

Development of an ISFET based heparin sensor using the ion-step measuring method

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Abstract: The ion-step measuring method was used to determine heparin concentrations in PBS and blood plasma. Heparin is a sulphated polysaccharide which is clinically used as a drug to prevent the clotting of blood. The measuring method is based on detection of changes in charge density in a porous membrane which is deposited on the gate of an ISFET. Protamine was used as affinity ligand in the membrane. In PBS a linear relation was found between the heparin concentration and the ISFET response. The incubation time was reduced from 18 h to 15 min by increasing the porosity of the membrane. The results of the measurements in blood plasma show a significant nonspecific binding of plasma components in the membrane. Suggestions are given to prevent this nonspecific adsorption. The results described in this paper show a detection limit for the ion-step measuring method of at least 5 ± 10^{-11} Mol/l which is promising for future practical applications.

Keywords: biosensor, ISFET, heparin, protamine.

1. INTRODUCTION

In 1990 a new measuring method was introduced to detect changes in charge density in a porous membrane deposited on top of an ISFET (Schasfoort *et al.*, 1990). With this new method it is possible to determine concentrations of large charged molecules such as proteins Schasfoort

et al. already showed the possibility of determining aHSA concentrations by using HSA as affinity ligand in the membrane.

A new research project has been started to develop a simple sensor system based on the ion-step measuring method. This project is aimed at the determination of heparin concentrations in blood as a first practical application of the ion-step measuring method (van Kerkhof *et al.*, 1992).

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Heparin

Heparin is a sulphated glycosaminoglycan (GAG) found in liver, lung and other tissues which has several pharmacological properties (Nader and Dietrich, 1989). The property of heparin which has attracted most attention and resulted in its widespread use is its ability to prolong the clotting time of blood by preventing the formation of fibrin. This anticoagulant activity of heparin is used in vascular surgery and in treatment of postoperative thrombosis and embolism.

The coagulation enzyme thrombin which converts fibrinogen into fibrin (the insoluble basis of a blood clot), is inhibited by antithrombin III, another plasma protein. Heparin binds to antithrombin III and produces a conformational change, thereby converting antithrombin III from a progressive, slow inhibitor to a very rapid inhibitor. After binding of this antithrombin III-heparin complex to a thrombin molecule, heparin dissociates from the complex and is reusable (Björk *et al.*, 1989; Rosenberg, 1987).

Heparin is one of a large variety of drugs extracted from natural sources whose activity cannot be adequately described or predicted by chemical and physical methods. In spite of the enormous advances made in recent years of the knowledge of the chemical basis for the anticoagulant activity of heparin (Petitou, 1989; Casu, 1989), the assessment of heparin for quality control purposes by manufacturers and for dosage by clinicians is still made in units of biological activity. Quantity of heparin is expressed in units. The heparin unit is defined as the anticoagulant activity of 1/130 mg of the International Standard heparin preparation (Barrowcliffe, 1989). Unlike other drugs in other systems, the activity for a given commercial preparation relative to the International Standard heparin preparation changes with the method of comparison in coagulation tests. Hence the values will not necessarily be the same for any two preparations when compared by different procedures. Equivalence figures can be expressed in units qualified by the name of the procedure but because this means a number of different values for a given preparation, the result can be confusing.

A heparin treatment must result in heparin levels in the blood that are sufficient to control thrombosis and yet avoid undue risk of bleeding. The antithrombotic effect and the risk of bleeding varies, however, not only with the dose but also

with the individual response. This variation in response to heparin calls for an individualization of the heparin dose regimen and a careful clinical monitoring by laboratory tests (Abildgaard, 1989). The activated partial thromboplastin time (APTT) is the most widely used clinical laboratory test for heparin monitoring. In this test, the clotting time is measured of a plasma sample to which an initiator of the bloodclotting process is added. In the presence of the initiator, coagulation is greatly accelerated and anticoagulant activity can then be measured by the delay of appearance of a clot, with clotting times in the order of minutes.

Commercial preparations of heparin are heterogeneous, and their components have molecular weights ranging from 3000 to 30000 (mean 15000). The primary chemical structure of heparin consists of alternating hexuronic acid (D-glucuronic or L-iduronic) and D-glucosamine residues, which are variably sulphated at amino and hydroxyl groups (Casu, 1989). At physiological pH values the three acid functional groups in heparin are fully dissociated to yield $-\text{OSO}_3^-$, $-\text{NHSO}_3^-$ and $-\text{COO}^-$ groups thus resulting in a highly negatively charged heparin molecule.

