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SPECIAL ISSUE PAPER

Wei Qin · Wei Zhang · Kang Ping Xiao
Mark E. Meyerhoff

Enhanced sensitivity electrochemical assay of low-molecular-weight heparins using rotating polyion-sensitive membrane electrodes

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Abstract Use of a novel rotating polycation-sensitive polymer membrane electrode yields sensors that can serve as simple potentiometric titration endpoint detectors for the determination of three FDA approved low-molecular-weight heparin (LMWH) anticoagulant drugs (Fragmin, Normiflo, and Lovenox). The rotating electrode configuration dramatically improves the reproducibility and increases the sensitivity for LMWH determinations by protamine titration. At a rotation speed of 3000 rpm, electrodes with optimized thin (50 μm) polymer membranes doped with dinonylnaphthalene sulfonate (DNNS) respond to low levels of protamine (<2 μg mL^{-1}) with good precision (±1 mV, N=10), when protamine is infused continuously into a Tris-buffer solution, pH 7.4. When infusing protamine (at 5 μg min^{-1}) continuously into solutions containing Fragmin, a clear endpoint is obtained, with the amount of protamine required to reach this endpoint proportional to the level of Fragmin present. A detection limit of less than 0.02 U mL^{-1} Fragmin can be obtained via this new method, approximately one order of magnitude lower than that previously reported based on a non-rotating polycation electrode. Similar low detection limits can be achieved for potentiometric titrations of Normiflo and Lovenox. Such titrations can also be carried out in undiluted plasma samples containing the various LMWH species. In this case, detection of the LMWHs at clinically relevant concentrations (>0.2 U mL^{-1}) can be readily achieved.

Keywords Polyion sensor · Low-molecular-weight heparins · Rotating electrode

Introduction

Over the past thirty years, considerable effort has been made to adapt modern polymer membrane type ion-selective electrodes for developing simple potentiometric measurement methods for pharmaceutical agents, both for quality control purposes and for potential clinical monitoring of physiological samples [1, 2, 3, 4]. More recently, a new potentiometric polyion sensor/membrane electrode technology has been developed that enables the measurement of important polyanionic (e.g., heparin, low-molecular-weight heparins (LMWHs), dermatan sulfate, etc.) and polycationic (protamine and synthetic cationic polypeptides) drug species in samples as complex as whole blood [5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17]. The bioanalytical applications of such polyion sensors have been further extended to include the selective assay of protease activities [18, 19], detection of protease inhibitors [20] and activators [21] using natural or synthetic polyionic peptides as substrates, and the development of novel homogeneous immunoassay schemes in which protease enzymes or synthetic polyionic species serve as labels [22, 23].

Potentiometric measurements of low levels of polyionic pharmaceuticals can be achieved by using electrodes in which the polymer membranes are doped with appropriate lipophilic anion or cation exchangers (tridodecylmethylammonium (TDMA) for polyanions; tetraphenylborate (TPB) or dinonylnapthalene sulfonate (DNNS) for polycations). The potentiometric polyion response of such electrodes is governed by a non-equilibrium steady-state extraction of the polyion into the organic membrane phase of the electrodes via formation of cooperative ion pairs with lipophilic exchanger in the membrane phase. This results in a significant change in the phase boundary potential (EMF) at the membrane/sample interface [24, 25]. This potential change over the initial detectable concentration range of polyions is governed by the following equation [26]:

$$\Delta EMF = \pm\frac{RT}{F}\ln\left(1-\frac{zD_a\delta_m}{R_T D_m \delta_a}C_{polyion}\right) \quad (1)$$

where ΔEMF is the change (+ sign for polyanion yields a negative EMF change, and a – sign for polycation yields a positive EMF change) in the membrane potential after addition of the polyion with concentration $C_{polyion}$; z is the

W. Qin · W. Zhang · K. P. Xiao · M. E. Meyerhoff (✉)
Department of Chemistry, University of Michigan,
Ann Arbor, MI 48109–1055, USA
e-mail: mmeyerho@umich.edu

charge number on the analyte polyion; R_T is the total concentration of ion-exchanger sites within the organic membrane phase; D_a and D_m are the diffusion coefficients of the polyion in the aqueous and membrane phases, respectively; and δ_a and δ_m are the diffusion layer thicknesses for the polyion in each phase. This quasi steady-state model provides a semi-quantitative explanation for the influence of various experimental parameters on the polyion electrode's response properties, including the nature and content of the polymer and plasticizer that comprise the organic membrane phase, the geometric configuration of the electrode (e.g., planar vs. cylindrical), the concentrations of lipophilic ion-exchanger in the organic membrane phase, and the degree of convection of the sample solution [26].

