is connected to a Nicolet 310 digital oscilloscope which stores output curves on a floppy disk. The data can then be modified on a PC using Vupoint software. For presentation purposes, the curves can be filtered with a software low-pass filter using a cut-off frequency of 40 Hz for elimination of the 50 Hz main supply interference which has a typical top-top amplitude of 0.2 mV.

3.3 *Measurement devices*

ISFETs with a Ta_2O_5 gate insulator were fabricated in the MESA cleanroom laboratory following the usual ISFET processing steps. The ISFETs showed a response of about –58 mV/pH. The ISFET chips were mounted on a piece of printed circuit board and encapsulated with Hysol epoxy. Around the gate a circular area of diameter 2.5 mm and depth of about 150 μm were left uncovered.

The methods below were used to silylate Ta_2O_5 ISFETs with APS.[7,13]

Method 1: The ISFETs are immersed in a solution of 2% (v/v) APS in acetone for 3 h, and subsequently rinsed in acetone and dried for 2 h at 115°C.

Method 2: A 10% (v/v) APS aqueous solution is adjusted to pH 3 – 4 with 6 M HCl directly after preparation. The ISFETs are immersed for 2 h in this solution which is placed in a water bath at 75°C. After this, the ISFETs are rinsed in water and dried for 2 h at 115°C.

Method 3: The ISFETs are immersed for 3 – 6 h in a 10% (v/v) APS solution in toluene and subsequently rinsed in acetone and dried for 2 h at 115°C.

Method 4: The ISFETs are immersed for 3 h in a mixture of 19 ml methanol, 1 ml water and 100 μl APS which was stored for at least one day to ensure the formation of oligomers. After rinsing in ethanol, the ISFETs are dried at 115°C for 2 h or more.

Method 5: The ISFETs are immersed for 1 h in a 0.25% (v/v) solution of APS in 50 mM HAc, pH 4 and subsequently rinsed in water and dried at 115°C for 1 h.

Method 6: The ISFETs are immersed for 1 h in a 0.25% (v/v) solution of APS in acetone. After rinsing in acetone the ISFETs are dried at 115°C for 1 h.

Method 7: The ISFETs are immersed for 1 h in a 0.25% (v/v) solution of APS in toluene placed in a water bath at 75°C. Afterward, the ISFETs are rinsed in toluene.

Method 8: The ISFETs are placed in a vessel surrounded by an oil bath at 115°C. Nitrogen flow is led through a bottle containing water or through a bottle containing pure APS, and subsequently through the heated vessel which contains the ISFETs. The nitrogen flow enters the vessel through the bottom which consists of sintered glass, and leaves the vessel through the cover. First the nitrogen flow is directed through a bottle containing demineralized water (for 1 h) to ensure a certain degree of moisture at the ISFET surfaces. Then the nitrogen is led through a bottle containing pure APS (at room temperature) for 1 h. The nitrogen transports APS vapor which can react with the Ta_2O_5 surface of the ISFETs in the heated vessel.

The membranes of polystyrene beads were made by 1:1 mixing and ultrasonication of a 0.25% agarose solution with a 2.5% suspension of polystyrene beads in water at 40–50°C and subsequently casting 3 μl portions on top of the gate area of the ISFETs. After drying overnight at 4°C and then increasing the temperature to 55°C for 1 h, membranes with a thickness of about 10–15 μm (in dry condition) were fabricated.

3.4 *Measurement protocol*

In each determination of an ion-step response, 3 – 5 responses were successively recorded, and the amplitude of the ion-step response was defined as the mean value of these responses. For the determination of heparin concentrations in PBS solutions, 15 ml vessels were used in which the ISFET was placed for 10 min. The solution was not stirred. After incubation the ISFET was rinsed with PBS and mounted in the wall-jet cell of the measurement setup. The ion-step response was recorded, and the change in the amplitude with respect to the response before incubation was taken as a parameter.

For the measurements in normal plasma, a test tube containing 2 ml of normal plasma was used to which small amounts of a 100 U/ml heparin solution (in 0.9% NaCl) were added to obtain the different concentrations. The ISFET was incubated in the plasma for 10 minutes (without stirring), rinsed in PBS, and subsequently the ion-step response was determined.