The ion-step measuring method

The device used in this measuring method consists of a porous membrane of polystyrene beads linked together with agarose, which is deposited on the gate of an ISFET (Fig. 1). An affinity ligand can be immobilized in the membrane via physical adsorption or covalent binding. The charged groups of the polystyrene beads together with the charged groups of the affinity ligand, result in a net charge density c_x in the membrane

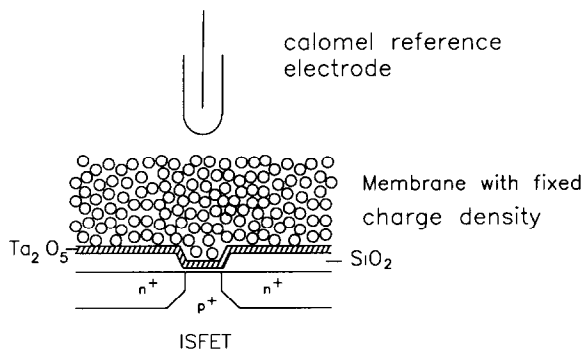


Fig. 1. The device.

which is pH dependent in case the charged groups are titratable. If the sensor is actuated by a stepwise change of the electrolyte concentration c_s at $t = t_0$ (Fig. 2A), the ISFET shows a transient response of which the amplitude ΔV is a function of the net charge density in the membrane (Fig. 2B). The ISFET response is positive if c_x is negative and vice versa. The origin of the response is investigated in detail by Eijkel *et al.* (1992). Here we would like to confine ourselves to a short description of the mechanism.

When a layer of protein molecules is in thermodynamical equilibrium with a solution with a certain pH and ion concentration, the ions in the solution and in the protein layer will be distributed according to the Donnan ratio (the ratio of the ion activities in the two phases). As a result, there will exist an electrical potential difference between the protein layer and the bulk solution which is known as the Donnan potential.

The dissociation of titratable groups of a protein molecule is influenced by the electrolyte concentration. A positively charged protein will contain more bound protons per molecule at higher electrolyte concentrations and a negatively

charged protein will contain less bound protons per molecule at higher concentrations.

When the membrane of polystyrene beads is in thermodynamical equilibrium, Donnan potentials exist at the pore walls of the porous membrane, where the protein is immobilized. At high charge densities the resulting fields can extend to the pore centres, and consequently the membrane itself will have a potential with respect to the bulk solution.

As a result of a stepwise increase in electrolyte concentration, the Donnan potentials of the protein layer will diminish, thereby also diminishing the potential of the membrane itself versus the bulk solution. Furthermore, protons will be released by negatively charged protein molecules or taken up by positively charged protein molecules because the dissociation is changed with the ion concentration. The underlying ISFET measures the change in the potential of the membrane with respect to the bulk solution as well as the pH effect of the protons released or taken up. The pH effect of the protons is responsible for almost the entire response on an ion-step.

Measuring heparin concentrations with the ion-step measuring method

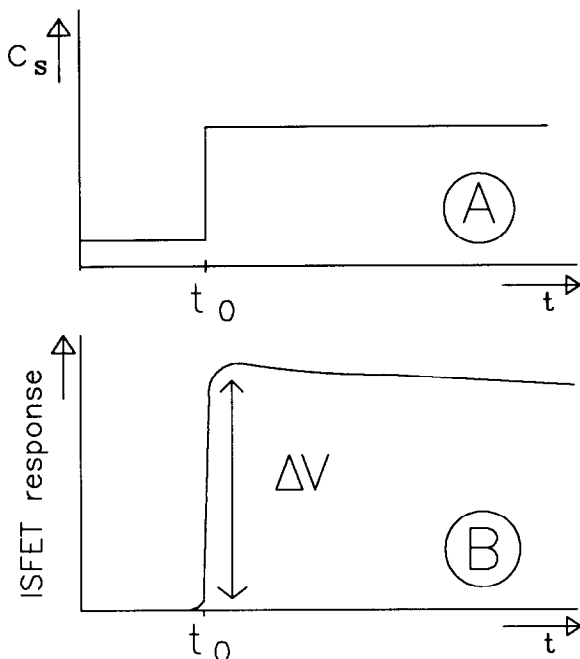


Fig. 2. A: Electrolyte concentration c_s as a function of time. B: ISFET response as a function of time for c_x is negative.

In clinical practice a heparin treatment is monitored by coagulation tests which determine the biological activity of heparin. It is shown that only about one third of a commercial heparin preparation has anticoagulant activity (Hirsch, 1991). The relevance of *in vitro* heparin assays (measuring the activity) to *in vivo* events is however still unclear. Other studies have shown that extra anticoagulant activity of injected heparin is generated *in vivo* (Levy and Jaques, 1978). In these studies the total absolute amount of heparin in plasma is measured instead of the biological activity (Jaques *et al.*, 1990).