A significant recent advance in potentiometric polyion sensor technology involves the use of a novel rotating membrane electrode configuration to greatly improve the detection limits of both potentiometric polycation and polyanion sensors by effectively decreasing the diffusion layer thickness of aqueous phase and thus enhancing mass transfer of the analyte polyion to the membrane/sample interface [27]. Indeed, operation of planar polyion sensors at 3000–5000 rpm results in an enhancement in the detection limits toward heparin and protamine of at least one order of magnitude relative to the same sensors employed with standard magnetic stir-bar sample convection. Moreover, when used as an endpoint detector for polyion titrations, a dramatic improvement in the precision of the EMF responses to polyionic species can be achieved using the rotating electrode configuration owing to the more reproducible control of the mass transfer of the polyion to the surface of the membrane [27].

In this report, we demonstrate the application of this new rotating polyion electrode concept to devise an improved electrochemical assay method for detecting low levels of three FDA approved LMWHs (Fragmin, Normiflo, and Lovenox) via a simple titration method. The approach is based on use of a rotating polycation sensor to detect the endpoint of titrations of LMWHs in which polycationic protamine is employed as the titrant. The strong binding of protamine to the various LMWHs enables relatively sharp endpoints to be observed [14]. The LMWHs have been developed as alternative anticoagulants (to unfractionated heparin (UFH)) for prophylaxis and treatment of deep vein thrombosis (DVT) [28, 29]. They function by inhibiting Factor Xa and, to a lesser extent, Factor IIa activities in blood [30]. Compared with UFH, LMWHs show a much better efficacy/safety ratio [31]. Further, they possess greater bioavailability when given by subcutaneous injection and longer duration of anticoagulant effect, which permits once or twice daily administration. It will be shown here that a rotating polycation sensitive membrane electrode using DNNS as the exchanger provides a means to assay lower levels of various LMWHs (vs. that reported previously by Ramamurthy et al. with non-rotating sensor [14]), with potential applications in quality control of these products, as well as for clinical monitoring of these drugs in undiluted plasma samples.

Experimental

Reagents

Protamine sulfate (from herring), tris-[(hydroxymethyl)aminomethane] (Tris) and Rhodamine 6G were obtained from Sigma (St Louis, MO, USA). 2-Nitrophenyloctyl ether (*o*-NPOE), and tetrahydrofuran (THF) were purchased from Fluka Chemika Biochemika (Ronkonkoma, NY, USA). Dinonylnaphthalene sulfonate (DNNS) was a gift from King Industries (Norwalk, CT, USA). Polyurethanes M48 and Pellethane 2363–80AE were kindly provided by Medtronic (Minneapolis, MN, USA) and Dow (Midland, MI, USA), respectively. Normiflo (Ardeparin sodium; Wyeth Ayerst, Philadelphia, PA, USA), Fragmin (Dalteparin sodium; Pharmacia, Columbus, OH, USA), and Lovenox (Enoxaparin sodium; Rhone–Poulenc Rorer, Collegeville, PA, USA) were provided by the University of Michigan College of Pharmacy. All solutions were prepared with deionized water (18 MΩ). Unless otherwise noted, the working buffer used for all the experiments was 50 mmol L^{-1} Tris-HCl, pH 7.4, containing 0.12 mol L^{-1} NaCl. Citrated sheep blood was obtained from Colorado Serum (Denver, CO, USA) and the plasma was prepared by centrifuging at a speed of 1500 rpm for 15 min.

Membrane and polycation-sensitive membrane electrode preparation

Disposable protamine-sensitive membrane electrodes were prepared as described previously [27]. Membranes contained (in wt %) DNNS (1), *o*-NPOE (49), Pellethane (30) and M48 (20) [14]. The membrane components (totaling 600 mg) were dissolved in THF (10 mL) and poured into a glass ring (i.d. 10 cm) fixed on a glass plate. Overnight evaporation of the solvent yielded a membrane of ~50 µm thickness, visually determined by an optical microscope. For each protamine-sensitive electrode, a disk of the membrane with a 7-mm diameter was punched from the larger membrane and glued at one end of a 1-cm-long Tygon tube (i.d. 4.8 mm, o.d. 7.9 mm; Fisher Scientific, Pittsburgh, PA, USA).