4. Results and Discussion

4.1 *Characterization of silylated ISFETs using ion-step response*

Figure 3 shows typical ion-step responses (10 to 100 mM KCl, pH 7) of an unmodified Ta_2O_5 ISFET (curve 1) and of some different silylated ISFETs (curves 2–4). It is obvious from the figure that the effect of silylation on the ion-step response varies. The ISFETs showing ion-step responses 2–4, were silylated by method 4 using solutions of different ages and after storage of the silylated ISFETs for different times in PBS. Later in this section, it will become clear which effects cause the differences between the ion-step responses. The fact that the responses can become negative indicates that the negative charge of Ta_2O_5 can be fully compensated by the positive charge of APS.

The time constants of the ion-step responses of the different silylated ISFETs are the same as those of the untreated ISFET response. This indicates that the ion-step response of a silylated Ta_2O_5 ISFET can be described in the same way as that of a bare ISFET, as described earlier,[6] assuming a different surface charge density. In other words, the silylation procedure can be seen as a surface modification. Most methods used for silylating Ta_2O_5 ISFETs will result in multilayer coverage of the oxide, as explained in section 2. However, if it is assumed that these layers remain relatively thin (<100 nm), the time to reach thermodynamic equilibrium in these layers (*e.g.* after an ion step), is very short in comparison with those in other methods. In the case of much thicker layers, membrane effects, as described earlier,[2] become important and certainly would affect the time constant of the transient ion-step responses.

To evaluate the different silylation methods, several series of ISFETs (all the same type of Ta_2O_5 ISFETs) were silylated according to the different methods mentioned previously.

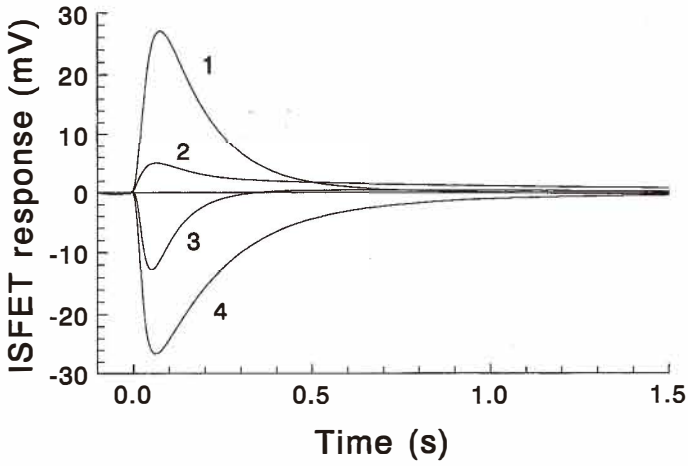


Fig. 3. Ion-step responses (10 to 100 mM KCl, pH 7) of a bare ISFET (curve 1), and different silylated ISFETs (curves 2–4).

After silylation, the response to an ion step of 10 to 100 mM KCl at pH 7 was recorded. Table 1 shows the method of silylation, the number of ISFETs in each series, the moment of measurement and the amplitude range of the ion-step responses of the different ISFETs in each series. The measured amplitudes of the ISFET responses in each series were, in most cases, randomly divided in the range bounded by the limits given in Table 1. The differences between the amplitudes of the ion-step responses within one series are very large in some cases and it seems that silylation from an aqueous solution (method 2 and to some extent method 4), results in more variance in the amplitudes than the nonaqueous methods. In an aqueous solution, the siloxane oligomers that are formed have several hydroxyl groups by which they can bind to the oxide surface. It may be expected that these oligomers form a layer which shows more variance in structure and thickness than a layer which is fabricated from much smaller APS molecules, as is the case in the nonaqueous methods.

4.2 *Stability of the APS layer with respect to hydrolysis*

During the experiments, it was noted that ion-step responses changed after the ISFETs were stored in aqueous solutions. This was not unexpected and most likely the result of hydrolysis of the tantalum-oxane bonds as described in section 2. To characterize this hydrolysis, the amplitude of the ion-step response (10 to 100 mM KCl, pH 7) was recorded as a function of storage time in PBS for different types of silylated ISFETs. Some typical results are shown in Fig. 4. The slowest change in amplitude (curve 3) is observed with APS layers obtained by silylation according to method 4 using a 13-day-old silane bath and a drying time of 16 h at 115°C. A 13-day-old silane bath has a high concentration of siloxane oligomers which results in a relatively thick layer. The long drying time in this

Table 1
Range of amplitudes of ion-step responses (10 to 100 mM KCl, pH 7) within one series for different methods of silylation.

