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Improving measurement stability and reproducibility of potentiometric sensors for polyions such as heparin

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Abstract

The growing importance of polymer membrane-based potentiometric polyion sensors in biomedical research and clinical measurements has brought up the question of how accurate and reproducible these sensors are. Indeed, recent research has revealed that these sensors behave quite differently than classical ion-selective electrodes. This paper explores ways to improve measurement reproducibility and long term potential stability by considering the unique pseudo steady-state response mechanism of the polyion sensors developed so far. Heparin may be stripped out of the phase boundary membrane surface with a high sample NaCl concentration and this characteristic is used to modify the calibration procedure in order to avoid memory effects. It is also attempted to reduce long term potential drifts by continuously stripping heparin out of the membrane at the membrane-inner filling solution side.

Keywords: Potentiometric sensors; Ion-selective electrode; Polyion; Heparin

1. Introduction

Polymeric membrane-based potentiometric electrodes have become an interesting and important area of research due to their direct application in biomedical analysis. Rapid and accurate analysis of polyions in whole blood samples continues to be challenging and the discovery that polymer membranes similar to those that have been used in the direct analysis of small ions also give a response to polyions has opened a new branch of research in the field [1,2]. Potentiometric sensors of this type have been developed for heparin as well as for protamine [3,4] and the development of similar membrane-based sensors for many other polyions is in progress [5].

Both heparin, a natural anticoagulant with an average MW of about 15000 Da and an average charge of -70 and protamine, a heparin antagonist with an average MW of 4500 Da, are used in clinical settings. Heparin is a parenteral drug of which about 500 million doses are administered in the world every year [3]. It is the anticoagulant of

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choice for acute treatment during pregnancy [6] and in infants [7], is regularly used in children [8] and is also safe for nursing mothers [6]. Because of the rapid onset of anticoagulation seen with heparin, it is used in overlap therapy with oral anticoagulants such as warfarin, which has a much slower onset of action [9]. Heparin is also extremely important in surgical procedures, especially cardiac and vascular surgery, where it is used to avoid thrombus formation [9]. Protamine is given post-surgically to neutralize heparin activity and avoid prolonged bleeding. Since much of the heparin is metabolized in the body and the extent of metabolism varies with age [8], not only do the heparin levels in the patient's blood need to be monitored during surgery, but the post-surgical heparin levels must be known to give an accurate dose of protamine.

The analysis of heparin in blood has always been performed via indirect clotting time measurements such as activated clotting time (ACT) and activated partial thromboplastin time (APTT). Since different commercial reagents respond differently to heparin [10,11] and other biological factors also influence clotting times, giving variations for the same concentration of heparin [9], the accuracy of the methods toward detecting true concentrations of heparin is reduced. A more accurate but time consuming method of analysis that has been used is to measure heparin levels by titrating the patient's blood with protamine and comparing the results with the ones obtained from APTT measurement [9].

Recently, a polymer membrane-based potentiometric sensor has been developed that contains the heparin carrier known as TDMAC (tridodecylmethylammonium chloride). With a quaternary ammonium structure very similar to polybrene, a strong heparin antagonist, the $TDMA^+$ ion is known to associate strongly with the negatively charged heparin molecule [12]. Polymeric membranes containing TDMAC have been shown to readily extract heparin from the sample under simultaneous release of chloride ions from the membrane [13]. However, owing to the high charge of the polyion heparin, a very low electrode sensitivity of about -1 mV per 10-fold heparin concentration change would ordinarily be expected for such a sensor. Therefore, these sensors must operate via a nonclassical steady-state mechanism where the extent of heparin uptake depends directly on the sample heparin concentration [13]. This leads to a sigmoidal response shape of the electrode with large and analytically useful potential changes. Bovine heparin, which has a higher charge density than porcine heparin, has been shown to give a somewhat larger electrode response [14], but the sensor may be used successfully for both types of heparin.

Direct measurement of heparin with this type of sensor is more rapid than current methods of measurement and avoids the inaccuracy caused by indirect assays. Although there are ions present in the blood such as Cl^- and HCO_3^- which interfere with the EMF response of these sensors, the interferences could be corrected for experimentally if the concentrations of these interfering ions are determined simultaneously. In this manner, direct measurements of heparin may be performed. An alternate method of heparin analysis which avoids interference from other ions involves the use of the heparin sensor as an endpoint indicator for the titration of the heparinized blood with protamine [2]. Other applications of the heparin sensor include the use of heparin titration to measure heparin binding characteristics with macromolecules [15].

