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Stabilized potentiometric solid-state polyion sensors using silver-calixarene complexes as additives within ion-exchanger-based polymeric films

Abstract Novel solid-state sensors for biomedically important polyions (i.e., heparin and protamine) that exhibit significantly enhanced initial EMF stability are prepared by incorporation of lipophilic silver-calixarene complexes along with the required ion-exchangers within polymeric films. A dithioether of tertiary butyl calix[4]arene together with its silver complex are added to the polyion sensing membranes which are deposited on a silver-epoxy conductor of a solid-state transducer. The silver-complex serves as a reversible electron transfer agent between the organic polymer film and the underlying solid-state conductor, resulting in highly reproducible starting EMF values and improved initial signal stability. This approach is further employed to devise a heparin sensing cartridge for blood measurements based on a novel differential measurement mode. One sensor responds to heparin, while the second identical solid-state sensor serves as a pseudo reference electrode with all heparin present in the sample within this half-cell complexed by the addition of excess protamine. The cartridges are evaluated by monitoring heparin response in spiked dog blood using poly(vinyl chloride) (PVC) blends and fluorinated silicone rubber (Dow Corning RTV 730) films containing appropriate levels of ion-exchanger and Ag^+-calixarene/free calixarene additives.

Introduction

It has been shown recently that polymeric membranes doped with appropriate lipophilic ion-exchangers can be used to conveniently detect low levels of polyion species by simple potentiometry using a classical ion-selective electrode (ISE) configuration [1]. In these sensors, the polyions are favorably extracted into the polymeric membranes as tight ion-pairs with the exchangers [e.g., tridodecylmethylammonium (TDMA) for polyanions, and dinonylnaphthalenesulfonate (DNNS) for polycations] yielding non-equilibrium steady-state EMF changes at the membrane/sample interface. This basic measurement principle has now been demonstrated for the detection of a wide range of polyionic species at sub-micromolar levels, including the highly sulfonated polysaccharide anticoagulant heparin [2, 3], and its polycationic antidote, protamine [4]. It has further been shown that such potentiometric polyion sensors can serve as useful endpoint detectors for monitoring heparin levels in undiluted blood samples via titration with protamine [5], as detectors for monitoring enzyme assays of serine proteases (and their inhibitors) [6, 7], and in the design of novel immunoassay schemes [8].

With these practical applications already demonstrated using traditional ion-selective membrane electrode geometries (i.e., with internal reference electrolyte solutions), it is of interest to develop inexpensive, single-use versions of the same sensors in which the polyion sensing polymeric films are deposited directly on solid internal contacts. In the past, such so-called "coated wire" electrode designs have been prone to significant limitations owing to the ill-defined phase boundary potential that exists at the membrane/conductor interface. This thermodynamically ill-defined boundary leads to significant EMF instability which, in turn, causes irreproducible starting potentials of sensors and high EMF drift rates of the output signals (> 100 mV) especially immediately after initial wetup. Recently, Liu et al. [9] have demonstrated that the addition of a lipophilic silver-ligand complex within sodium and ammonium ion sensing films (prepared with suitable neutral carrier type ionophores) can establish a reversible electron transfer between the membrane and a solid silver contact resulting in greatly enhanced EMF stability of solid-state ion sensors fabricated in this way.

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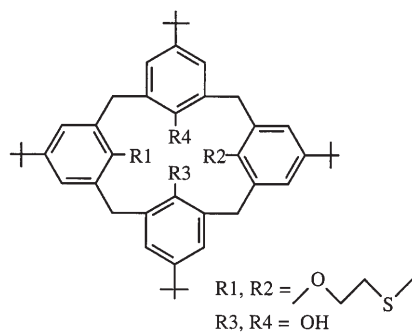The University of Michigan, Ann Arbor, MI 48109-2122, USA

Fig. 1 Structure of the dithioether of tertiary butyl calix[4]arene used in this work