Potentiometric measurements with rotating electrode system

The rotating potentiometric electrode system was described previously [27]. It consists of a Pine Instrument (Grove City, PA, USA) analytical rotator (model ASR) and an ASR motor control box (1000 rpm V^{-1}, 200–10,000 rpm range). Both the internal and external reference electrodes were made of silver wires with diameters of 0.076 and 1.0 mm, respectively, and chloridized with a solution of 1 mol L^{-1} HCl containing 0.1 mol L^{-1} $FeCl_3$. The internal reference electrode was inserted through the central void space of the rotator and down to near the surface of the electrode membrane.

Automated potentiometric titrations of LMWHs were performed by continuous infusion of a standard protamine solution into a sample solution using a syringe pump (model MD-1001, BAS, West Lafayette, IN, USA). The EMF responses were measured every second at ambient temperature via a Macintosh IIcx computer equipped with a LAB-MIO-16XL-42 16 bit A/D I/O board (National Instruments, Austin, TX, USA) and VF-4 electrode interface module (World Precision Instruments, Sarasota, FL, USA), controlled by LabView 2 software (National Instruments). Calibration or titration curves were obtained by infusing protamine into sample solutions in the absence or presence of LMWH, respectively, and plotting the change in the EMF response vs. the time or concentration of protamine infused. The endpoint of the titrations was determined as the protamine concentration or time required to achieve half of the maximum EMF response ($EMF_{1/2,\,max}$).

AC impedance spectroscopy studies

Polymer membrane impedance measurements were carried out to further understand the noise behavior of the polycation sensors when employed in a rotating electrode configuration. Such measurements were made using an in-house designed PTFE cell containing two parallel Pt electrodes with an exposed area of 1.8 cm^2. The polymer films containing DNNS were placed between two Pt electrodes. The AC impedance of the cell comprising sensor membrane bathed symmetrically with electrolyte solutions was obtained with a Model 6310 impedance analyzer from EG&G Instruments (Princeton, NJ, USA). A version of 1.11 M398 electrochemical software provided by the manufacturer was used to control the experimental conditions. The AC impedance was measured by applying 5 mV peak-to-peak AC signal over a frequency range from 100 kHz to 10 Hz. The data were collected via a "single-sine' mode. The resistances for the bulk membranes were obtained from the complex plane impedance plots (Cole–Cole plot), using the intercepts of the semicircles with the real impedance axis [32, 33, 34].

Diffusion measurement of small ion through polycation-sensitive membrane

A 7-mm diameter disk was cut from the parent membrane and glued to a Tygon tube (i.d. 4.8 mm, o.d. 7.9 mm). This tube was connected with a pipette tip containing 1.0 mL of 0.56 mmol L^{-1} Rhodamine 6G aqueous solution, and immersed into 2 mL of deionized water in a glass vial. With continual stirring of the water solution in the glass vial, the concentration of Rhodamine 6G in the recipient solution was assayed at various time intervals by measuring the fluorescence intensity at 545 nm, using an excitation wavelength of 480 nm with a spectrofluorimeter (RF-1501, Shimadzu Scientific Instruments, Columbia, MD, USA). The apparent diffusion coefficient of Rhodamine 6G in the membrane phase was then related to the rate of concentration increase in the glass vial.

Results and discussion

Characterization and optimization of the rotating polycation-sensitive membrane electrode

For disk type rotating polymer membrane electrodes, the diffusion layer thickness in the aqueous phase δ_a is related to the angular rotating frequency, ω, according to the following equation:

$$\delta_a = 1.61/D_a^{1/3}\nu^{1/6}\omega^{-1/2} \quad (2)$$

where ν is the kinematic viscosity of solution. Figure 1a shows the calibration curves toward polycationic protamine obtained by using several different membrane electrode rotation speeds. As expected, potentiometric response curves are shifted toward lower concentrations as the rotation speed increases, an observation that is consistent with the results reported previously by Ye and Meyerhoff [27]. By defining the concentration of protamine that corresponds to $EMF_{1/2,\,max}$ as $C_{1/2}$, which is proportional to the detection limit of protamine, a linear relationship was observed between $C_{1/2}$ and $\omega^{-1/2}$ (see Fig. 1b). This is expected based on the Eqs. (1) and (2) above. Figure 1a also illustrates that much more reproducible EMF responses can be obtained with the rotating electrode configuration than those with non-rotation mode (0 rpm; only a magnetic stir bar used to convect the solution) (see s.d. values for N=4 experiments for non-rotation experiment vs. those observed by rotating the electrode). Given the improved detection limit toward protamine coupled with this enhanced precision, it should be possible to detect LMWHs at concentrations lower than the 0.2 U mL^{-1} reported by Ramamurthy et al. [14] for protamine titrations of LMWHs as followed with a non-rotating DNNS based sensor.