Because of the importance of potentiometric sensors in biomedical analysis and the small errors permitted in clinical measurements, it is important to have knowledge of sources of error in the sensors and take steps to alleviate these sources. In this paper a brief discussion on the response mechanism of these sensors and on how it differs from small ion sensors, is given. The mechanism by which polyions are extracted from the membrane with high inner electrolyte concentrations is discussed and applied to long term drift experiments as well as calibration methods. It is shown how the classical calibration techniques neglect to reflect the polyion response mechanism, and that the accuracy and reproducibility of the sensors can be improved simply by overcoming a source of error that is caused by the nature of the polyion response. The results from this study are a step toward making these polyion sensors more applicable to biomedical analysis.

2. Experimental section

2.1. Reagents

Tridodecylmethylammonium chloride (TDMAC), bis(2-ethylhexyl)sebacate (DOS), high molecular weight poly(vinyl chloride) (PVC), tetrahydrofuran and sodium chloride (puriss. grade) were purchased from Fluka Chemica-Biochemica (Ronkonkoma, NY). Heparin sodium salt (from bovine intestinal mucosa) was purchased from Sigma (St. Louis, MO). Aqueous solutions were prepared using Nanopure® (Millipore) purified distilled water.

2.2. Membranes and electrodes preparation

The heparin sensitive polymer membranes were prepared by dissolving the heparin carrier (TDMAC), the plasticizer (DOS), and the polymer matrix (PVC) in approximately 1.5 ml THF. The exact membrane composition [3] can be described as high plasticizer or low plasticizer membranes (specified high DOS and low DOS, respectively). The high DOS membrane used in the drift measurement was composed of 1.5 wt% TDMAC, 66 wt% DOS and 32.5 wt% PVC for a total membrane mass of 140 mg. The low DOS membrane used in the calibration and reproducibility measurements was composed of 1.5 wt% TDMAC, 32.5 wt% DOS, and 66 wt% PVC for a total membrane mass of 140 mg. After dissolving in ca. 1.5 ml THF, the membrane cocktails were poured into a glass ring (2.2 cm i.d.) affixed onto a microscopic glass slide and allowed to evaporate over night. A disk 6 mm in diameter was cut with a cork borer and incorporated into a Philips electrode body (IS-561, Glasbläserei Möller, Zürich, Switzerland). A sodium chloride inner filling solution was added to each electrode and the electrodes were conditioned in 0.01 M NaCl overnight.

2.3. EMF measurements

Potentiometric measurements were made in unstirred solutions versus a double junction Teflon® sleeve Ag/AgCl reference electrode (Ingold, Wilmington, MA) in a cell of the type

Ag|AgCl|KCl|1 M LiOAc||sample|
|membrane||inner electrolyte|AgCl|Ag

All potentials were measured at 22 ± 1.0°C via a Macintosh computer equipped with a LAB-MIO-16XL-42 16 bit A/D I/O board (National Instruments, Austin, TX) and up to four battery powered 4-channel high Z interface modules with built in low pass filters (World Precision Instruments, Sarasota, FL) controlled by LabView software (National Instruments) at a software adjustable gain of 10. To minimize the noise, 100 consecutive EMF data values were acquired per measurement interval and averaged, and the measurement interval was 5 s. When plotting the change in EMF versus the log of heparin concentration the EMF value was calculated as the mean of the individual data over the last minute of measurement. When tracing the response over the entire measurement, the averaged data from each measurement interval was plotted versus time.

2.3.1. Calibration method

All electrode membranes were backfilled with inner filling solutions of 0.01 M NaCl. After a 5-min baseline measurement of the background 0.01 M NaCl sample was taken, the classical calibration curve was obtained by consecutively adding small aliquots from a heparin stock solution (1.58×10^{-4} M) to the sample. The change in EMF resulting after each addition was plotted against the log of the sample heparin concentration. After stripping the heparin from the membranes for 2.5 min using 2 M NaCl, followed by reconditioning the membranes in 0.01 M NaCl for 2.5 min, four heparin samples (2 U ml^{-1}, 4 U ml^{-1}, 6 U ml^{-1}, and 8 U ml^{-1} heparin, where 1 U $ml^{-1} = 4 \times 10^{-7}$ M) were measured individually, repeating the stripping and reconditioning procedures between each sample measurement. The sample data points were plotted with the classical calibration curve.

After performing the 2.5-min stripping and reconditioning procedures described above, a 5-min baseline measurement of the background electrolyte solution (0.01 M NaCl) was taken, and the new calibration curve measurements were

immediately begun. The first aliquot was placed in the background solution and measured, followed by the 2.5-min stripping and reconditioning procedures. The second measurement was made by adding a volume of heparin stock solution, equal to the sum of the first two aliquots in the classical method, to a 50 ml 0.01 M NaCl (heparin-free) background solution. In this manner each calibration point of the new calibration curve exactly matched the concentration that was measured for the corresponding calibration point in the classical method. The change in EMF after each addition was plotted against the log of the sample heparin concentration. Four heparin samples were again measured using the same procedure as described above and these sample data points were plotted with the new calibration curve.