Method of silylation	Number of devices	Moment of measurement	Amplitudes of ion-step responses (mV)
1	8	direct	–25 to –16
1	11	overnight in pH 7	–20 to –12
1 (dried for 16 h at 115°C)	8	direct	–45 to –23
2	8	direct	–29 to –3
3	4	direct	–51 to –48
3	5	overnight in pH 7	–48 to –38
4 (1 day)*	10	direct	–20 to –5
4 (1 day)*	15	overnight in pH 7	–5 to +4
4 (3 days)*	19	overnight in pH 5	–32 to –6
4 (3 days)*	20	overnight in pH 7	–20 to +2
4 (4 days)*	14	direct	–30 to –5
4 (4 days)*	14	overnight in pH 7	–23 to –3
4 (5 days)*	8	direct	–30 to –15
4 (5 days)*	10	overnight in pH 5	–27 to –11
4 (10 days)*	7	overnight in pH 7	–39 to +3
4 (13 days, 16 h at 115°C)*	20	direct	–41 to –22
5	5	direct	–10 to –6
6	5	direct	–25 to –15
7	10	direct	–48 to –38
8	5	direct	–23 to –16

* Number of days that the APS/methanol/water mixture was stored before use.

case (16 h) results in a high degree of condensation and thus cross-linking in the polysiloxane layer. In this way, the layer protects the ISFET against water and thus prevents hydrolysis at the Ta_2O_5 surface.

Silylation method 3 also resulted in very stable layers (with respect to hydrolysis over time), as shown in curve 3. In this case, dense layers of APS are formed from a 10% APS solution in toluene, resulting in large ion-step responses, as shown in Table 1. Since no siloxanes are formed in the toluene, most APS molecules will form oxane bonds with Ta_2O_5 and a high degree of condensation will be achieved. We can assume that with this method, a relatively thick layer is formed because of the high APS concentration. This highly condensed layer will also protect the interface with the Ta_2O_5 surface against water. A less concentrated solution of APS in toluene, as used in method 7, initially also resulted in very large ion-step responses with comparable amplitudes (see Table 1), but the ISFETs showed a faster hydrolysis over time (comparable to curve 2 in Fig. 4) in comparison with the ISFETs silylated by method 3. This might be explained by the fact that these layers were not dried at 115°C and hence, they are thinner due to the low APS concentration. The layers which were fabricated according to method 1 (2% APS in acetone) also showed a

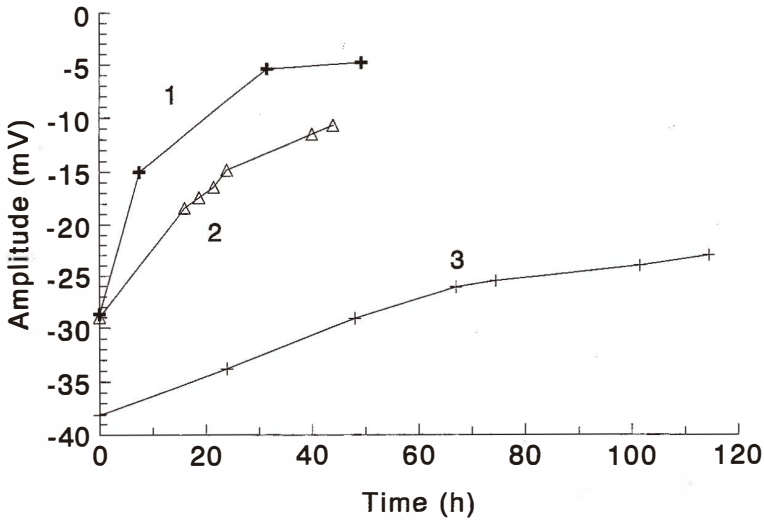


Fig. 4 Amplitude of ISFET response to ion step from 10 to 100 mM KCl at pH 7 as a function of storage time in PBS solution. Curve 1: ISFET silylated according to method 2. Curve 2: ISFET silylated according to method 1; also typical result for silylation methods 4 (up to 5-day storage of APS solution) to 8. Curve 3: ISFET silylated according to method 4 using a silane bath 13 days old and a drying time of 16 h at 115°C; also typical result for silylation method 3.

faster hydrolysis than the layers deposited from APS in toluene by method 3. It is known that acetone contains more water than toluene, which together with the lower concentration of APS used in method 1 in comparison to method 3, explains the poorer stability of APS layers deposited from acetone.

Method 2 resulted in layers which show a relatively fast hydrolysis, as shown by curve 1. The other methods yielded layers of which the hydrolysis, as a function of time, was almost comparable to curve 2.

