Potentiometric flow injection sensing system for determination of heparin based on current-controlled release of protamine

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HIGHLIGHTS

- A potentiometric flow injection system for determination of heparin is described.
- An external current is applied for controlled release of protamine.
- The system has been employed for detection of heparin in whole blood.

GRAPHICAL ABSTRACT
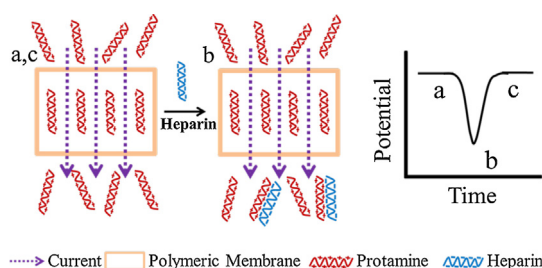
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ABSTRACT

A flow injection system incorporated with a polycation-sensitive polymeric membrane electrode in the flow cell is proposed for potentiometric determination of heparin. An external current in nano-ampere scale is continuously applied across the polymeric membrane for controlled release of protamine from the inner filling solution to the sample solution, which makes the electrode membrane regenerate quickly after each measurement. The protamine released at membrane–sample interface is consumed by heparin injected into the flow cell via their strong electrostatic interaction, thus decreasing the measured potential, by which heparin can be detected. Under optimized conditions, a linear relationship between the potential peak height and the concentration of heparin in the sample solution can be obtained in the range of 0.1–2.0 U mL^{-1}, and the detection limit is 0.06 U mL^{-1}. The proposed potentiometric sensing system has been successfully applied to the determination of heparin in undiluted sheep whole blood.

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1. Introduction

Heparin is a highly-sulfated polysaccharide with an average molecular weight of 15,000 and an average valence of 70. It is widely used as an anticoagulant drug in a variety of surgical procedures, such as kidney dialysis and open heart surgery, via the accelerating effect on inactivating coagulation factors [1,2]. Rapid and accurate measurement of heparin levels during clinical procedures are of crucial importance to avoid significant detrimental effects caused by heparin overdosing, such as hemorrhages and thrombocytopenia [3,4].

The activated clotting time (ACT) or the activated partial-thromboplastin time (aPTT) has been widely used for the quantification of heparin in clinical analysis. However, these assays are indirect and not always reliable [5]. Various methods including colorimetry [6], Raman spectroscopy [7,8], liquid chromatography [9], electrochemistry [10], and fluorimetry [11] have also been developed for measuring heparin. Unfortunately, these methods may not be suitable for whole blood analysis. In

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recent years, potentiometric sensors based on polyion-sensitive membranes have made significant contributions to heparin quantification, even in undiluted blood, via direct [12–15] or indirect [16–20] detection modes. Since the spontaneous extraction of polyions into membrane phase can form cooperative ion pairs with the lipophilic ion exchangers in the membranes, it is unfortunate that these electrodes are naturally irreversible [14,15,21]. More recently, reversible polyion-sensitive membrane electrodes based on the chronopotentiometric sensing strategy have been developed [22–26]. In this method, a lipophilic salt with a carefully matched cation and anion is added into the polymeric membrane to suppress the spontaneous extraction of polyions and a current pulse is applied to control the cation or anion fluxes into the membrane. This method can also be used for on-line rapid determination of heparin via flow injection analysis (FIA) [27]. However, the determination of heparin could be disturbed by the lipophilic anions co-existing in samples.