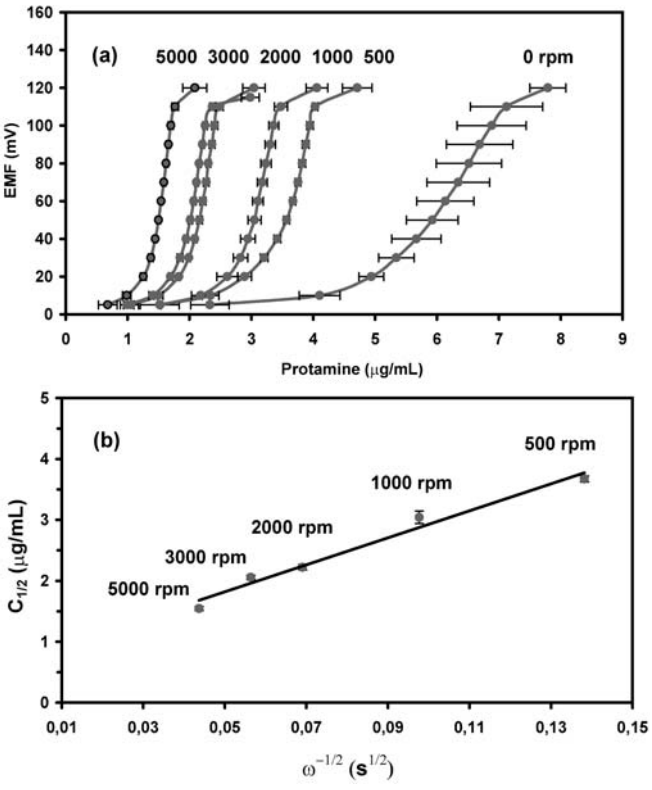

Fig. 1 (**a**) Protamine calibration curves obtained with rotating polycation-sensitive membrane electrodes, at different rotation speeds. Calibration data for 0 rpm were obtained by convection of sample with a magnetic stir bar. A protamine solution (1.0 mg mL^{-1}) was continuously infused (5 μL min^{-1}) into 6 mL of Tris buffer. (**b**) Relationship between measured $C_{1/2}$, the polycation concentration corresponding to half the total maximum ΔEMF response toward protamine, and $\omega^{-1/2}$, where ω is the rotation angular frequency. Error bars represent one standard deviation for four separate measurements

Although the membrane composition (specific polymer/plasticizer) of the DNNS-based polycation sensor had been optimized previously for titrimetric determination of UFH and LMWHs [13, 14], in preliminary experiments for the present work, additional fundamental studies were undertaken to further understand and ultimately enhance the analytical performance of the rotating polycation sensor for the proposed application. For example, reliable measurements of polyions via simple potentiometric titration require that the rotating polymer membrane electrodes exhibit relatively low noise levels. This is complicated by the fact that the membranes used are highly resistive, and rotation of the polymer membrane electrode causes some vibrations of the sensing membrane along with additional electronic noise from the rotating equip-

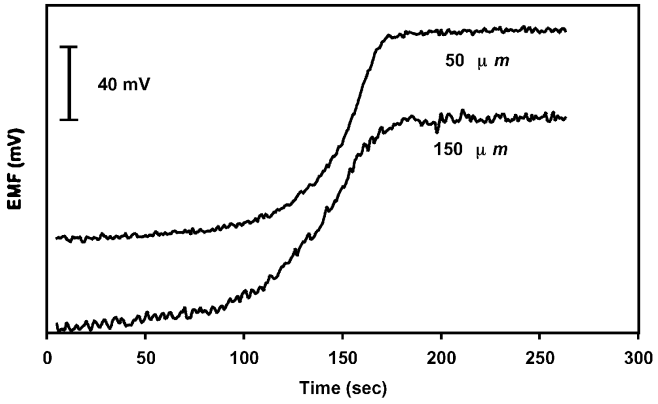

Fig. 2 Effect of membrane thickness (50 μm vs. 150 μm) on noise level of the protamine EMF response using rotating polycation-sensitive membrane electrodes as the detector. Rotation speed, 3000 rpm; medium, Tris buffer, pH 7.4; sample volume, 6 mL; titrant concentration, 1.0 mg mL^{-1}; infusion rate, 5 μL min^{-1}