Without taking part in the discussion about how to monitor a heparin treatment, the present paper describes the possibility of measuring heparin concentrations (total amount) with the ion-step measuring method. The method can probably also be used to measure biological activity of heparin by using another affinity ligand binding to another analyte, which interaction depends on the biological activity of heparin (e.g. thrombin to bind antithrombin III). This will be a subject for future research.

The range which is of physiological importance for monitoring is 0.1–1.0 units/ml. The heparin we used in our experiments was a commercial heparin solution for clinical application. The concentration was defined in anticoagulant activity units/ml. The results of our experiments are therefore given as a function of this concentration.

To determine heparin concentrations protamine has been chosen as an affinity ligand. Protamine is clinically used to neutralize heparin and to counteract its anticoagulant effects. The protamines are a family of basic proteins which typically contain 22 arginines out of 32 amino acids (MW \pm 4000). It is found that the interaction of protamine and heparin consists of 1:1 pairing of anionic heparin sites with cationic protamine sites (Cundall *et al.*, 1982).

The use of the positively charged protamine as affinity ligand for the negatively charged heparin will result in a substantial change in charge density in the membrane which is very beneficial when using the ion-step measuring method. Therefore, the protamine-heparin couple seems to be a good realistic example for the development of the first sensor system based on the ion-step measuring method. The protocol to realize the calibration curve of the sensor response as a function of heparin concentration is as follows:

- Immobilize protamine in the porous membrane.
- Calibrate the device by measuring the response to an ion-step.
- Expose the device to a known heparin concentration for a certain incubation time.
- Determine the response to an ion-step.
- Determine the shift in response with respect to the calibration.
- Repeat the previous steps for different heparin concentrations.
- Plot the shift in response as a function of heparin concentration.

2. MATERIALS AND METHODS

The measurement set-up consists of a computer-controlled flow through system in which the pH can be varied by mixing two buffers with variable ratio. The electrolyte concentration at the surface of the membrane-covered ISFET can be changed

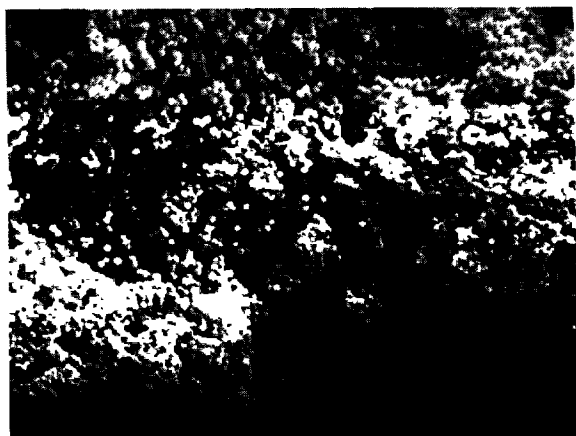
within 50 msec from a 10 mM KCl to a 50 mM KCl solution by switching a valve. For some of the experiments the 10 mM KCl solution was buffered with a mixture of 0.2 mM citric acid and 0.5 mM TRIS to vary the pH between 4 and 9 while the 50 mM KCl solution was not buffered. Other experiments were performed with 10 and 50 mM KCl solutions which were both buffered with respectively 0.5 and 0.2 mM HEPES (pH 7.55).

The responses of the Ta₂O₅-ISFETs were measured with a source and drain follower (Bergveld, 1981) and recorded with a Nicolet 310 digital storage oscilloscope.

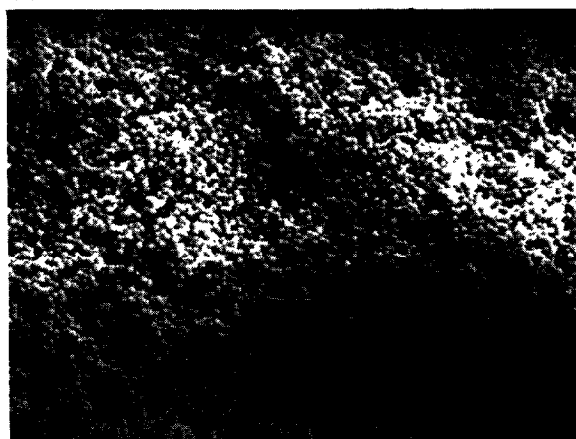
The membranes were made according to the process described by Schasfoort *et al.* (1990). A 0.25% agarose solution (low IEE, zero M_r, Biorad) was mixed 1:1 with a suspension of polystyrene beads (2.5% solids-Latex, Polysciences inc.) at 40–50°C and portions of 3 microlitres were cast on the ISFETs. After overnight drying at 4°C and a temperature step of 55°C (for one hour), membranes with a thickness of about 10–15 micron (in dry condition) resulted. An additional step with respect to the original process was ultrasonication of the mixture for 1–2 min before casting it on the ISFETs. This resulted in more homogeneous membranes as is shown on the SEM-photographs in Fig. 3.