Herein, we examine whether the same concept can be adapted to prepare solid-state polyion sensors with more reproducible starting EMF values and reduced initial drift rates. To achieve this goal, the dithioether derivatized tertiary butyl calix[4]arene shown in Fig. 1 and its silver complex were prepared and used as membrane additives. The incorporation of both compounds in the organic membrane allows the following electron transfer reaction between the membrane and a silver-epoxy contact:

$$AgL^+(m)+e^- \leftrightarrow Ag^0(s)+L(m) \tag{1}$$
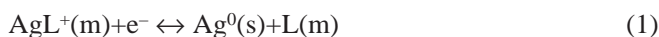
where $L(m)$ denotes the free derivatized calixarene ligand and $AgL^+(m)$ the silver-calixarene complex in the membrane phase. $Ag^0(s)$ represents the silver of the solid epoxy contact. Therefore, the phase boundary potential $E_{Ag/m}$ can be described by the following equation:

$$E_{Ag/m} = E^0_{AgL} - \frac{RT}{F} \ln \frac{C_L}{C_{AgL^+}}$$

with

$$E^0_{AgL^+} = E^0_{Ag} - \frac{RT}{F} \ln K_{AgL^+} \tag{2}$$

where C_L denotes the concentration of the calixarene in the membrane, C_{AgL^+} the concentration of the silver-calixarene complex in the membrane and K_{AgL^+} is the equilibrium constant for the formation of the silver-calixarene complex within the membrane phase.

In the studies reported here, addition of silver-calixarene complex and free calixarene were made to sensing films based on the ion-exchangers TDMA and DNNS for the detection of heparin and protamine (an arginine rich peptide), respectively. As the presence of the calixarene compounds could potentially interfere with the extraction of the polyions into the membranes, the content of free calixarene and its silver complex within the films were optimized. It will be shown that the potentiometric response of solid-state polyion sensors with membrane polymers in the form of PVC/ter-poly(vinyl chloride)/(vinyl acetate)/hydroxypropyl acrylate) blends (denoted as PVC/ter-polymer) and room temperature vulcanizing silicone rubber (Dow Corning RTV 730; denoted as DC 730) are essentially equivalent to conventional ISE designs, and that the initial starting EMF values and drift are

dramatically decreased compared to solid-state devices prepared without the silver-calixarene added to the membrane phase. The improvement in the reproducibility of the starting potentials enables fabrication of a novel heparin detection cartridge based on a differential measurement between two identical solid-state heparin sensitive electrodes.

Experimental

Reagents

The ter-polymer of poly(vinyl chloride) (80%)/poly(vinyl acetate) (15%)/poly(hydroxypropyl acrylate) (5%) and the plasticizer octyldiphenylphosphate (ODP) were purchased from Scientific Polymer Products (Ontario, NY). Dioctyladipate was obtained from Aldrich (Milwaukee, WI). The silicone rubber DC 730 was a product of Dow Corning (Midland, MI). The ion-exchanger dinonylnaphthalenesulfonate (DNNS) was a kind gift from King Ind. (Norwalk, CT). Tridodecylmethylammonium chloride (TDMAC), tetradodecylammonium tetrakis(4-chlorophenyl)borate (ETH 500), high molecular weight poly(vinyl chloride) and dioctylsebacate (DOS) were from Fluka (Ronkonkoma, NY). Heparin (from porcine mucosa) and protamine (from herring) were purchased from Sigma Chemical Co. (St. Louis, MO).

The dithioether of tertiary butyl calix[4]arene was synthesized as previously reported by Cobben et al. [10]. The silver complex of the calixarene derivative was formed by shaking a 0.05 M solution of the ligand with a 0.1 M AgNO$_3$ solution as described in [9]. The formation of the complex was verified by elemental analysis of the white silver salt and by NMR studies which showed a conformational change of the ligand structure after the complex formation.