ment. Preliminary experiments indicated that the thickness of the polymer membrane plays an important role in the noise level of the sensor once rotated. As illustrated in Fig. 2, a thicker membrane (150 μm) yields a noisier EMF response compared to a thinner membrane (50 μm) when a Tris-buffer solution is infused (via syringe pump) with a standard protamine solution, thereby continuously increasing the bulk concentration of protamine in the buffer solution. This difference in noise level is likely due to an increase in membrane resistance for the thicker polymer film. Indeed, separate impedance measurements of the two membranes indicated that the thicker membranes have resistances on the order of 1200±50 KΩ, compared to approx. 350±20 KΩ for thinner membranes. Using sensor membranes that were 50±10 μm thick and rotating the resulting electrodes at 3000 rpm, the typical s.d. for steady-state EMF responses toward a given level of protamine over a 100 s period was found to be ±1 mV (N=10), more than adequate to clearly define endpoints of protamine titrations.

In these preliminary studies, it was also desirable to assess how the sensitivity of the rotating polycation sensor may change as a function of its storage time, both dry and wet. It has been reported that the bulk membrane resistance of organic polymeric ion-selective membranes is dependent on the soaking time [35]. AC impedance data for the DNNS-based polycation membrane (composed of *o*-NPOE and polyurethanes) bathed symmetrically in Tris buffer, pH 7.4, were measured after different soaking times. Results indicate that the bulk membrane resistance decreases with time and becomes relatively stable after 24 h exposure to the buffer solution. During this period, the membrane impedance changes from 350±20 to 150±10 KΩ. This resistance decrease with soaking may be due to the uptake of water by the membrane, which causes a change in the physical properties of the polymeric phase and a concomitant change in the local environment for the mobile ions within the membrane [36]. It can be anticipated that the uptake of water inside the membrane phase may also change the diffusion coefficient of the protamine and or protamine-DNNS complex in this phase and this could further influence the detection limits of the sensor (see D_m in Eq. 1). Since protamine and its DNNS complex diffuse slowly in the membrane, Rhodamine 6G, a small cation, was employed as an independent probe to determine whether diffusion changes with soaking time. The diffusion of Rhodamine 6G across the membrane can be monitored by a fluorimetric assay of Rhodamine 6G in the recipient solution as a function of time. The rate of the concentration increase of Rhodamine 6G is related to the membrane–phase diffusion coefficient for Rhodamine 6G. Fig. 3 indicates that the diffusion coefficient in conditioned membranes is larger than in unconditioned mem-

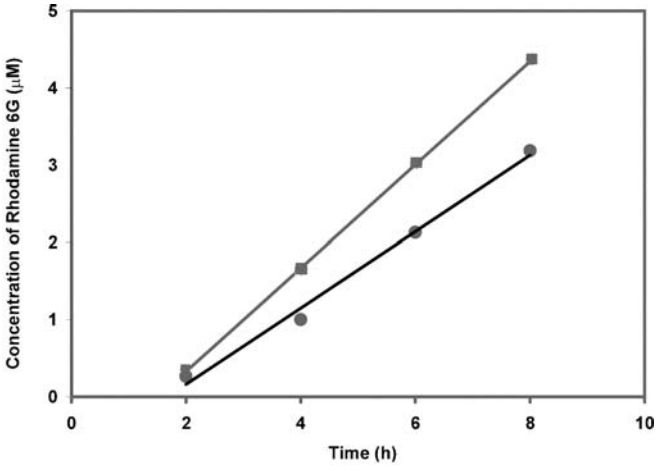

Fig. 3 Dependence of the concentration of Rhodamine 6G in the receiving phase as a function of time for membranes unconditioned (*circles*) and conditioned with buffer for one day (*squares*). The average of three measurements is shown