2.3.2. Reproducibility

All electrodes were filled with inner filling solutions of 0.01 M NaCl. Measurement reproducibility was studied by repeating measurements of a 1.19×10^{-6} M heparin sample using two different measurement methods. In the first method, electrodes which had been previously stripped of heparin were reconditioned and stabilized in 50 ml of a 0.01 M NaCl background electrolyte solution. Following a 5-min baseline measurement of the background electrolyte solution an aliquot of the heparin stock solution was added to the background solution and measured for 5 min. A total of five samples of this concentration were measured with a 5-min reconditioning period (0.01 M NaCl) between each. After the final sample was measured, the electrodes were placed in reconditioning solution for 1 h to observe restabilization times. The data collected from each 5-s measurement interval was plotted against time.

Electrodes were stripped of heparin and reconditioned for 2.5 min each before beginning the second method of sample measurements. This method differed from the first only in that the previously described stripping and reconditioning procedures preceded each sample measurement instead of the 5-min reconditioning period. After five samples had been measured the membranes were stripped of heparin for 2.5 min and the electrodes were placed in the reconditioning solution for 1 h to observe restabilization times. The data collected from each 5 s measurement interval was plotted against time.

2.3.3. Long term drift

The drift study was performed using electrodes containing high DOS membranes and inner filling solutions varying in concentration from 1×10^{-4} to 2 M NaCl. After conditioning the electrodes were stabilized in 100 ml 0.1 M NaCl background electrolyte solution for 20 min before the electrodes were exposed to heparin. The heparin stock solution was then added to the background electrolyte solution to give 100 ml of 8.4×10^{-5} M heparin solution. The sample was stirred using a Corning magnetic stirrer, although similar experiments without stirring were also performed for comparison purposes. The response was traced over the entire measurement and the data was plotted versus time.

3. Results and discussion

Polyion-selective electrodes (polyion-ISEs) have a mechanism of response that differs from that of small ion-selective electrodes (ISEs). Because of this difference, as will be explained in more detail, the polyion-ISEs require a membrane renewal step between sample measurements that is not necessary with classical ISEs. This step is known to be important for achieving reproducible results [13]. Until now the extent of the error caused by not renewing the membrane between measurements has not been documented. More importantly, the calibration method which has been used classically for ISEs has so far been adopted for use with polyion-ISEs without investigating how the difference in response mechanisms may affect the calibration procedure. The formation of a calibration curve is actually a series of sample measurements of known, increasing analyte activities. This study reveals that the membrane renewal step used between samples of unknown analyte activity should also be used between measurements when creating a calibration curve for the polyion-ISE systems. The error caused by skipping the membrane renewal step in the cali-

bration process may be larger than is clinically acceptable and this study therefore directs researchers toward achieving more accurate measurements with these instruments. To explain why this error occurs and how the membrane renewal is accomplished, a brief overview of the response mechanism of ISEs and polyion-ISEs must first be given.

During the preparation of ISEs the membranes are conditioned with a solution which contains the salt of the target ion, known as the primary ion. Upon conditioning, the primary ion will diffuse into the membrane and replace the original counterions of the lipophilic ionic sites or carriers within the membrane. When the conditioned ISE is allowed to contact a sample containing the primary ion, a local equilibrium is established between the target ions in the sample and the same ion in the membrane. Under these conditions, a change in sample concentration has no significant effect on the concentration of the ion in the membrane. This so-called equilibrium response at the sample-membrane interface gives a phase-boundary potential that obeys the Nernst equation [16]. Hence, the slope of the response curve will decrease as the magnitude of the charge of the primary ion increases. For a polyion such as heparin, which has a large average charge of -70, the slope of the response curve is predicted by the Nernst equation to be too small to be analytically useful (less than 1 mV change per 10-fold change in polyion concentration).Polyion ISEs, therefore, will not give an analytically useful response if prepared in the same manner as classical ISEs. The response mechanism describing this behavior of polyions in polyion-ISEs has been investigated [14], and the response mechanism has been developed and explained specifically for the heparin sensor [2,13]. Polyion sensitive membranes are typically conditioned in a solution containing only background electrolyte (chloride ion in the case of heparin) with no polyion present. Upon exposure of the membrane to a sample containing the polyion, a non-equilibrium response is established which is due to the polyion only partly exchanging with the chloride ions in the membrane. Such processes are described for small ion sensors as well [17,18]. In effect, this results in an activity change of the chloride ion in the phase boundary region of the membrane. The measured potential shows a super-Nernstian slope that allows analytical measurements to be performed. This response is reproducible if the membranes are initially void of the polyion at the sample-membrane interface, i.e. the activity decrease of the chloride ion in the membrane remains the same for a given sample heparin concentration.