Transducer and cartridge set-up

The solid-state transducer used throughout this study was produced by a screen printing process described elsewhere [11] (see Fig. 2, as example of design). Two silver-epoxy electrodes were printed on a glass wafer. In a second printing step, an epoxy encapsulation was printed on top of the silver-epoxy layers leaving only the contact pads of the two electrodes and the active round sensing spot of the two sensors uncovered. Glass plates of the size of 1×2.5 cm with two imprinted electrodes were separated from each wafer by an automated dicer. The polymeric sensing films were then hand-pipetted on the uncovered round area of the two silver-epoxy electrodes. This manual step can be easily automated
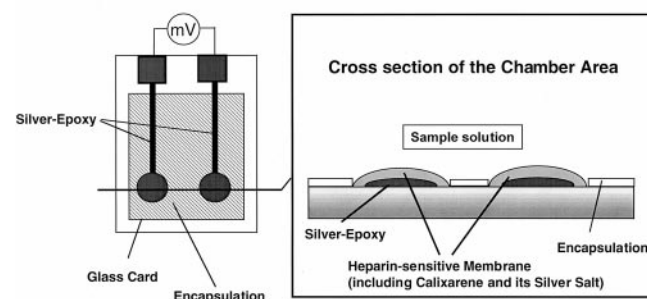

Fig. 2 Schematic representation of the heparin sensing cartridge based on two solid-state heparin sensors

by using a spin coating technique for future fabrication of such glass chips.

The differential heparin sensing cartridges (see Fig. 2) were assembled using the glass chips described above. A plexiglass cover was fixed on the chips using vacuum grease. In this way two chambers were formed. The two chambers of the chips were connected by a channel filled with silver-epoxy which was chloridized on both ends for contact with each chamber. For the measurement of heparin in spiked dog blood samples, 0.3 mg of protamine was dried in one of the chambers (reference chamber) from 5 μL of an aqueous protamine solution. In the second chamber (measuring chamber) 5 μL of doubly distilled water was evaporated in order to guarantee a similar treatment of both sensing films.

Preparation of polymeric polyion sensing films

Membrane cocktails containing the free calixarene ligand and its silver salt were prepared by weighing out 2 mg of the free ligand (2 wt. % relative to the total weight of all membrane components) and 1 mg of the silver-ligand complex before adding the other membrane components. The additional membrane components were then added in the following order: polymer, ion-exchanger, ETH 500 (if necessary) and plasticizer. Optimized membrane formulations for the detection of heparin, based on silicone rubber, are plasticizer free and contained 95.5 wt. % of the silicone rubber and 1.5 wt. % TDMAC; those based on PVC contained 57.7 wt. % PVC, 7.7 wt. % ter-polymer, 1.5% wt. TDMAC and 30.1 wt. % dioctylsebacate (DOS). For protamine detection, the membrane was formulated with 2.2 wt. % DNNS, 42.0 wt. % PVC, 5.2 wt. % ter-polymer and 47.6% DOS. In all cases, 100 mg total of the membrane components (including the calixarene additives) were subsequently dissolved in 1.5 mL of freshly distilled tetrahydrofuran by shaking the mixture for at least 3 h. Five μL of the membrane cocktail was then hand-cast on the sensing areas of the solid-state wafers. All sensing sites were evaluated after curing the membranes for at least 24 h by evaporation of the THF under a hood.

EMF measurements

The EMF values of the solid-state ion sensors were recorded with a Macintosh IIcx computer coupled with a NB-MIO-16x analog/digital input/output board obtained from National Instruments (Austin, TX) and a custom built electrode interface/amplifier module controlled by Labview 2 software from National Instruments, as described previously [12]. When not measured in a differential mode, all EMF values were recorded vs. a miniature external Ag/AgCl reference electrode purchased from Bioanalytical Systems Inc. (West Lafayette, IN) in a stirred 0.12 M NaCl solution after adding aliquots of a given polyion stock solution every 5 min. Differential measurements were performed by connecting the solid-state electrode of the reference chamber (electrode with deposited protamine; see Fig. 2) with the reference electrode input of the amplifier module. Different solutions with or without the analyte heparin were filled in the chambers as described in the following section and the EMF value was recorded after 100 s.