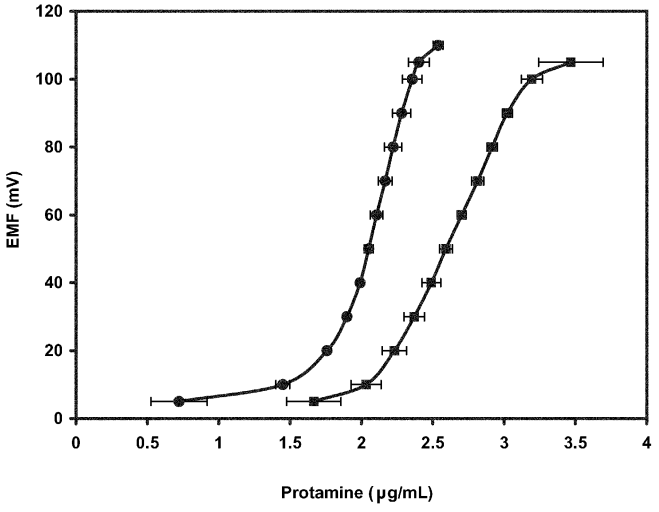

Fig. 4 Protamine EMF responses of rotating polycation-sensitive membrane electrodes for membranes unconditioned (*circles*) and conditioned with buffer for one day (*squares*). Rotation speed, 3000 rpm; medium, Tris buffer; sample volume, 6 mL; titrant concentration, 1.0 mg mL^{-1}; infusion rate, 5 μL min^{-1}. Error bars represent one standard deviation for three measurements

branes. The membrane electrodes' responses to protamine for both membranes are shown in Fig. 4. It can be seen that the unconditioned membrane exhibits a more sensitive dose-response toward protamine than the membrane that is first conditioned for 1 day in buffer. This result is consistent with the behavior predicted by Eq. (1), i.e. a lower diffusion coefficient in the membrane phase should yield a shift in the non-equilibrium polyion EMF response towards a lower concentration range. Thus, for all analytical data reported here, fresh, unconditioned membrane electrodes were used for each titration experiment.

Additional preliminary experiments demonstrated that the polycation sensitive membranes were stable over a one-month period when kept on glass slides; however, the membrane compositions could change with time when the discs of the parent membrane were glued on the Tygon tubes required for mounting into the rotating electrode assembly. As illustrated in Fig. 5, a significant shift in the response toward protamine is observed after the membrane is in contact with the Tygon tube for one week. Since the detection limits toward protamine shift to a lower concentration range, and the total equilibrium EMF change is decreased, it can be assumed that the concentration of DNNS in the membrane is likely reduced during this contact period with the Tygon tube (see Eq. 1). This reduction is likely due to the diffusion of DNNS from membrane into the Tygon tubing. The loss of DNNS from the membrane was further confirmed by the observation that membrane impedance increased with time when the membrane was in contact with the Tygon tube (data not shown). Experiments also showed that much more noise appeared in the response of the membrane toward protamine after long-term contact with the tubing. Again, this is likely due to the decrease of DNNS concentration in the polymeric membrane phase with time. For this reason, all measurements of LMWHs were carried out using freshly prepared membrane electrodes, in which the active membrane was glued to the Tygon tubing on the day of use, before fixing this tubing in the rotating electrode assembly.

Determination of LMWHs with the rotating polycation-sensitive membrane electrode in buffered saline and undiluted plasma samples

Detection of LMWHs using a polycation-sensitive electrode as the endpoint detector can be performed via classical potentiometric titrations using protamine as titrant. Protamine binds stoichiometrically, via electrostatic interactions, with such heparin species [7, 14]. Since the protamine-sensitive rotating electrode responds only to free protamine, not the heparin–protamine complex, it can be used as an endpoint detector to follow the titrations. Indeed, for real sample measurements, titrations using polyion sensor as endpoint detectors yield more reliable analytical results than the direct potentiometric measurements of heparin (with polyanion sensors) due to less interference from the endogenous ionic species present in real samples [5].