Protamine sulphate (grade X, from salmon) was purchased from Sigma and heparin from Organon Teknika (Thromboliquine[®], 5 ml ampoules containing 25000 units). Protamine was immobilized in the membrane by physical adsorption or by covalent binding. In the case of immobilization by physical adsorption, ISFETs with membranes were exposed to a solution of 0.1 mg ml⁻¹ protamine sulphate in phosphate buffered saline (PBS) for 1–17 h. In the case of immobilization by covalent binding, polystyrene beads with functional amino groups were washed in a buffer solution and subsequently the amino groups were activated by glutaraldehyde. Next, the beads were washed again and exposed to a solution of 0.25 mg ml⁻¹ protamine sulphate for about 4–5 h. After washing, the beads were used to make membranes.

For the experiments in blood plasma, citrated normal plasma was used which was a gratefully accepted gift from the laboratory of the 'Medisch Spectrum Twente' hospital. Small amounts of the concentrated heparin solution were added to



(a)



(b)

Fig. 3. (a) SEM photograph of a membrane of 0.12 μm beads without ultrasonication of the membrane mixture. (b) SEM photograph of a membrane of 0.12 μm beads after ultrasonication of the membrane mixture.

the citrated normal plasma to obtain plasma samples with different heparin concentrations.

3. RESULTS

A typical ion-step response of an ISFET provided with a membrane of 0.12 micron polystyrene beads is given in Fig. 4. In this case the 10 mM KCl and the 50 mM KCl solutions were both buffered at pH 7.3. The top curve represents a typical ion-step response of an ISFET with a bare membrane without immobilized proteins. The negative surface charge on the beads (and possibly the agarose) result in a positive response.

The bottom curve in Fig. 4 represents a typical ion-step response of an ISFET with a membrane (0.12 micron beads) with immobilized protamine.

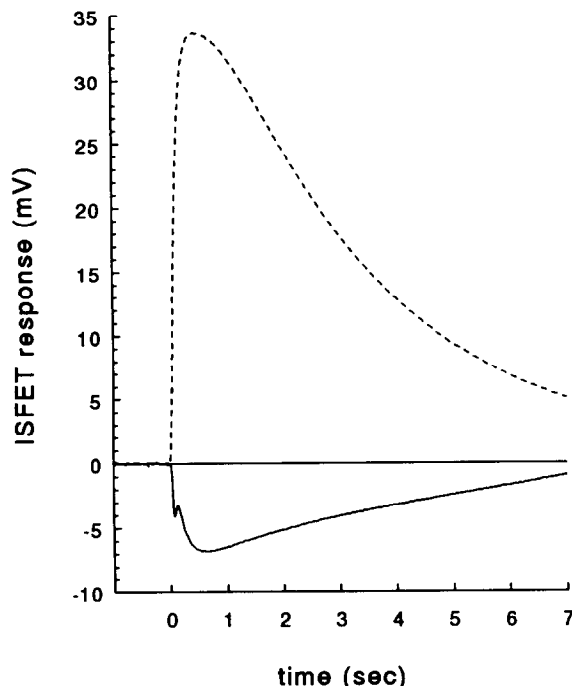


Fig. 4. Top curve: typical ion-step response of an ISFET with a membrane of 0.12 μm beads without coupled proteins. Bottom curve: typical ion-step response of an ISFET with a membrane of 0.12 μm beads with adsorbed protamine.

To immobilize the protamine, the device was incubated for 15 h in 0.1 mg ml⁻¹ protamine sulphate. The positively charged groups of the protamine result in a net positive charge in the membrane and thus in a negative ion-step response. The small negative peak that precedes the larger response is a typical boundary potential of the membrane while the larger response is caused by a proton uptake by titratable groups resulting in a temporary increase of the pH in the membrane. Experiments showed that already after two hours of incubation in 0.1 mg ml⁻¹ protamine sulphate, the ion-step response reaches a maximum value indicating that the membrane is saturated with protamine. Yet the reason for incubating the membranes for 15 hours is the experience that protamine which was immobilized by long incubation times did not desorb at all for at least 48 h, while short incubation times did result in some desorption of the protamine from the polystyrene beads.