Results and discussion

Preliminary studies

The concept of stabilizing the inner Ag⁰/polymer membrane interface was first evaluated by measuring the potentials of the two electrodes on each sensor chip vs. a miniature external reference electrode. The optimized membrane compositions, as given in the Experimental Section for PVC/ter-polymer films, were employed for these ini-
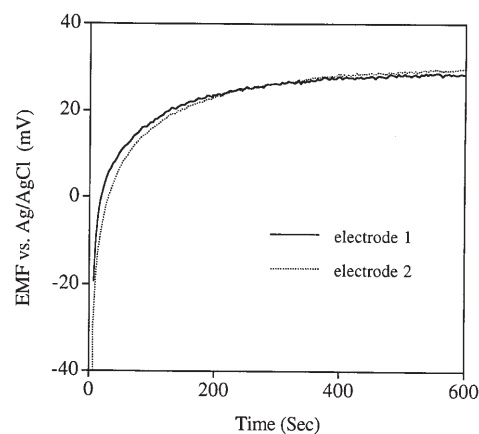

Fig. 3 Typical EMF response of two heparin electrodes on a sensing chip during the first 600 s after wet-up in 0.12 M NaCl

tial studies. Indeed, a very high reproducibility of the starting EMF values for both types of polyion sensors (TDMA- and DNNS-based polyanion and polycation, respectively) was found. The absolute starting potentials of the electrodes on the glass wafers could be reproduced in 0.12 M NaCl solution with a standard deviation of ± 1.4 mV (n = 4) for the heparin sensitive sensors and ± 1.1 mV (n = 4) for the protamine sensitive devices.

Figure 3 shows the typical EMF output values upon equilibration of two heparin sensitive solid-state sensors on a given wafer during the first 10 min after wet-up (upon exposure to 0.12 M NaCl). It is clear that the potential difference of the two electrodes is always less than 2 mV suggesting that a reproducible phase boundary potential exists at the membrane/silver-epoxy interface. Nonetheless, the EMF values did drift significantly during this time period (up to 50 mV for both electrodes). This can be explained by the fact that water diffuses into the membrane on the side of the film that is contacting the test solution. This hydration effect changes the activity of the ions within the surface layer of the membrane. This change in activity of the primary ion (chloride in the case of polyanion sensors) alters the phase boundary potential between the membrane and the aqueous solution. Consequently, a drift is observed during the duration of the membrane hydration. Such a drift due to water uptake, which has been studied in detail by Li et al. [13], is not observed when conventional electrodes with an inner filling solution (where the ion-selective membrane is contacted with an aqueous solution on both sides of the membrane) are utilized since the change in the phase boundary potential on both sides of the membrane cancel each other out. For the development of disposable heparin measuring cartridges based on solid-state sensors, this inherent drift due to hydration of the film can be reduced by appropriate choice of plasticizer [9]. However, the magnitude of such drift is not of great importance since, if it occurs for both electrodes in a nearly identical fashion, the drift will thus cancel out in the differential mode of operation (see below).

Solid-state polycation sensor

After proving via the above preliminary studies that the EMF values of solid-state polyion sensors can be made to be more consistent from sensor to sensor using the silver-calixarenes as membrane additives, the starting EMF reproducibility and initial drift characteristics of such sensors were studied in more detail using a variety of membrane formulations designed for sensing polycationic protamine (see Table 1). All the films included the cation-exchanger dinonylnaphthalenesulfonate (DNNS) as the active compound, but employed different plasticizers and membrane polymers. Liu et al. [9] have already shown that the choice of the plasticizer is crucial with respect to initial water uptake and drift rates. The most promising plasticizer combination was found to be a mixture of dioctyladipate (DOA) and octyldiphenylphosphate (ODP) (50:50 v/v). Therefore, membrane compositions based on PVC/ter-polymer using this plasticizer combination as well as 100% DOS plasticizer were examined. All films contained 1 to 1.1 wt. % of the lipophilic salt ETH 500 to reduce the membrane resistance of the DNNS-containing films [14]. This yielded solid-state sensors that exhibited less noise even when the sensing membrane contained reduced plasticizer levels (30 wt.%).