The hydrolysis of APS layers which were fabricated according to method 4 was found to be dependent on the age of the silane bath. Table 2 lists the range of change in amplitude within each series after one night (about 16 h) in a buffer solution at pH 7, for ISFETs silylated by method 4 using silane baths of different ages. It can be seen that the layers deposited from an older silane bath show a smaller change after one night than layers deposited from a fresher silane bath. An older silane bath probably contains more siloxane oligomers, resulting in a thicker layer which gives better protection against water after cross-linking during baking.

4.3 *Heparin sensitivity of silylated ISFETs*

Since APS treatment introduces functional amine groups at the surface, we examined whether or not the APS layers could be used directly as an affinity ligand for binding heparin to the surface. For stable binding of heparin to the surface, the density of amine

Table 2
Range of the change in ion-step responses (10 to 100 mM KCl, pH 7) of ISFETs silylated according to method 4, for different ages of the APS/methanol/water solution.

Age of solution	Number of devices	Range of change in amplitude after overnight storage in buffer at pH 7
1 day	10	min 9, max 15 mV
4 or 5 days	22	min 1.5, max 9 mV

groups available for binding to heparin must be as high as possible. When heparin is bound to the surface, the charge of the heparin molecules must compensate for a substantial part of the APS charge before it can be detected by a change in the ion-step response.

ISFETs which were silylated according to method 3, did not show a different ion-step response after incubation in a heparin solution. Increasing the heparin concentration up to 5000 U/ml did not change the ion-step response. Also in silylation method 4, using a silane bath 13 days old and a drying time of 16 h at 115°C was not successful in this regard. A possible explanation might be that the structure of these layers results in a density of surface NH_2 groups which is insufficient for firm binding of heparin molecules. The ion-step responses of these ISFETs indicate highly positively charged surfaces (see Table 1), but a significant number of these amine groups might not be accessible to heparin molecules because they are enclosed in the polysiloxane layer. Another possibility is that the amount of bound heparin does not significantly change the total charge density which is determined by the ion-step response.

Using the other silylation methods, including method 4 with shorter drying times and fresher baths, resulted in some sensitivity to heparin. After incubation in a heparin solution, ion-step responses with smaller (negative) amplitudes were obtained, indicating a decrease in positive charge density at the surface caused by the binding of negatively charged heparin molecules. In some cases, the ion-step responses even changed from a negative transient into a positive one.

To test whether different silylated ISFETs of one series showed a comparable sensitivity to heparin, several ISFETs of one series, silylated by method 4, were incubated in a buffer solution with a specific heparin concentration. Before incubation, all ISFETs showed very different initial responses after silylation and additional storage in the buffer solution. Table 3 shows the amplitudes of the ion-step responses (10 to 100 mM KCl, pH 7) of two series of silylated ISFETs (all Ta_2O_5 ISFETs silylated by method 4 in a 5-day-old bath) before and after incubation in a heparin solution. The only difference between the two series is that the ISFETs are incubated with different heparin concentrations. The error which is given for each value of the amplitude indicates the range of amplitude of the 3–5 responses successively recorded in each determination of the ion-step response, as described in the measurement protocol. Although the initial responses of the ISFETs are very different, the changes in the responses after incubation in a heparin solution are almost the

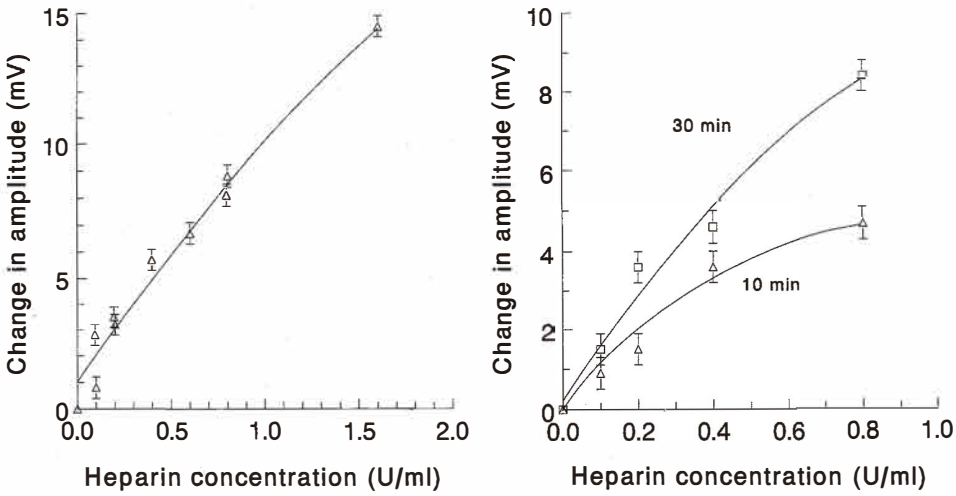
Table 3
Changes in amplitude of ion-step responses of two series of silylated ISFETs (method 4, 5 days) after incubation for 10 min in 0.4 or 0.5 U/ml heparin in PBS.