Ion-step responses were also recorded as a function of pH. Fig. 5 shows the amplitude ΔV of the ion-step response as a function of pH for

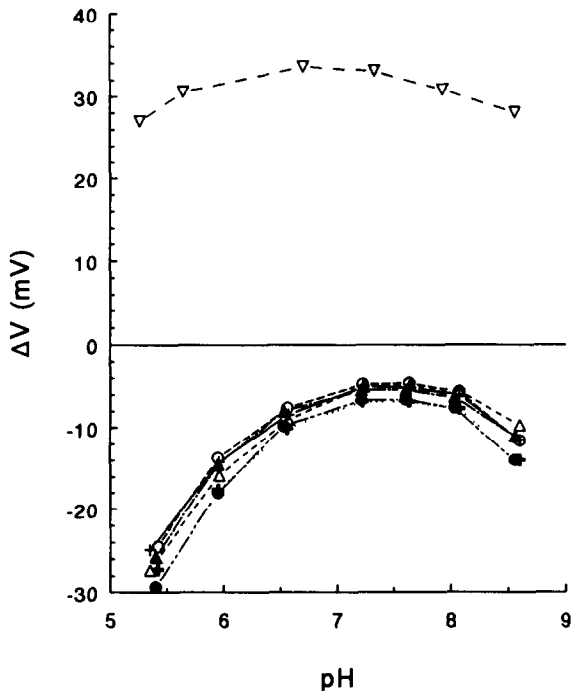


Fig. 5. Top curve: amplitude ΔV of an ion-step response of an ISFET with a bare membrane as a function of pH. Bottom curves: amplitude ΔV of ion-step responses of six ISFETs with a protamine loaded membrane as a function of pH.

an ISFET with a bare membrane (upper curve, typical result) and for a series of six ISFETs provided with a protamine loaded membrane (lower curves). The pH of the 10 mM KCl solution was varied by mixing a citric acid and a TRIS buffer solution. The 50 mM KCl step solution was not buffered ($\text{pH} \pm 5.5$) because the transient response of the membrane on an ion-step is much faster than the establishment of the new pH equilibrium in the membrane.

As a result of the dissociation of titratable groups, the charge density in a positively charged membrane should decrease with increasing pH until the charge density becomes zero at the iso-electric point of the charged membrane and then changes sign. If the ion-step response should only be a function of the charge density, the bottom curves in Fig. 5 should continuously rise with increasing pH and eventually cross the X-axis at the iso-electric point of the membrane. This effect is however not seen. The reason for this deviation might be that because the ion-step response is mainly caused by a release or uptake of protons, which result in a temporary pH

change in the membrane, the buffer capacity of the solutions and the membrane also play a very important role. Further experiments did indeed show a different response when using different buffer capacities. More experiments need to be done to determine the exact influence of the buffer capacity and to interpret the shape of the ΔV -pH curves. Up to now we decided that, in order to compare ion-step responses before and after modulation of the charge density in the membrane, the buffer capacity must be kept constant. In a practical application of the sensor, the buffer capacity as well as the pH will be kept constant before and after modulation of the charge in the membrane and only one ion-step response is needed (after calibration) to determine the concentration. The exact shape and nature of the ΔV -pH curve is then of minor importance.

Each of the six ISFETs with protamine loaded membranes was subsequently immersed in a PBS solution with a different heparin concentration. After 18 and 40 h of incubation, the ion-step responses were again recorded as a function of pH. In Fig. 6 the amplitudes of the transient ion-step responses after 40 h of incubation are shown as a function of pH. The shape of the ΔV -pH curves does not significantly change after incubation in different heparin solutions. It is therefore concluded that the change in the ion-step response as a function of heparin concentration does practically not depend on pH. In Fig. 7 we have shown the change in the amplitude of the ion-step response at pH 7.4 for the six different ISFETs with respect to the response before incubation in the heparin solutions. The change in the amplitude of the ion-step response is plotted as a function of heparin concentration. The curves which are fitted through the points show a linear relation between the change in response and the heparin concentration in the range 0.1–1.0 units/ml.