If a polyion-ISE has been exposed to its target polyion for a long period of time so that the sample-membrane interface becomes saturated with the polyion, the sensor will begin to show equilibrium behavior similar to a classical ISE. At this point the phase-boundary potential will start to obey the Nernst equation and the electrode is no longer analytically useful for polyion detection. Fig. 1 shows how repetitive use of a heparin responsive electrode without renewing the membrane will slowly approach these conditions. Another situation that would cause Nernstian behavior is exposure of the membrane to polyion concentrations that are high enough to completely displace the smaller ion from the membrane surface [2].

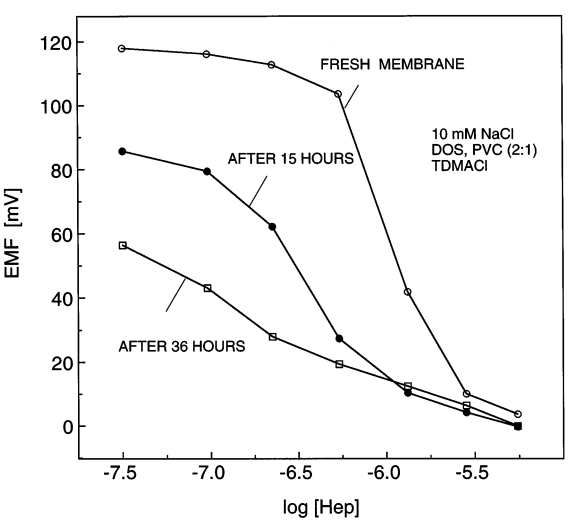

Fig. 1. Three calibration curves of a DOS–PVC (2:1) heparin sensor immediately after conditioning and after a specified time of soaking in the highest measured heparin solution. The sensitivity decreases over time due to slow heparin accumulation in the sensing film.

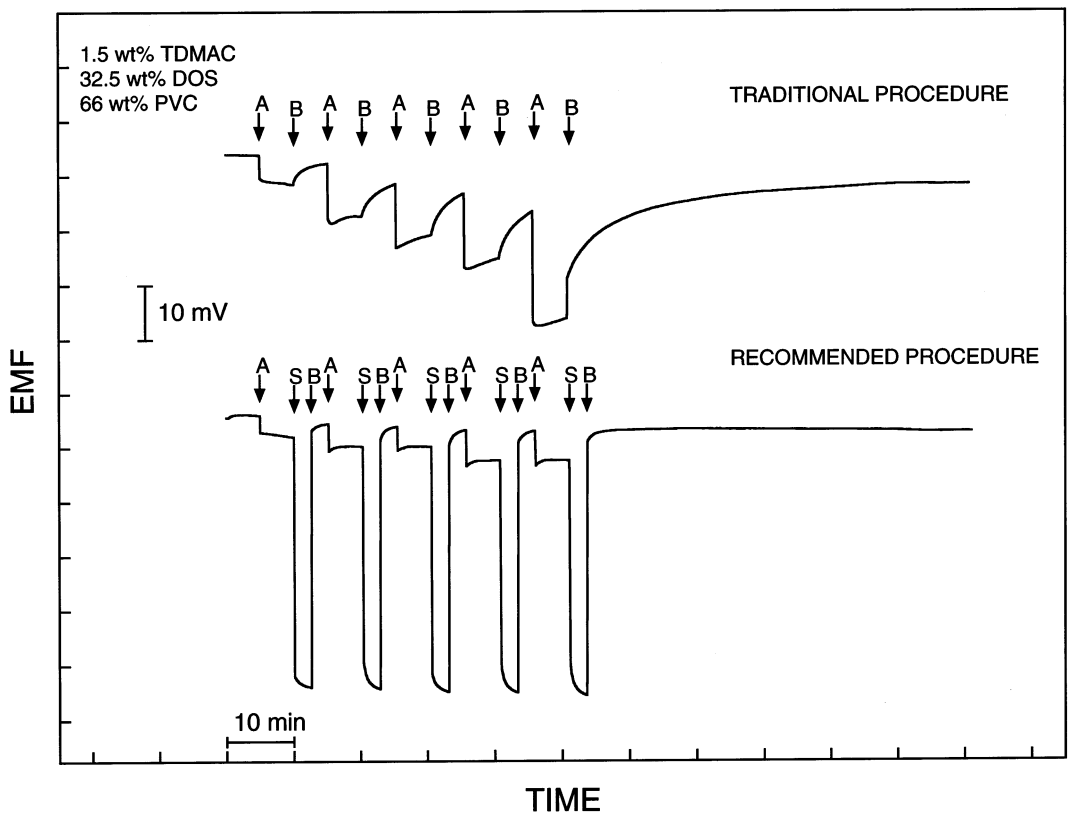

Fig. 2. Consecutive measurements of identical 1.19×10^{-6} M heparin samples. Upper plot: traditional method with the sequence: A, heparin sample; B, 0.01 M NaCl reconditioning step. Lower Plot: proposed method following the sequence: A, heparin sample; B, 0.01 M NaCl reconditioning; S, stripping in 2 M NaCl. Final reconditioning time is 1 h in 0.01 M NaCl for both experiments.