Recently, we developed a polycation-sensitive membrane electrode for reagentless determination of heparin by using the zero current ion fluxes of protamine released from the inner filling solution to the sample solution [28]. The electrode can be reused by conditioning at a high concentration of protamine for ca. 10 min before next measurement. Such an additional regeneration procedure will prolong the analysis time and make continuous monitoring difficult. In the present work, an external anodic current is continuously applied across the polymeric membrane to drive the ion fluxes of protamine from the inner filling solution to the sample solution, which could make the electrode membrane regenerate quickly after each measurement. By integrating the FIA and galvanostatic techniques, a rapid and continuous sensing platform for heparin has been constructed. As shown in Fig. 1a, the applied current drives the fluxes of protamine through the polycation-sensitive membrane with a stable potential baseline. When heparin is injected into the flow cell with the carrier solution, it can electrostatically bind to protamine released at the sample–membrane interface. The consumption of free protamine could facilitate the stripping of protamine out of the membrane surface via the ion-exchange process with sodium ions, thus decreasing the membrane potential (Fig. 1b) [28]. With the current-controlled reagent delivery, the membrane electrode can be regenerated on line shortly after heparin flows out of the flow cell, which allows continuous sensing of heparin in the flow injection mode (Fig. 1c).
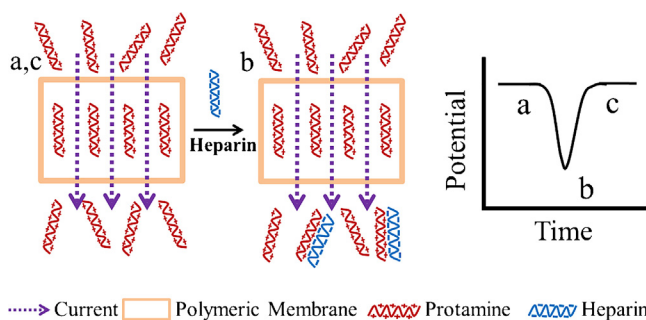
Fig. 1. Illustration of potentiometric flow injection determination of heparin with a polycation-sensitive membrane electrode based on the current-controlled release of protamine from the inner filling solution to the sample solution: (a) potential baseline (without heparin); (b) sample injection (with heparin); (c) membrane recovery (without heparin).

2. Experimental

2.1. Reagents

Dinonylnaphthalene sulfonic acid (DNNS) as a 50% solution in heptane, protamine sulfate from herring, heparin sodium salt from bovine intestinal mucosa (198 U mg^{-1}), Trizma base (Tris) were purchased from Sigma–Aldrich (St. Louis, MO, USA). High molecular weight poly(vinyl chloride) (PVC), 2-nitrophenyl octyl ether (o-NPOE) and tetradodecylammonium tetrakis(4-chlorophenyl) borate (ETH 500) were purchased from Fluka AG (Buchs, Switzerland). All the chemicals were of selectophore or analytical grade. Aqueous solution was prepared with freshly deionized water (18.2 MΩ cm^{-1} specific resistance) obtained with a Pall Cascada laboratory water system. Unless stated otherwise, 0.05 mol L^{-1} Tris–HCl buffer (pH 7.4) containing 0.12 mol L^{-1} NaCl was used as sample medium and carrier solution.

2.2. Apparatus

The FIA system for determination of heparin was controlled by a flow injection analyzer (FIS-D, Xi'an Remex Analyze Instrument Co., Ltd., China). The system contains two peristaltic pumps (1.8 mL min^{-1}), a six-way injection valve (500 μL loop) and a wall-jet flow cell (60 μL) [29]. The detection chamber has a three-electrode system. The working electrode (polycation-sensitive membrane electrode, i.d. 4 mm, o.d. 6 mm) and the reference electrode (Ag/AgCl electrode with an inner filling solution of 3 mol L^{-1} KCl, i.d. 2 mm, o.d. 4 mm) were embedded in the cell body, with a distance of 10 mm. A platinum wire as the counter electrode was placed between the working and the reference electrodes. Tygon and PTFE tubes were used to assemble the flow-through system. A CHI-660C electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China) was used to perform potentiometric measurements.

2.3. Membranes and electrode preparation

The polycation-sensitive membranes contained 3 wt% DNNS, 6 wt% ETH 500, 30 wt% PVC and 61 wt% o-NPOE. The membranes (~100 μm) were obtained by casting a membrane cocktail (386 mg) dissolved in 6.0 mL THF into a glass ring of 50 mm diameter fixed on a glass plate and evaporating the solvent overnight. Disks of 6 mm diameter punched from the parent membrane were glued to plasticized PVC tubes (i.d. 4 mm, o.d. 6 mm) to fabricate the polycation-sensitive membrane electrodes. 0.05 mg mL^{-1} protamine in 1 mL of 0.05 mol L^{-1} Tris–HCl buffer (pH 7.4) containing 0.12 mol L^{-1} NaCl was used as the inner filling solution. During the polarization, a stable potential on the inner side of the membrane can be obtained in the presence of a high concentration of sodium chloride. Before measurements, all the electrodes were conditioned in the solution identical to the inner filling solution for 12 h at 25 ± 1 °C, which ensures the stable ion fluxes of protamine from the inner solution to the sample solution.