Aiming at a practical application of the sensor, incubation times of 18 or 40 h are not very auspicious. To determine if more porous membranes result in shorter incubation times, ISFETs were provided with membranes of three different sizes of polystyrene beads (0.12, 0.43 and 1.14 micron diameter). Protamine was immobilized by physical adsorption and the response to an ion-step was recorded. The devices were subsequently immersed in a PBS solution containing 0.5 unit/ml heparin and the ion-step response

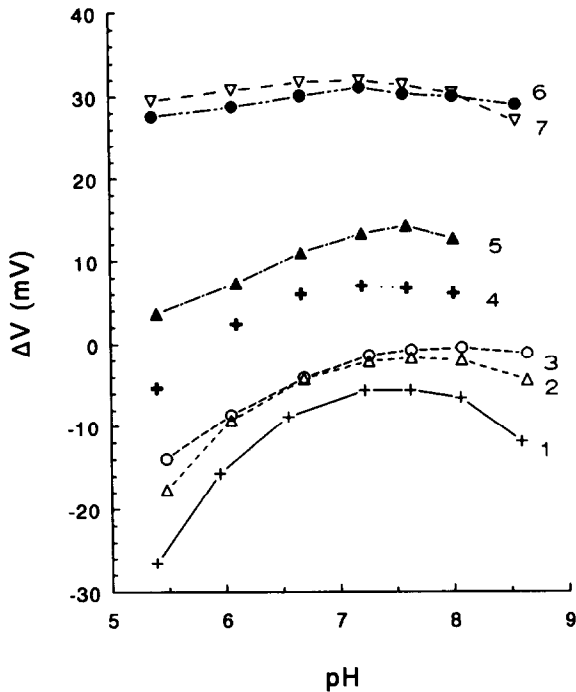


Fig. 6. Amplitude ΔV of ion-step responses after incubation for 40 hours in heparin as a function of pH. Curve 1 is the average of the six bottom curves of Fig. 5. Curves 2-7 are recorded after incubation in respectively 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 unit/ml heparin in PBS.

was recorded as a function of incubation time. Figure 8 shows the change in the amplitude of the ion-step response (with respect to the response before incubation in the heparin solution) for the different ISFETs as a function of incubation time (typical result). From this figure it is obvious that larger beads lead to more porous membranes and facilitate the diffusion of heparin into the membrane.

Based on these results we decided to use 1.14 micron beads for a new series of devices and determine the change in the ion-step response as function of a heparin concentration. Protamine was again immobilized by physical adsorption and before exposing the device to a heparin solution, a calibration ion-step response was recorded. Figure 9 shows the change of the amplitude of the ion-step response after 30, 60 and 170 min of incubation in the heparin solutions.

Another series of six ISFETs was provided with a membrane of 1.0 micron polystyrene beads containing functional amino groups. Protamine was immobilized by covalent binding via glutaral-

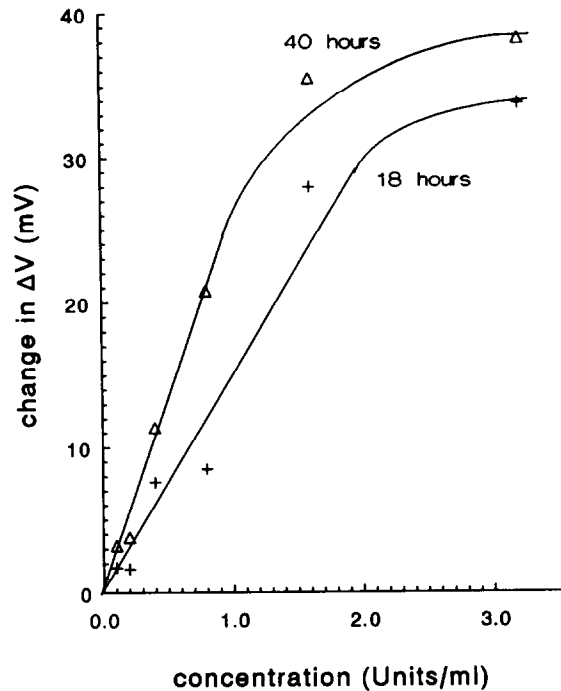


Fig. 7. Change in amplitude ΔV of the ion-step responses at pH 7.4 as a function of heparin concentration after 18 h and 40 h of incubation.

dehyde after which the devices were immersed in different heparin solutions. Figure 10 shows the results of this experiment. The same type of devices were used to determine the ion-step response in blood plasma with different heparin concentrations. In Fig. 11 the change in the amplitude of the ion-step response with respect to the response before incubation is shown as function of a heparin concentration in blood plasma.