The reversibility of polyion-ISEs by removing the heparin from the membrane surface between measurements has been discussed [3] and is known as the 'stripping' of the polyion from the sample-membrane interface. This is accomplished by placing the electrodes in a high concentration of the background electrolyte, typically 2 M NaCl.

3.1. Reproducibility of sample measurements

It is known that stripping the polyion from the membrane between sample measurements will make the sensor reversible, but this has not yet been documented. The upper traces in Fig. 2 show the plotted data from a repeated 5-min measurement of heparin where the heparin-selective electrode was simply allowed to recondition in 0.01 M NaCl for 5 min between measurements. Not only does the potential not return to the baseline after each measurement, but the point it does return to (higher points) decreases after each measurement. In addition, the response to the same sample concentration (lower points) also decreases. Even when the electrodes are allowed to recondition for an hour, as seen at the end of the plot, the baseline still does not return to the starting potential. The lower traces in Fig. 2 show data from a heparin measurement that was repeated five times with a 2.5-min stripping period using 2 M NaCl followed by a 2.5-min reconditioning time in 0.01 M NaCl between each measurement. The plot shows that both the baseline potential (top points) and the response (middle points) are much more reproducible when the heparin is stripped from the membrane between measurements (lowest

points). When allowed to recondition for an hour at the end of the measurement, the membrane which had been stripped of heparin for 2.5 min returned near the initial baseline potential.

In both cases the potential increases upon reconditioning, quickly at first and then slowly until it levels out. The potential stabilizes at about 15 mV below the starting potential for the unstripped case, whereas the potential approaches about 5 mV from the starting potential for the stripped case. A longer stripping period of 7 min gave even more reproducible results because the potential was able to completely return to baseline (data not shown), but since this length of time may be impractical for real world measurements, the shorter time of 2.5 min was chosen here.

3.2. Calibration method

The calibration method which has been used to obtain calibration curves for all ion selective electrodes has so far also been used to calibrate polyion selective electrodes, not yet taking into account the differences in the response mechanism of the two types of sensors. As already discussed, because of the non-equilibrium response of polyion-ISEs, a change in the sample concentration of the polyion will affect the amount of the polyion that diffuses into the membrane. When obtaining a calibration curve for a polyion-ISE via the classical calibration method, a greater amount of the polyion accumulates within the membrane during each calibration point measurement. For each subsequent measurement the starting baseline potential will be lowered due to the amount of polyion initially present in the membrane, resulting in an apparent increase in ΔEMF relative to that expected from a membrane completely void of heparin (similar to the effect described in the measurement reproducibility section). If the polyion sensor is calibrated this way, the sensor will give biased results when used to determine the concentration of the polyion in a real sample, showing a lower concentration than what is actually present in the sample. These errors are likely unacceptable in clinical measurements and must be alleviated.

An alternate calibration method in which the membrane is stripped of the polyion between each calibration point measurement should give a more accurate curve. Fig. 3a shows the raw data of a calibration curve which was formed using the classical calibration method, i.e. by consecutive addition of heparin aliquots to the sample. Fig. 3b shows an overlay of the calibration curve using the classical calibration method shown in Fig. 3a and data points from four heparin samples within the clinical range. Each of these four samples was measured after stripping residual heparin from the membrane with 2 M sodium chloride. The sample points deviate from the calibration curve by up to 0.25 orders of magnitude. In Fig. 4a the raw data of a heparin calibration curve using the stripping procedure between measurements is shown. In this procedure the heparin was stripped from the membrane surface and the membrane reconditioned as in the reproducibility measurement. In Fig. 4b an overlay of the calibration curve using the proposed calibration method and the same four data points as in Fig. 3b is shown. The maximum deviation seems to be less than 0.1 order of magnitude. These two figures show data from the same electrode and membrane. It must be pointed out that the deviations varied between electrodes, but the general trend was as described for this electrode.