2.4. EMF measurements

Ion-selective chronopotentiometry was used in this work. An anodic current of 20 nA was applied across the polymeric membrane polycation-sensitive electrode to release protamine from the inner filling solution into the sample solution to get a stable baseline. Potentiometric measurements of the interactions of protamine with injected heparin at the membrane–sample interface were performed at room temperature in the galvanic cell: Ag/AgCl/3 mol L^{-1} KCl/sample solution/polycation-sensitive membrane/inner filling solution/AgCl/Ag. A 0.05 mol L^{-1} Tris–HCl buffer

(pH 7.4) containing 0.12 mol L^{-1} NaCl was used as the carrier solution for sample injection. The potential peak heights were used for heparin quantification.

### 2.5. Real sample analysis

The sheep blood samples were obtained from the local market. 0.01 mol L^{-1} citrate was added to the samples to prevent blood coagulation. Heparin was measured in spiked undiluted sheep blood samples with the potentiometric flow injection sensing system.

3. Results and discussion

3.1. Current-controlled release of protamine for the FIA potentiometric sensing system

The traditional protamine-sensitive membrane electrode can generate zero-current protamine ion fluxes from the membrane into the sample solution, which have been used for reagentless determination of heparin [28]. As shown in Fig. 2a, under zero-current conditions, a large potential decrease is observed in the presence of 1.0 U mL^{-1} heparin in the FIA system. However, the spontaneous release of protamine across the membrane to the sample solution cannot recover the membrane rapidly, thus inducing a large potential drift and a long recovery time. This effect may be due to the fact that the protamine molecules with high molecular weights need a rather long time to cross the membrane [13]. Recently, it has been found that the ion fluxes across the membrane can be modulated and controlled precisely by applying an external current [23,30–32]. Previous reports have shown that ions across the polymeric membrane ion-selective electrode can be selectively and precisely delivered to the sample solution for calibration-free titrations [33]. Recently, Crespo et al. developed a technique based on a thin layer Coulometry and revealed that protamine can be selectively transferred from a thin layer to the outer solution at a fixed applied potential [34]. In this work, an anodic current is continuously applied to promote the diffusion of protamine through the polymeric membrane and maintain a constant release of protamine across the membrane. As shown in Fig. 2b, a stable baseline as well as a short recovery time can be obtained in the FIA system with the current-controlled reagent delivery.
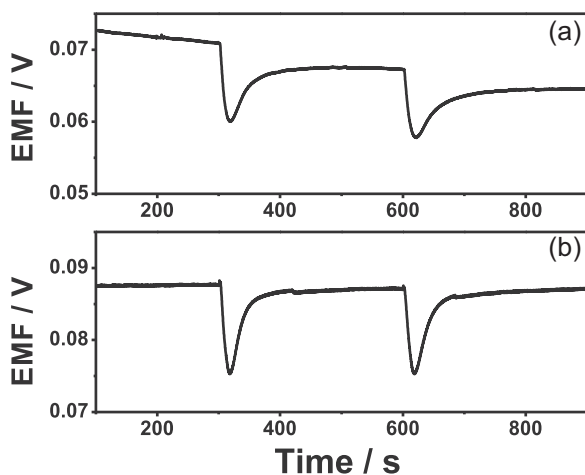
Fig. 2. Comparison of the potential responses of the polycation-sensitive membrane electrode in FIA system to 1.0 U mL^{-1} heparin under zero-current conditions (a) and with an anodic current of 20 nA (b). Unless stated otherwise, the following conditions were employed: membrane composition, 3 wt% DNNS/6 wt% ETH 500/31 wt% PVC/60 wt% *o*-NPOE; heparin, 1.0 U mL^{-1}; carrier solution, 0.05 M Tris–HCl buffer (pH 7.4) containing 0.12 M NaCl; applied current, 20 nA; sample volume, 500 μL; flow rate, 1.8 mL min^{-1}.

3.2. Optimization of membrane components

Since there is a sigmoidal relationship between the membrane potential and protamine concentration, the concentration of protamine released at membrane surface should be modulated in the most sensitive region so that a sensitive response curve can be obtained. The ratio of membrane components, which determines the diffusion coefficient and the cation-exchanger sites of protamine in the membrane phase, play an important role in controlling the concentration of protamine released at the membrane surface [13,28]. It is known that higher plasticizer contents increase the diffusion coefficient of protamine, and more anionic sits promote the ion-exchange process in the membrane phase. Both effects can facilitate the ion fluxes of protamine through the membrane, therefore increasing the concentration of protamine released at the membrane surface. In addition, higher lipophilic salt contents can also promote the diffusion of protamine by softening the membrane [21]. However, the membrane electrode may not be sensitive to heparin if too much protamine is released at the membrane surface (i.e., outside the most sensitive region). As shown in Table 1, the membrane containing 3 wt% DNNS, 6 wt% ETH 500, 31 wt% PVC, and 60 wt% *o*-NPOE shows the best performance in the FIA potential response in terms of high sensitivity and wide linear range. Therefore, this membrane composition was selected for the present system.