4. DISCUSSION AND CONCLUSION

The results of the experiments as described in the previous section show that it is possible to determine heparin concentrations by the ion-step measuring method by using protamine as affinity ligand. The relation between the change in the amplitude of the ion-step response and the heparin concentration in PBS can be described as linear in the range 0.1-1.0 units/ml. As described in the previous section, the measurements of the different heparin concentrations were performed with different devices because the ion-step measuring method is a 'single use'

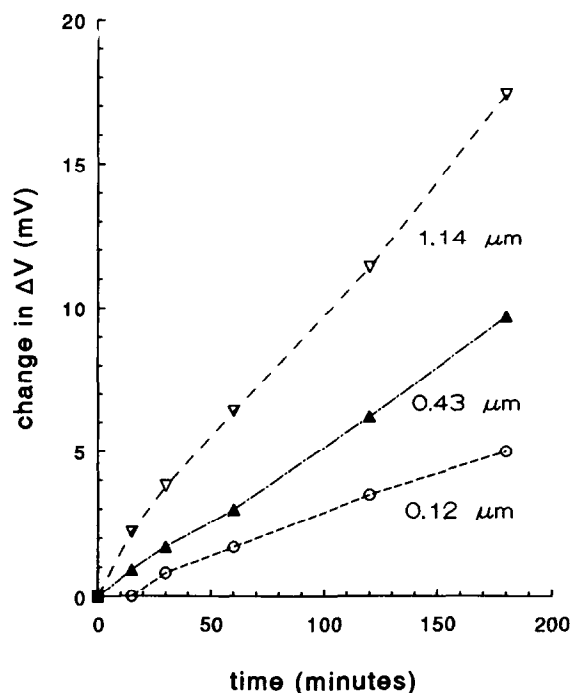


Fig. 8. Change in amplitude ΔV of ion-step responses of ISFETs with protamine loaded membranes of different beads as a function of incubation time in 0.5 unit/ml heparin.

method. Due to the fabrication process, the membrane thickness of the different devices varies by about 20%. This is the main reason for the individual deviations of the measured heparin concentrations from the fitted curve. Another fabrication process which controls the membrane thickness, for instance spinning the membrane mixture on a wafer of ISFETs, will reduce these deviations. For higher concentrations and longer incubation times, the curves deviate from the linear relation as a result of saturation of the membrane with heparin.

The slope of the response-concentration curve is a function of the incubation time, the bead diameter and the way of immobilization of the protamine. The slope varies from 26 $\text{mV unit}^{-1} \text{ ml}^{-1}$ for 0.12 micron beads after 40 h of incubation to about 3 $\text{mV unit}^{-1} \text{ ml}^{-1}$ for 1.0 micron beads after 15 min of incubation. The minimum acceptable slope of the response-concentration curve is determined by the specifications for a practical application together with the technical specifications of the measurement system. If we assume that the required accuracy for determining heparin concentrations is 0.05

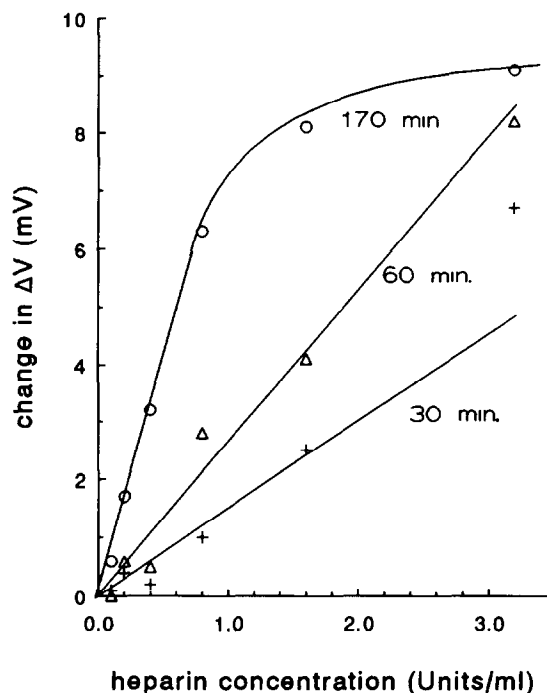


Fig. 9. Change in amplitude ΔV of ion-step responses of ISFETs with membranes of 1.14 μm polystyrene beads with adsorbed protamine as a function of heparin concentration in PBS after three incubation times.

unit/ml and that the ion-step response is reproducible within 0.1 mV, the minimum acceptable slope is 2 $\text{mV unit}^{-1} \text{ ml}^{-1}$.

Immobilization of protamine by covalent binding results (after a certain incubation time) in a larger slope of the response-concentration curve than physical adsorption (see Fig. 9 and Fig. 10). Covalent coupling by glutaraldehyde results in protein binding 6–10 atoms from the surface while coupling by adsorption results in protein binding on the surface. The covalently coupled protamine is therefore better attainable for heparin than the adsorbed protamine.