Initial response of silylated ISFET (mV)	Response after 10 min in 0.4 U/ml heparin (mV)	Change in response (mV)
-5.3 ± 0.2	-2.1 ± 0.2	3.2 ± 0.4
$+12.4 \pm 0.1$	$+16.0 \pm 0.7$	3.6 ± 0.8
-12.1 ± 0.2	-7.6 ± 0.2	4.5 ± 0.4
$+2.1 \pm 0.2$	$+7.1 \pm 0.2$	5.0 ± 0.4
$+12.6 \pm 0.1$	$+17.9 \pm 0.2$	5.3 ± 0.3
Initial response of silylated ISFET (mV)	**Response after 10 min in 0.5 U/ml heparin (mV)**	**Change in response (mV)**
-17.2 ± 0.4	-12.1 ± 0.3	5.1 ± 0.7
-23.4 ± 0.3	-19.5 ± 0.2	3.9 ± 0.5
$+1.0 \pm 0.1$	$+5.8 \pm 0.4$	4.8 ± 0.5
-6.8 ± 0.3	-2.3 ± 0.2	4.5 ± 0.5
-2.0 ± 0.2	$+7.1 \pm 0.2$	9.1 ± 0.4

same, taking into account the error. The last ISFET of the second series mentioned in Table 3 is an exception because the change in the amplitude of the ion-step response is much larger than for the other ISFETs within that series.

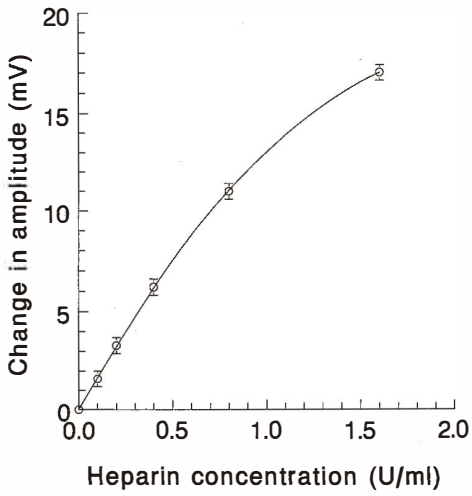
The results in Table 3 show that the change in the ion-step response after incubation in a heparin solution is comparable for most devices, although the silylated ISFETs seem to have very different net surface charge densities. Because of the relatively short incubation time, equilibrium will not be reached and the amount of heparin which binds to the surface will be determined by diffusion of heparin towards the surface (solution is not stirred). This might explain why the amount of bound heparin is about the same for the different surfaces, even if the surfaces have different initial charge densities. Another plausible explanation is that the density of NH_2 groups accessible to heparin is about the same for the different silylated ISFETs in Table 3. The significant differences in the initial responses might be explained by the different thicknesses of the APS layers resulting in different amounts of NH_2 groups in the polysiloxane layer which are not accessible to heparin.

The next step was to examine whether the ISFETs showed different changes in ion-step response after incubation in different heparin concentrations. Figure 5(a) shows the change in the amplitude of the ion-step response (10 to 100 mM KCl, pH 7) as a result of incubation of silylated ISFETs (according to method 1) as a function of the heparin concentration in PBS. The incubation time during which the ISFETs were exposed to the heparin solutions was 10 min. The error bars indicate the variance in the values of the amplitude of the 3–5 responses which were recorded each time. Each measurement shown in the figure was carried out with a different silylated ISFET; the ISFETs were only used once.



(a)

(b)



(c)

Fig. 5 (a) Change in amplitude of ion-step response (10 to 100 mM KCl, pH 7) of silylated ISFETs (method 1) after 10 min of incubation as function of heparin concentration in PBS. Each measurement was carried out with a different device. (b) Change in amplitude of ion-step response (10 to 100 mM KCl, pH 7) of silylated ISFETs (method 2) after 10 and 30 min of incubation, as function of heparin concentration in PBS. Each measurement was carried out with a different device. (c) Change in amplitude of ion-step response (10 to 100 mM KCl, pH 7) of silylated ISFETs (method 4, silane bath 5 days old, drying time 2 h at 115°C) after 10 min of incubation, as function of heparin concentration in PBS. Each measurement was carried out with a different device.