The solid-state polycation sensitive electrodes prepared with the membrane compositions defined in Table 1 were characterized with respect to the magnitude of response towards the polycation protamine and their EMF stability (reproducibility of the initial EMF value and drift rate after 1000 s of conditioning). The results of this extensive study are tabulated in Table 2. Except for membrane composition M5, which is the calixarene-free blank,

all polymer film compositions yield sensors with more reproducible starting potential after 500 s of wet-up in 0.12 M NaCl solution (compared to the M5 blank). Further, it can be seen that silicone rubber can also be used effectively as a membrane matrix (M2), yielding relatively reproducible starting potentials and significant protamine response, albeit significantly higher drift rates than PVC/ter-polymer films containing the silver calixarene/free calixarene species. Note that the protamine sensitivity for the sensors prepared with silicone rubber films is actually the highest of any of the formulations examined. Such enhanced sensitivity is due, in part, to the reduced diffusion coefficient of the protamine within the non-plasticized crosslinked silicone films. Indeed, it is well known from the reported mechanism of polyion response [1] that a reduced mass transfer rate within the sensing polymer film leads to more sensitive non-equilibrium potentiometric polyion responses. This is further exemplified by the results presented for PVC/ter-polymer membranes M1 and M3, which differ only in their plasticizer content. As shown, the concentration of protamine required to achieve half of the maximum EMF response is much higher for membrane M1 than M3 due to the difference in plasticizer content, and hence, diffusion rates of the polycation within these two films.

From the data presented in Table 2, it can be seen that PVC/ter-polymer-based films containing 30 wt. % of a DOA/ODP mixture as the plasticizer show the best overall performance in terms of the total combination of response parameters: reproducible starting EMF values, extremely low drift rates after 1000 s, good sensitivity and total response to protamine.

Table 1 Compositions of polymeric membranes examined for the preparation of protamine-responsive solid-state sensors (in wt. %)

	PVC	DC 730	Ter-polymer	Silver Calix.	Free Calix.	ETH 500	DNNS	Plasticizer
M 1	41.9	–	5.2	1.0	2.2	1.0	2.2	46.5 (DOA/ODP: 50/50 v/v)
M 2	–	92.8	–	1.0	2.1	1.0	3.1	–
M 3	55.9	–	8.2	1.0	2.2	1.0	2.2	29.5 (DOA/ODP: 50/50 v/v)
M 4	55.9	–	8.2	1.0	2.2	1.0	2.2	29.5 DOS
M 5	57.9	–	8.3	–	–	1.0	2.2	30.6 DOS

Table 2 EMF response characteristics of various polymer membrane compositions used to prepare solid-state protamine sensors

	Reproducibility of starting potential after 500 s [mV ± SD]	EMF drift after 1000 s [mV/h ± SD]	Sensitivity (Half total response) [µg/mL ± SD]	EMF Response towards 30 µg/mL of protamine [mV ± SD]
M 1	126.0 ± 3.0	4.9 ± 0.3	30.4 ± 13.2	101.5 ± 16.4
M 2	110.6 ± 1.8	27.8 ± 8.5	0.6 ± 0.2	113.8 ± 11.8
M 3	113.3 ± 2.6	1.8 ± 0.6	2.1 ± 0.4	99.1 ± 1.5
M 4	102.9 ± 3.9	12.2 ± 2.4	1.7 ± 0.3	101.9 ± 2.6
M 5	41.2 ± 33.5	42.3 ± 5.9	4.1 ± 2.9	93.2 ± 2.7

Solid-state differential electrochemical cell
for heparin measurements

The same enhanced performance in terms of reproducible starting EMF values, low drift after 1000 s, and nearly equivalent response to polyions was observed for solid-state heparin sensitive electrodes formulated with polymer films containing TDMAC and both the silver complex and free calixarene (data not shown). This suggested the possibility of preparing a simple heparin sensitive differential measurement cartridge depicted in Fig. 2. The cartridge consists of two silver-epoxy based electrodes covered with heparin sensitive polymeric films of an identical composition including the free calixarene and its silver salt. Membrane formulations based on PVC/ter-polymer and silicone rubber were evaluated. The two chambers were connected by a silver-epoxy filled channel, which was chloridized on both ends. The cell notation for this arrangement can be given as follows:

Ag / polyion sensitive membrane / sample solution in chamber 1 / AgCl(s) / Ag / AgCl(s) / sample solution in chamber 2 / polyion sensitive membrane / Ag.