3.3. Optimization of FIA parameters

The influence of applied current on the sensitivity of the polycation-sensitive electrode is shown in Fig. 3a. The peak height with a lower current of 10 nA is higher than that with zero current, which is due to the increasing amount of protamine released at the membrane–sample interface. Higher currents can also lead to shorter recovery times. However, for currents higher than 20 nA, the concentration of protamine released at the membrane surface could be out of the most sensitive reaction region for measuring heparin, which decreases the potential peak heights. A current of 20 nA was selected for sensitive determination of heparin and rapid recovery of the membrane electrode. The amount of protamine released per hour is negligible as compared to that in the inner filling solution (see Supporting information).

As shown in Fig. 3b, the potential response is correlated with the injected sample volume in the range of 100–500 μL. Heparin of larger sample volumes could consume more protamine released at the membrane–sample interface via the electrostatic interaction, thus causing larger potential changes. However, with an injected sample volume larger than 500 μL, the protamine consumption could not increase significantly, and the potential change would level off. Therefore, 500 μL was used as the sample injection volume.

The effect of the flow rate on the potential response was investigated in the range of 0.9–2.8 mL min^{-1}. As shown in Fig. 3c, the potential response to heparin increases with increasing the flow rate up to 1.8 mL min^{-1}, which is probably due to the fact that more vigorous mixing in the flow cell could induce more efficient interaction between protamine and heparin. However, at flow rates higher than 1.8 mL min^{-1}, the increase in flow rate would decrease the potential response, which might be attributed to the short duration for the protamine–heparin interaction in the flow cell. Therefore, the flow rate of 1.8 mL min^{-1} was chosen for the potentiometric FIA sensing system.

3.4. Characteristics of the present potentiometric flow injection sensing system

Under the optimal conditions, the potential response to heparin was tested for the FIA system. Since the consumed protamine at

Table 1
Optimization of the membrane components of the polycation-sensitive electrode.[a]

PVC (wt%)	o-NPOE (wt%)	DNNS (wt%)	ETH 500 (wt%)	Linear range ($U\,mL^{-1}$)	Detection limit ($U\,mL^{-1}$)	Slope ($mV\,U^{-1}$)
29.0	58.0	3.0	10.0	0.5–0.0	0.09	3.0 ± 0.2
31.0	60.0	3.0	6.0	0.1–2.0	0.06	10.8 ± 0.3
45.5	45.5	3.0	6.0	0.2–2.0	0.16	9.4 ± 0.7
31.0	62.0	1.0	6.0	0.5–2.0	0.10	3.8 ± 0.7
29.7	59.3	5.0	6.0	0.2–2.0	0.19	9.0 ± 0.1

[a] Experimental conditions: carrier solution, 0.05 M Tris–HCl buffer (pH 7.4) containing 0.12 M NaCl; applied current, 20 nA; sample volume, 500 µL; flow rate, 1.8 mL min^{-1}.