Figure 5(b) shows the changes in amplitude of the ion-step responses of ISFETs silylated according to method 2, as a function of the heparin concentration in PBS. The two curves correspond to the change in ion-step response after 10 and 30 min of incubation. Again, for each different concentration, a different ISFET was used. Note that the slope of the fitted curve recorded after 10 min of incubation is much lower than that in Fig. 5(a). Apparently, these ISFETs show a smaller sensitivity to heparin, resulting in a smaller slope than for the silylated ISFETs the results for which are shown in Fig. 5(a). The structure of the layer seems to strongly affect heparin sensitivity.

In Fig. 5(c) the results for ISFETs silylated according to method 4 using a silane bath 5 days old and a drying time of 2 h at 115°C are presented. Each measurement was again carried out with a different ISFET from the same series.

It is obvious from Figs. 5(a) and 5(b) that not all measurements match the fitted curve within the error of measurement. This can be explained by the fact that the individual silylated ISFETs, which were used to determine the different heparin concentrations, will have slightly different heparin sensitivities as also follows from the results in Table 3. In Fig. 5(c), the match with the fitted curve is better, which might mean that the difference in heparin sensitivity between individual ISFETs is larger in the case of silylation methods 1 and 2 than in the case of method 4. It must be mentioned, however, that with ISFETs silylated by method 4, additional experiments were performed which resulted in fits not as good as that shown in Fig. 5(c), which is not unexpected considering the results of Table 3.

All the curves representing the change in the amplitude of the ion-step response as a function of the heparin concentration show a linear relationship for low concentrations and deviation from the linear relationship with decreasing slope for higher concentrations. This behaviour indicates a saturation effect at higher concentrations.

ISFETs which were silylated according to methods 5–8 did show any change in ion-step response after incubation in a heparin solution, but the relationship with the heparin concentration was not reproducible. It seems that binding of heparin to the APS layers was not very strong because bound heparin could be partly removed simply by extensive stirring in a buffer solution: particularly when the ISFET was incubated in a relatively high heparin concentration (>0.7 U/ml), the results were not reproducible. Apparently, the density of surface NH_2 groups accessible to heparin, was not sufficient to firmly bind heparin molecules in these cases.

The same types of silylated ISFETs as used in the case of Fig. 5(c), were used to determine the change in ion-step response after incubation in blood plasma samples containing different concentrations of heparin. The reproducibility of the results was, however, not very good and no clear relationship between the change in ion-step response and the heparin concentration could be determined. Some typical results for two series of ISFETs are shown in Fig. 6. Again, for each measurement, a different ISFET was used. The results suggest a nonspecific binding (in the case of a heparin concentration of 0 U/ml) which results in a change in the amplitude of the ion-step response of 5 mV. Although the presence of heparin seems to result in an increase in the change in ion-step response, no clear relationship between the change in ion-step response and the heparin concentration could be determined. This might be due to the nonspecific binding which results in a random number of bound molecules of different charges.

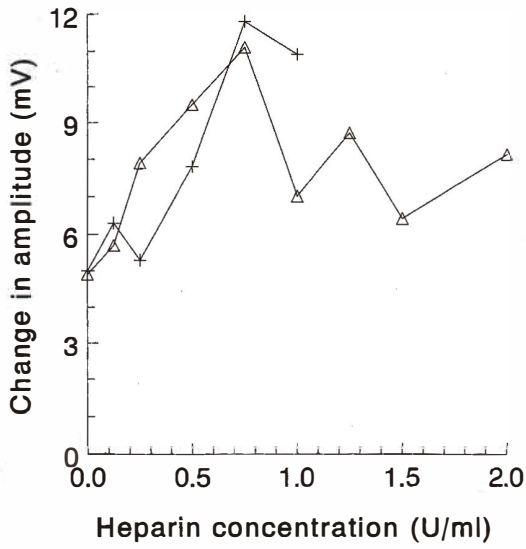


Fig. 6. Change in amplitude of ion-step response (10 to 100 mM KCl, pH 7) of silylated ISFETs (method 4, silane bath 5 days old, drying time 2 h at 115°C) after 10 min of incubation, as a function of heparin concentration in blood plasma. Each measurement was carried out with a different device.