3.3. Drift

With the heparin-selective ISE, some heparin will eventually reach the inner phase boundary of the membrane where it starts to displace chloride ions into the inner filling solution, causing a slow reversal in potential and long-term drift [13]. Since high concentrations of NaCl strip the heparin from the membrane, it can be expected that the magnitude of the drift may be decreased using high concentrations of NaCl in the inner filling solution. As heparin reaches the inner phase boundary an inner electrolyte composition of 2 M NaCl will remove heparin from the membrane surface and into the inner electrolyte. This phenomenon has been used to confirm transport of heparin across the polymer membrane [13]. To test the theory that heparin stripping at the inner

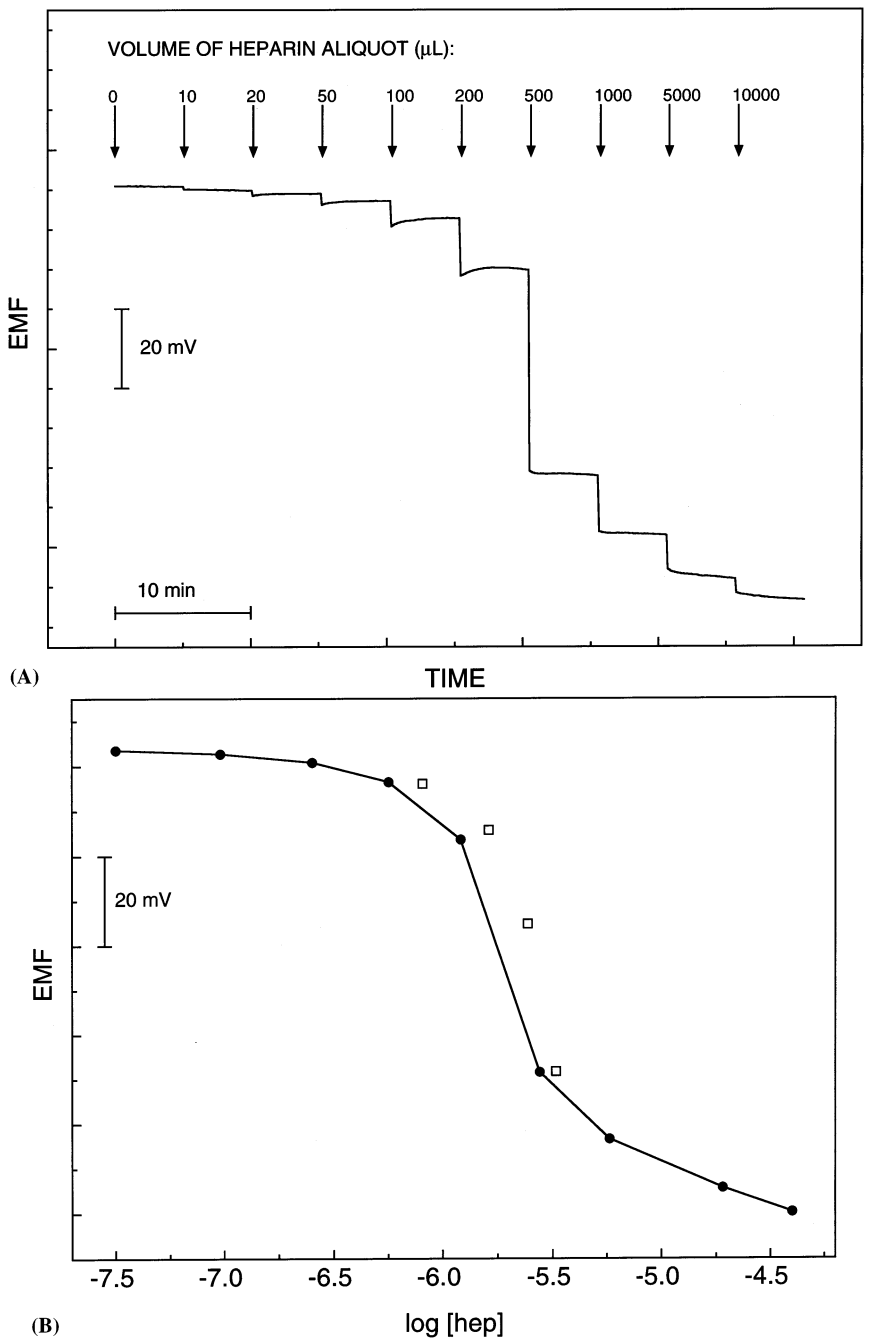

Fig. 3. Electrode potential as a function of time for the traditional calibration method of the heparin-responsive electrode, where heparin aliquots are continuously added to the sample. This method allows heparin to continuously accumulate within the sensing film. 3B. Classical calibration curve for the heparin selective electrode (Fig. 3A). Open squares (A) indicate four sample readings of varying concentrations of heparin. Calibration curve gives concentrations significantly lower than actual concentrations of samples.