To increase the response after only 15 min of incubation, there are a few parameters that still can be optimized. The use of larger beads will result in more porous membranes which facilitates diffusion of heparin in the membranes. However, larger beads will give a smaller charge density in the membrane which results in a smaller temporary pH change in the membrane in response to an ion-step. The thickness of the membranes can also be reduced to facilitate diffusion of heparin in the membrane but this will also result in a smaller ion-step response. More experiments

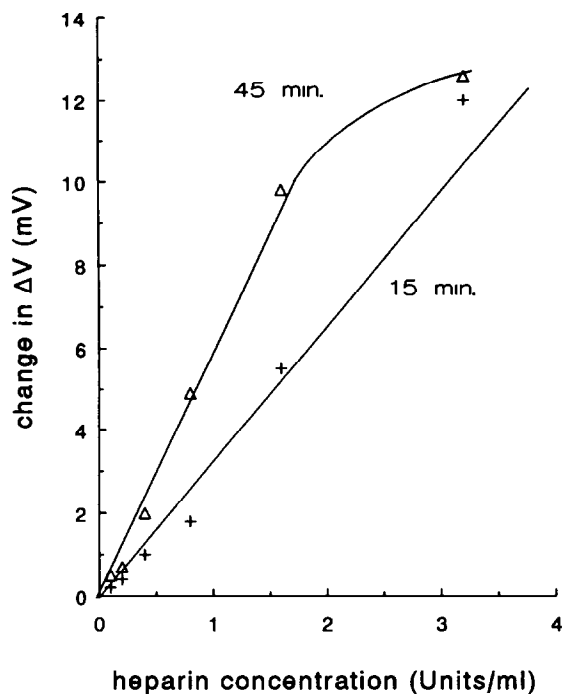


Fig. 10. Change in amplitude ΔV of ion-step responses of ISFETs with membranes of 1.0 micron polystyrene beads with covalently bound protamine as a function of heparin concentration in PBS after two incubation times.

have to be carried out to optimize these two parameters.

The curves in Fig. 11 which represent the results of the measurement in blood plasma show a significant offset caused by nonspecific adsorption of plasma components in the membrane. To reduce nonspecific adsorption it is common practice to block all redundant adsorption sites after coupling the affinity ligand. This is usually done with albumin or small polypeptides. We expect that also in our application, blocking of the adsorption sites will reduce the nonspecific adsorption of plasma components.

The measured relation between the shift in the ion-step response and the heparin concentration in blood plasma is not linear. It is unclear if this is caused by the nonspecific binding of other plasma components or that it is caused by the binding of plasma proteins to the heparin. More experiments will be necessary to determine the relation between the ion-step response and the heparin concentration in blood plasma.

The results presented in this paper show the possibility of detecting heparin concentrations of

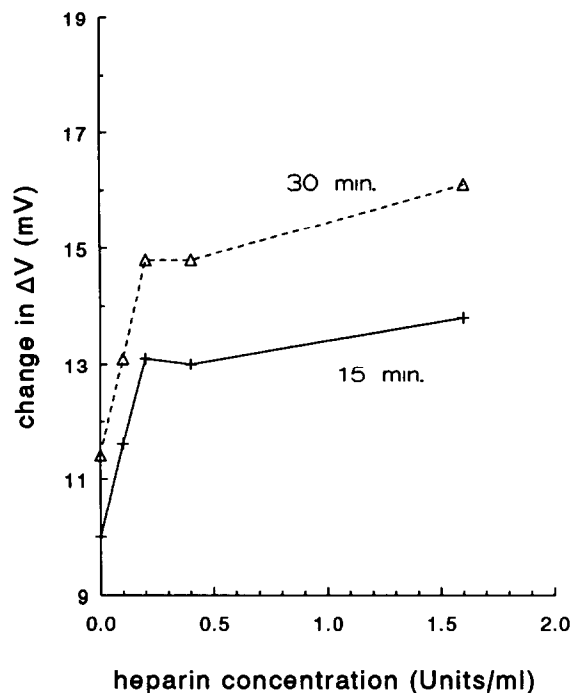


Fig. 11. Change in amplitude ΔV of ion-step responses of ISFETs with membranes of 1.0 micron polystyrene beads with covalently bound protamine as a function of heparin concentration in blood plasma after two incubation times.

0.1 units/ml using the ion-step measuring method. It has been reported that 1 unit of heparin corresponds with about 8 microgram for many commercial preparations (Jaques *et al.*, 1990). The average molecular weight of the heparin preparation used is about 15000; thus 0.1 unit/ml corresponds with 5×10^{-11} Mol/l. This makes the ion-step measuring method a very interesting method for a broad scale of applications including immunodiagnostics.

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