the membrane–sample interface can be replenished by the current-driven ion fluxes of protamine across the polycation-sensitive membrane, the membrane electrode is recovered rapidly
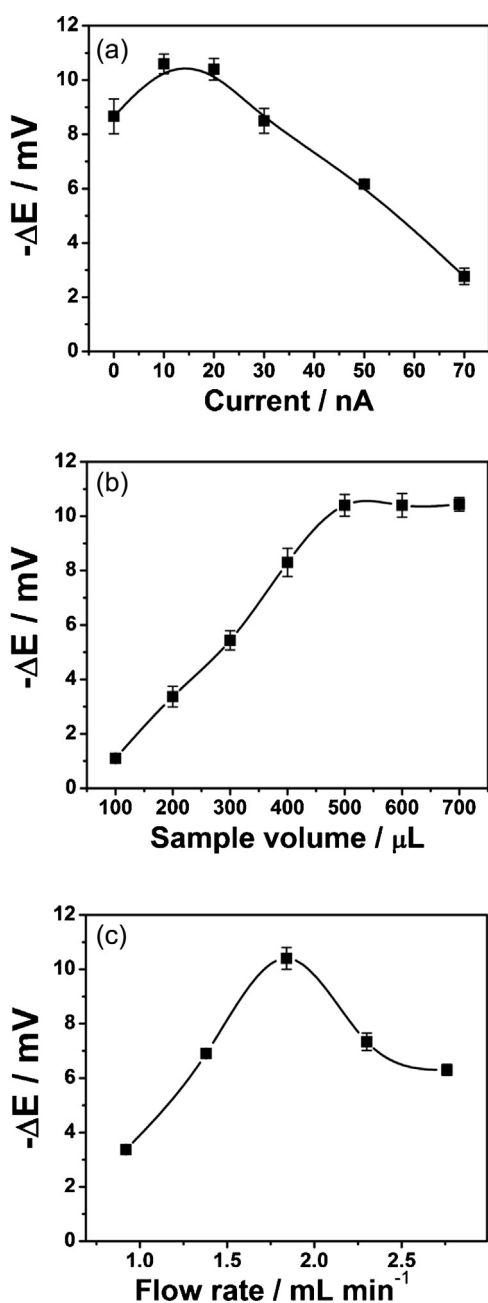

Fig. 3. Effects of the applied current (a), sample volume (b) and flow rate (c) on the potential response to 1.0 U mL^{-1} heparin in the FIA system. The other conditions are as given in Fig. 2. Each error bar represents one standard deviation for three measurements.

(within 200 s) in the flow system (Fig. 4). Compared with the batch mode [28], the FIA detection strategy demonstrated here is more practical especially for rapid analysis of a large number of samples. As shown in the inset of Fig. 4, there is a linear relationship between the potential peak height and the concentration of heparin in the sample solution in the range of 0.1–2.0 U mL^{-1} ($-\Delta E = 10.80C - 0.84$, $r = 0.9976$, ΔE in mV, C in U mL^{-1}). The detection limit was calculated to be 0.06 U mL^{-1} (3σ). A relative standard deviation of 4.2% could be obtained from multiple measurements of 1.0 U mL^{-1} heparin ($n = 7$). When not in use, the electrodes were stored in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.12 M NaCl and 0.05 mg mL^{-1} protamine at 4 °C. As illustrated in Fig. S1, the polymeric membrane electrode shows a good stability, and no significant change in potential response is observed over a period of 30 days.

3.5. Interference study

The lipophilic anions co-existing in biological samples, such as salicylate (Sal$^-$) and thiocyanate (SCN$^-$), can strongly interfere with the potential response to heparin when using the polyanion-sensitive membrane electrodes [22]. This problem can be effectively eliminated by the indirect measurements of heparin via the heparin–protamine interaction using the polycation-sensitive membrane electrodes [20,28]. For the present flow system, the indirect detection mode was employed, and an external current was continuously applied through the polymeric membrane for controlled release of protamine. As expected, Sal$^-$ and SCN$^-$ at concentrations ranging from 10^{-7} to 10^{-4} mol L^{-1} showed no significant potential responses on the membrane electrode (Fig. 5). It should be noted that chondroitin sulfate (ChS) and hyaluronic acid (HA) are both polyanions that can electrostatically interact with protamine. Although the electrostatic interaction between protamine and HA or ChS is weaker than that between protamine and heparin [8], HA and ChS may interfere with the detection of heparin.
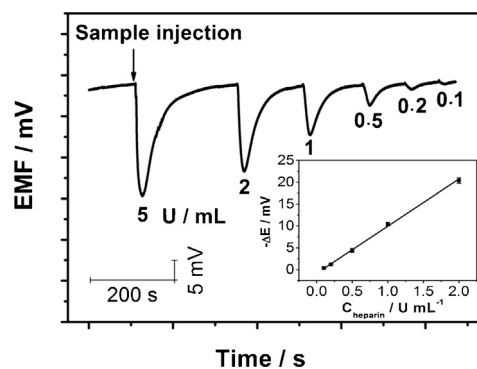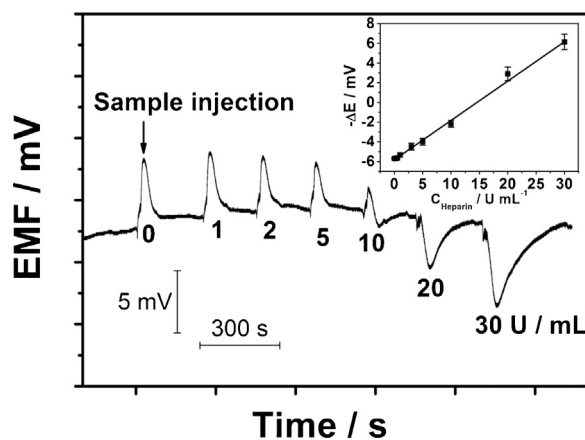