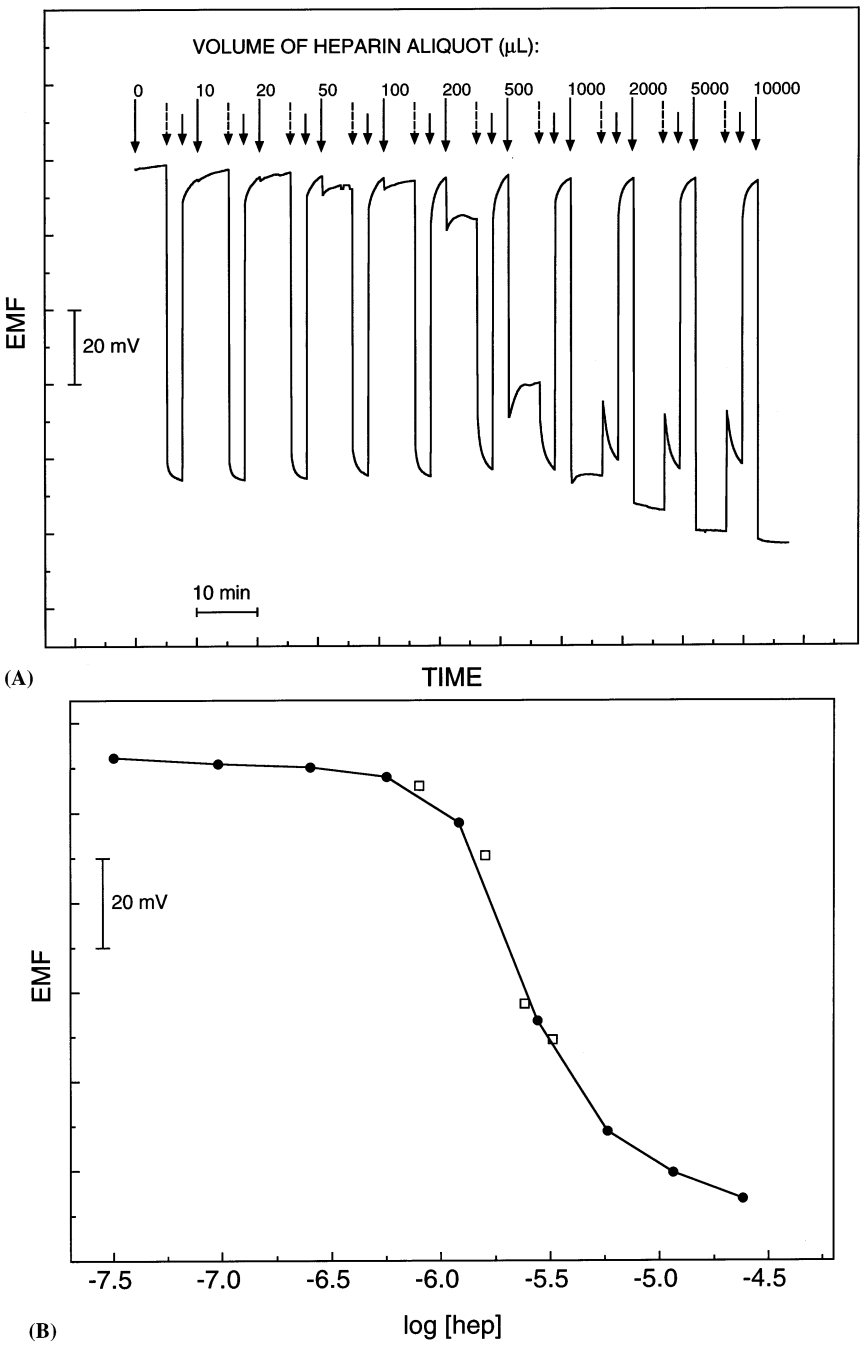

Fig. 4. Potential as a function of time for the proposed calibration method of the heparin-selective electrode where heparin is stripped from the membrane surface between measurements. Dashed arrows indicate stripping period in 2 M NaCl; short arrows represent reconditioning periods in 0.01 M NaCl. B. Recommended calibration curve for heparin selective electrode with heparin stripping between measurements (Fig. 4A). Open squares (A) indicate four sample readings of varying heparin concentrations. Calibration curve gives concentrations close to actual concentrations of samples.

phase boundary will alleviate the drift, long term measurements were made using several heparin-selective electrodes with various concentrations of NaCl in the inner filling solution, each having the same membrane composition.