4.4 *Silylation of Ta_2O_5 ISFETs to change the point of zero charge*

Because it was found that a silylation procedure can result in negative ion-step responses at pH 7 (see Fig. 3), whereas untreated ISFETs show positive ion-step responses, we examined whether the initial negative surface charge density of a Ta_2O_5 ISFET (at pH 7) can be compensated by an APS layer to obtain a zero ion-step response at pH 7. In this manner, these modified ISFETs can be used to determine the ion-step response of polystyrene bead membranes because no interfering ISFET response will occur.

Several Ta_2O_5 ISFETs were silylated according to method 4 and subsequently stored in PBS. Directly after the silylation procedure, the ISFETs showed responses of –20 to –25 mV to an ion step from 10 to 100 mM KCl at pH 7. The ion-step responses were subsequently measured after several periods of storage in PBS, in which the APS layer slowly hydrated resulting in decreasing amplitudes of the negative responses. When the responses were reduced to responses with an amplitude of <5 mV (but still negative), different membranes of polystyrene beads were deposited on the ISFETs as described in the experimental section. To determine the effect of the ISFET response on the total response, the same batches of agarose/polystyrene mixtures were used to fabricate membranes on top of untreated Ta_2O_5 ISFETs. Figure 7 shows an ion-step response (10 to 100 mM KCl, pH 7) of an untreated Ta_2O_5 ISFET with a membrane of positively charged 1.03 μm polystyrene beads with amidine surface groups. The positive ion-step response of the bare, untreated ISFET (before the membrane was mounted on it), is also shown in the figure. Although the membrane consists of positively charged beads, which should result

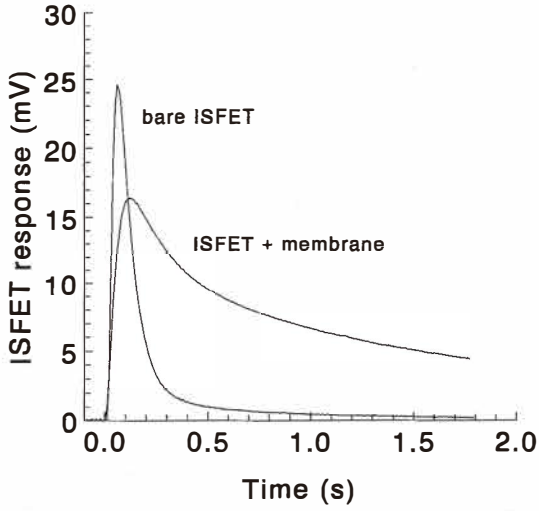


Fig. 7. Response to an ion step from 10 to 100 mM KCl at pH 7 of a bare, untreated ISFET and an untreated ISFET with a membrane of positively charged polystyrene beads of 1.03 μm in diameter.

in proton uptake and thus in a negative ISFET response, the ion-step response is still positive due to the interfering ISFET response.

Figure 8 shows the ion-step response of a silylated Ta_2O_5 ISFET with the same type of amidine polystyrene beads membrane. The small, negative ion-step response of the silylated ISFET before the membrane was deposited is also shown in the figure. The interfering ISFET response is now limited and the total response is negative due to proton uptake by the membrane groups.

Figure 9 shows two typical ion-step responses of Ta_2O_5 ISFETs with a membrane of 0.1 μm negatively charged (sulfate groups, pKa $\pm$0.8) polystyrene beads. Curve 1 is the response of an untreated Ta_2O_5 ISFET with a membrane, and curve 2 is the response of a silylated Ta_2O_5 ISFET with a membrane. The silylated ISFET showed a small ion-step response < -5 mV before the membrane was mounted on it. Curve 2 can therefore be considered solely as the response of the membrane, whereas curve 1 is the combined response of the ISFET and the membrane. It is obvious by comparing the two curves that the ISFET response has a significant effect on the total response. Even after the first few seconds, which is normally the maximum duration of the bare ISFET response, the total response is still influenced by the ISFET response. In this specific case, the buffer capacity of the membrane is very small because the sulfate groups at the beads have pK_a of about 0.7 and will all be protonated at pH 7. Therefore, the proton release by the membrane groups will be minimal and the membrane will hardly suppress the ISFET response. The protons which are released from the ISFET surface must diffuse from the surface, which will be a slower process in the membrane phase than in the solution phase. This might explain why the ISFET response seems to last longer in the experiments shown in Fig. 9 than that observed in the experiments shown in Fig. 3.