Fig. 4. Potential responses to heparin in Tris buffer at different concentrations ranging from 0.1 to 5.0 U mL^{-1}. The inset shows the calibration curve for the FIA system. The other conditions are as given in Fig. 2. Each error bar represents one standard deviation for three measurements.
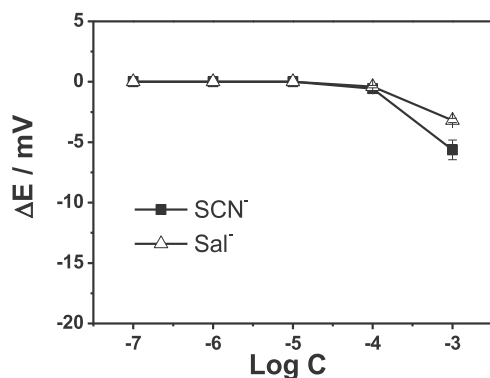
Fig. 5. Potential responses of the polycation-sensitive membrane electrode to interfering ions including Sal^- and SCN^- at concentrations ranging from 10^{-7} to 10^{-3} mol L^{-1}. The other conditions are as given in Fig. 2. Each error bar represents one standard deviation for three measurements.

3.6. Applications

The proposed FIA detection system was evaluated for determination of heparin in undiluted sheep whole blood, to which citrate (0.01 mol L^{-1}) was added as the anticoagulant. As shown in Fig. 6, an increase in the membrane potential occurs when the injected undiluted whole blood flows through the surface of the membrane electrode, and notably the first five peaks are above the baseline. Such an effect might be due to the change in sample viscosity. The high viscosity of whole blood could induce large resistance to the mass transport of protamine from the membrane phase to the sample solution, which results in an accumulation of protamine at the membrane surface and thus the increase in the membrane potential. These explanations on the potential responses to the whole blood samples may be confirmed by the diluted blood test. We also investigated the potential responses of the polycation-sensitive membrane electrode to heparin in 10-fold diluted pig blood samples at concentrations ranging from 0 to 8.0 U mL^{-1}. In that case, none of the peaks were above the baseline (see Fig. S2). As shown in Fig. 6, the proposed sensing system is useful for measuring heparin in undiluted whole blood in the range of 1–30 U mL^{-1} ($-\Delta E = 0.40C - 5.82$, $r = 0.9930$, ΔE in mV, C in U mL^{-1}) with a detection limit of 0.6 U mL^{-1}. A relative standard deviation of 9.8% could be obtained for multiple measurements of 5.0 U mL^{-1} heparin ($n = 5$). Compared with the potential response with buffer solution (Fig. 6), the response to heparin in undiluted whole blood

is less pronounced. Indeed, the elevated concentration of protamine at the membrane surface induced by the high viscosity of whole blood makes the membrane electrode insensitive to heparin in the sample solution at lower concentrations. In addition, the electrostatic interaction between protamine and heparin in the viscous medium could be less efficient as compared to that in the buffer solution.

4. Conclusions

In summary, a potentiometric flow injection sensing system integrated with a polycation-sensitive membrane electrode has been developed for rapid and continuous determination of heparin based on the current-controlled released of protamine. In contrast to the conventional polyion sensors which are irreversible, the proposed polycation-sensitive membrane electrode can be regenerated rapidly via the current-driven ion fluxes of protamine from the inner filling solution, which allows on-line measurements of heparin in the flow injection mode. The proposed system has been successfully used to determine heparin levels in undiluted sheep whole blood. In addition, the present method can be applied to monitor other species which can interact with protamine, such as aptamers and their targets. Further applications of this sensing configuration are currently in progress in our laboratory.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.12.018.

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Fig. 6. Potential responses of the polycation-sensitive membrane electrode to heparin in undiluted sheep blood at concentrations ranging from 0 to 40 U mL^{-1}. The inset shows the calibration curve. The other conditions are as given in Fig. 2. Each error bar represents one standard deviation for three measurements.

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