Long term drift experiments were performed using a high DOS membrane because it has a higher diffusion coefficient for heparin than the low DOS membrane by an order of magnitude [13], allowing for shorter measuring times. The inner electrolyte concentrations ranged from 1×10^{-4} to 2 M NaCl. The heparin sample contained 100 ml of 2.75×10^{-5} M heparin, which is the concentration of heparin needed to saturate 50 times the number of carrier sites based on a $[R_{70}^{+}Hep^{-70}]$ complex between the carrier and the polyion. This high concentration as well as sample stirring was used to avoid depletion of the sample at the membrane-sample interface, which otherwise causes irregular EMF values (data not shown).

As Fig. 5 shows, the response to heparin is very rapid, giving an immediate potential drop of about 60 mV. A drift of about 70 μV min^{-1} is then observed as the potential rises approximately 20 mV in the first 5 h after heparin was added to the sample. The drift then slows to about 12 mV over the next 5 h. At about 10 h after the addition of heparin the drifts of the different electrodes separate; the electrodes containing a lower concentration of NaCl in the inner electrolyte continue with an approximate 18 mV drift over the next 15 h while the potential for the higher concentrated inner filling solutions begin to level out. This measurement has been repeated several times with reproducible results.

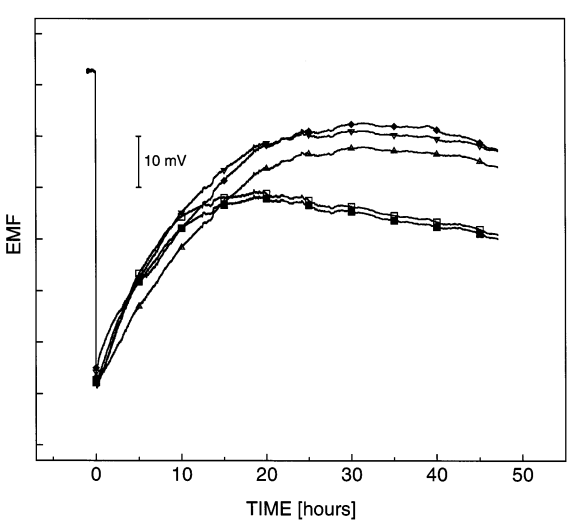

Fig. 5. Long-term response profile of heparin-sensitive DOS–PVC (2:1) membrane with the following inner filling solution NaCl concentrations: (V) 0.0001 M, (H) 0.01 M, (F) 0.1 M, (Q) 1 M, (A) 2 M. The initial heparin response of about 60 mV is followed by a significant rise in potential which is likely due to diffusion related processes in addition to heparin reaching the inner phase boundary of the membrane.

The response profile confirms that the concentration of the inner electrolyte does affect the magnitude of the drift, but the notion that the stripping of heparin from the inner phase boundary by 2 M NaCl would alleviate the drift was not confirmed. Clearly, the electrodes with the highest inner electrolyte concentrations were seen to have an unexpected and significant drift during the first 10 h of the measurement. It is likely that the drift during this time is not only caused by slow changes in interfacial membrane compositions due to heparin reaching the inner phase boundary, but by other diffusion related processes. Indeed, since heparin diffuses much more slowly across the membrane than chloride ions, a developing diffusion potential may explain the observed effects, although a concentration gradient of the ion exchanger sites within the membrane, equally caused by diffusing ions with different mobilities, may also cause potential drifts. These effects remain to be investigated for this case. Pretsch and coworkers performed spectropotentiometric studies on some electrode membranes selective for small ions, and potential drifts were observed in these experiments as well [19]. The drifts depended clearly on the membrane thickness, indicating that diffusion related processes were responsible for these effects.

4. Conclusions

We have shown experimentally how the potentiometric response of the heparin selective ISEs is much more reproducible when the membrane is renewed by heparin stripping with 2 M NaCl. Because of the low relative expense and simplicity of making the heparin selective membranes dis-

posable sensors may be viewed as an alternative to membrane renewal between every sample measurement. However, our results indicate that the apparent increase of the ΔEMF between sample measurements due to the potential not returning to baseline is also seen when creating a calibration curve using the classical method of calibration. The use of disposable sensors will not correct for calibration errors, but when the calibration measurements are separated by the simple stripping procedure the potential is shown to return to the baseline between measurements. This method gives a more accurate calibration curve so that future measurements with the sensor will show improved accuracy.

The drift measurement shows that there is some effect of the inner filling solution concentration on the drift due to the stripping of heparin from the inner surface of the membrane, but this did not remove the large initial drift. It must also be pointed out that the drift from the commonly used 0.01 M NaCl inner filling solution is not greatly influenced by raising the inner electrolyte composition to 2 M NaCl, and physiological levels of heparin would be much lower than in the experiments performed here, giving even less drift. The unexpected results in the drift experiment have brought up the question of what factors other than heparin reaching the inner phase boundary are causing the drift. Experiments determining the effect of diffusion related processes on these systems are planned to be performed in this laboratory.

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