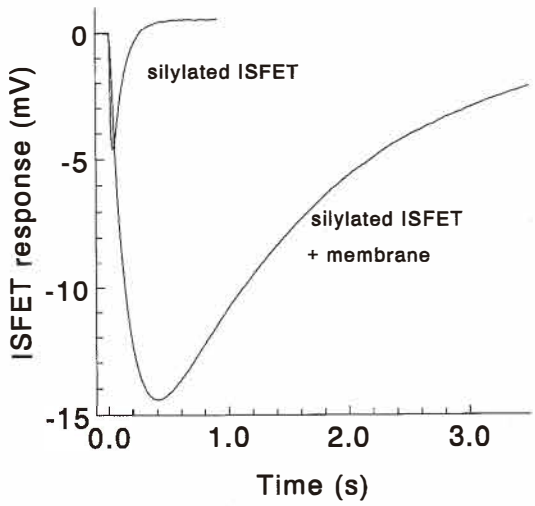


Fig. 8. Response to an ion step from 10 to 100 mM KCl at pH 7 of a 'tuned' silylated ISFET and the same silylated ISFET with a membrane of positively charged polystyrene beads of 1.03 μm in diameter.

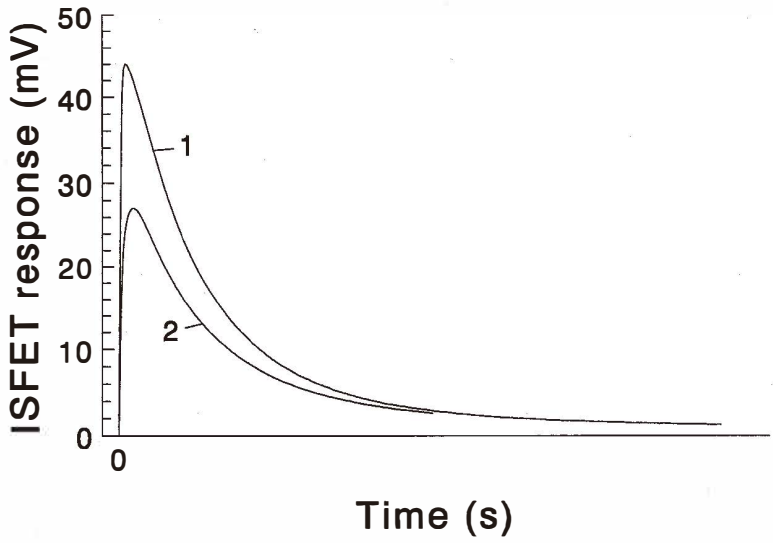


Fig. 9. Response to an ion step from 10 to 100 mM KCl at pH 7 of an untreated ISFET with a membrane of 0.1 μm negatively charged beads (curve 1) and of a silylated ISFET with the same type of membrane (curve 2).

5. Conclusions

In this paper it has been shown that modification of the Ta_2O_5 surface of an ISFET results in a change in the ion-step response. The amine-functionalized silane γ-APS was used to introduce amine groups at the surface. Although several silylation methods were used, it seems very difficult to realize reproducible layers of APS because the amplitudes of the ion-step responses of ISFETs in the same series showed large variances. The condensation reaction of the silanol groups and Ta_2O_5 (reaction 3) is an equilibrium reaction, the equiliblium point of which can only be shifted by preventing contact with water. However, since APS is hydrophilic, this seems to be an impossible condition to realize. It was also noted that the APS layers slowly hydrolyzed, as shown by the slow change in the ion-step response after storage in aqueous solutions. The rate of hydrolysis decreased with time, but equilibrium was not reached.

Because of this relative instability of the APS layers with respect to hydrolysis, it will be difficult to use an APS layer to covalently couple an affinity ligand. The coupling procedures often require long incubation times in aqueous solution, during which APS might become detached from the surface. Therefore it will be difficult to make reproducible and stable layers of bound affinity ligand based on APS.

APS-silylated ISFETs can be used directly to determine heparin concentrations in PBS solutions by the ion-step measuring method with an incubation time of 10 min. For each concentration, a different ISFET is used. In this manner, the contact of each ISFET with the aqueous solution is very limited, which implies that a slow loss of APS does not play a significant role. The sensitivities to heparin of the different ISFETs within one series, are comparable but show small variances which result in variance in the measurements. Determining heparin concentrations in blood plasma was difficult due to nonspecific binding of other plasma compounds.

Silylating ISFETs with APS can be used as an effective method to reduce the ISFET response to an ion step at pH 7 by 'tuning' the point of zero charge (as determined by the ion-step response) to pH 7. This option makes it possible to determine the ion-step response of a charged membrane without an interfering ISFET effect. However, using this option causes the APS layer to slowly hydrolyze which means that the point of zero charge, as determined by the ion-step response, will also slowly change.

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