When the same chloride activity is present in both chambers (when same sample is added), a zero potential difference should exist across the chloridized silver wire that provides electrical contact between the two half cells. Similarly, the phase boundary potential at the interface between the membranes and silver-epoxy contacts is also well defined by the addition of the lipophilic calixarene additives within the heparin sensing polymeric films, and is identical for both electrodes. In addition, the initial phase boundary potentials of the membrane/solution interfaces are identical because both sample solutions contain the same amount of primary salt (chloride) that define this phase boundary potential for the respective electrodes. Hence, in theory, when a small volume (75 μL) of sample solution that does not contain heparin is placed into each chamber, a zero potential should be measured between the two solid-state electrodes.

To assess whether all phase boundary potentials are indeed equivalent and cancel within this dual solid-state polyion sensor configuration, both chambers of the sensing cartridge were filled with 0.12 M NaCl solution. As shown in Table 3, results for a series of test cartridges (N = 15) indicate that a reproducible starting potential of close to 0 mV can be observed regardless whether PVC/ter-polymer or silicone rubber is used as the membrane polymer. Table 3 further illustrates that the incorporation of the calixarene and its silver complex greatly enhances the reproducibility of the starting potential difference for each of the electrodes in the differential cell. In the absence of the calixarene additives the basic theoretical assumptions for a zero potential are invalid due to the ill-defined phase boundary potential between the membrane and the silver-epoxy contacts. This leads to much larger standard deviations for the starting EMF values measured 100, 200, and 300 s after the sodium chloride solution is added to both chambers (see data in Table 3 for cartridges prepared without calixarenes in the membrane).

To ensure that the polymer films can still sense heparin, the sodium chloride solution in one chamber was exchanged with solutions containing different levels of the polyanion heparin (in 0.12 M NaCl). The resulting calibration curves for such an experiment are presented in Fig. 4. It can be seen that for both membrane polymers a heparin response over the range of 0.3 to 5 U/mL of heparin was obtained (within the clinically relevant concentration range for heparin in blood [15]). While the standard deviation of each measured differential potential (represented by the error bars in the figure) is lower for the PVC/ter-polymer membranes (for N = 5 cartridges), it can also be clearly observed that the overall EMF response towards heparin is much larger for the sensors prepared with doped silicone rubber membranes. Therefore, the disadvantage of poorer EMF reproducibility is partly compensated for by a significantly larger overall EMF response. It should be mentioned that, in the past, the use of silicone rubber for the detection of polyions was limited due to the high resistance of such polymer matrices. However, by using the fluorinated silicone rubber DC 730 which has a lower membrane resistance [16], in combination with ETH 500 additives, such problems can be overcome, making the silicone rubber films potentially more useful for preparing heparin sensing cartridges (noise level < 1 mV).

To test whether the heparin sensing cartridge prepared with two solid-state sensors can be used to detect clinically relevant levels of heparin in blood, canine blood spiked with different levels of heparin were used as test samples. Each blood sample was added in equal volume (75 μL) to both chambers of the cartridge. In the reference chamber, dried protamine (300 μg) was deposited on top of the sensing film to neutralize all the heparin in the sam-

Table 3 Effect of calixarene additives on the reproducibility of heparin-sensitive solid-state sensors after different wet-up times

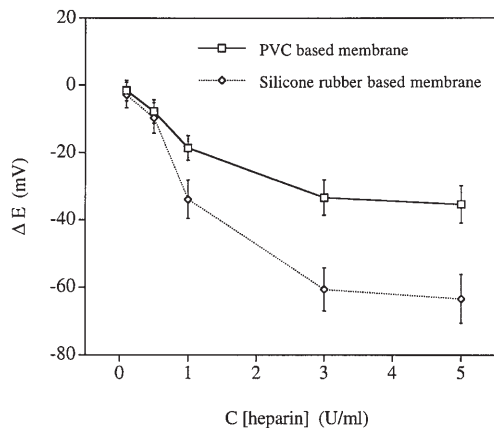|	Δ EMF, 100 s	Δ EMF, 200 s	Δ EMF, 300 s
PVC based membrane with calixarene additives (N = 15)	(−1.2 ± 3.7) mV	(−0.9 ± 3.2) mV	(−0.6 ± 3.3) mV
PVC based membrane without calixarene additives (N = 15)	(4.3 ± 18.1) mV	(4.1 ± 22.0) mV	(4.7 ± 16.9) mV
Silicone rubber based membrane with calixarene additives (N = 15)	(−0.7 ± 4.0) mV	(−1.1 ± 4.1) mV	(−1.2 ± 4.0) mV
Silicone rubber based membrane without calixarene additives (N = 15)	(2.6 ± 30.4) mV	(1.4 ± 25.7) mV	(1.9 ± 35.0) mV

Fig. 4 Calibration curves of the differential heparin sensing cartridges obtained by filling the measuring chamber with 0.12 M NaCl solutions containing different heparin concentrations (N = 5 cartridges). The reference chamber is filled with a heparin free 0.12 M NaCl solution
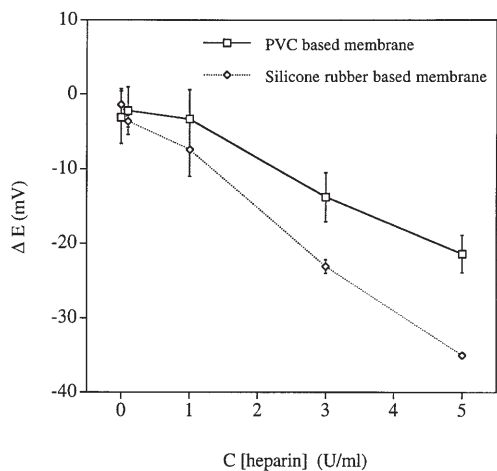

Fig. 5 Calibration curves of the differential heparin sensing cartridges obtained by filling the measuring chamber with undiluted canine blood spiked with different levels of heparin (N = 3 cartridges). Heparin was neutralized in the reference chamber by 0.3 mg of dried protamine

ple via formation of a strong electrostatic protamine-heparin complex which is not extracted into the membrane. As a consequence, the phase boundary potential at the membrane/blood interface of the reference half-cell chamber is only dependent on the activities of small inorganic ions (e.g., chloride, bicarbonate, etc.) present in the blood sample. However, the potential of the electrode in the measuring chamber does change depending on the concentration of the polyanion heparin in the blood, since the uncomplexed heparin present is extracted into the organic membrane phase, ion pairing with the TDMA exchanger. Since the chloride content in the blood is identical in both chambers, all other phase boundary potentials of the cell remain the same. Hence, the potential difference between the two solid-state electrodes is related only to the heparin concentration in the blood. Figure 5 shows the typical heparin calibration curves obtained for such an arrangement

in canine whole blood for cartridges prepared with both silicone rubber and PVC/ter-polymer films. Compared to the calibration curves observed with aqueous solutions of heparin in 0.12 M NaCl, the overall total response decreases for both polymer films. A loss in sensitivity can also be observed. Both effects can be attributed to the fact that whole blood contains various proteins that can partially neutralize heparin and further hinder the extraction of heparin into the membrane [3].

The proposed heparin sensing cartridge may find application as disposable devices for rapid determination of heparin levels in whole blood during open-heart surgery, where the therapeutic levels of heparin are typically 2–8 U/mL to prevent thrombosis [15]. Excessive doses of heparin must be avoided to prevent post-operative hemorrhage [17]. At the same time, the circulating levels of heparin must remain high enough to prevent thrombus formation on the extracorporeal tubings, oxygenator, etc. The proposed cartridge design would enable clinicians to conveniently assess the relative concentration of heparin present, and this information is desired for effectively managing the hemostatic state of the patient [18].

Conclusion

The initial EMF values of solid-state type polyion sensors can be made more reproducible and stable by incorporating a dithiocalixarene derivative and its silver ion complex within the polymeric sensing film. The silver calixarene complex provides a stable redox potential between the organic film and the underlying silver metal conductor without significantly altering the magnitude of EMF response of the polymer film towards the target polyions. This approach was valuable in the development of a simple dual solid-state sensor based cartridge design that responds to heparin levels in undiluted blood samples. The development of a number of dry chemistry, multi-chamber sensor based test systems capable of quantitating heparin as well as other biological species (e.g., proteases [8]) is envisioned using this basic technology.

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