With improved analytical sensitivity and precision toward detection of protamine (see Fig. 1), the rotating DNNS-based membrane electrode was first applied to monitor low levels of Fragmin. Typical EMF response curves obtained when titrating Fragmin with protamine in buffer solution are shown in Fig. 6. It can be seen that the use of rotating electrode (3000 rpm) enables the determination of Fragmin at concentrations down to 0.05 U mL^{-1} or less (via continuous infusion of 1.0 mg mL^{-1} protamine solution at 5 µg min^{-1}). The sensitivity can be further improved by employing a lower infusion rate of protamine into the test sample (to obtain higher titration resolution) (see Fig. 7). Indeed, when the infusion rate is reduced to 1 µg min^{-1} (into 6 mL buffer volume containing Fragmin), resolution

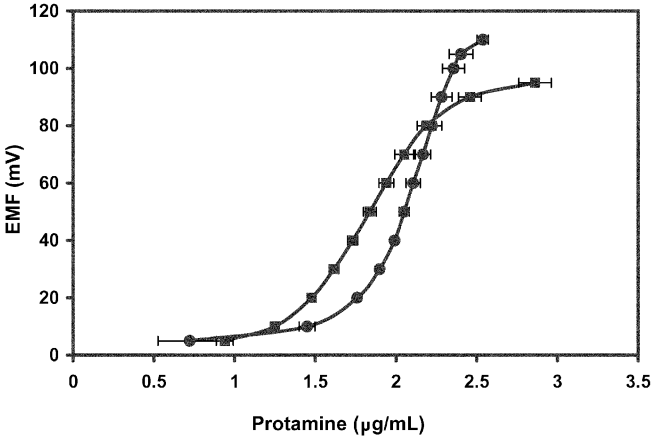

Fig. 5 Protamine EMF responses of rotating polycation-sensitive membrane electrodes for fresh membrane (*circles*) and membrane in contact with Tygon tube for one week (*squares*). Rotation speed, 3000 rpm; medium, Tris buffer; sample volume, 6 mL; titrant concentration, 1.0 mg mL^{-1}; infusion rate, 5 µg min^{-1}. Error bars represent one standard deviation for three measurements

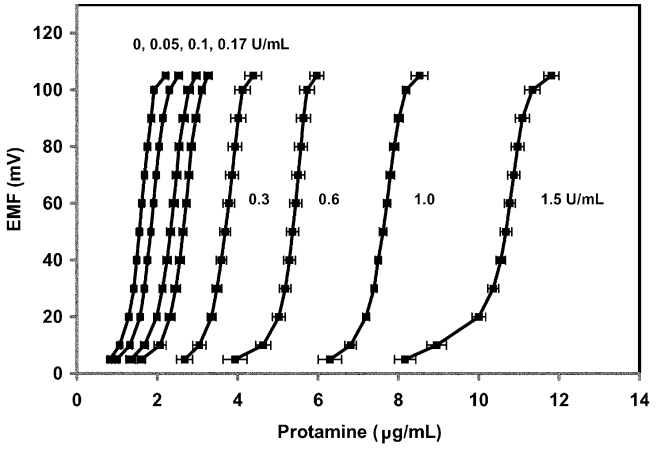

Fig. 6 Typical potentiometric titrations of varying amounts of Fragmin with protamine (1.0 mg mL^{-1}) at an infusion rate of 5 µg min^{-1} as monitored by rotating polycation-sensitive membrane electrodes (3000 rpm) in Tris buffer (6 mL). Error bars represent one standard deviation for five measurements

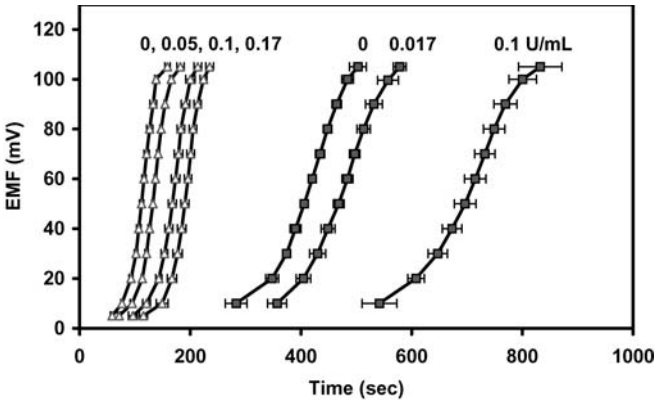

Fig. 7 Potentiometric titrations of Fragmin with protamine at a low infusion rate of 1 µg min^{-1} (*squares*) and at a high infusion rate 5 µg min^{-1} (*triangles*) as monitored by rotating polycation-sensitive membrane electrodes (3000 rpm) in Tris buffer (6 mL). Error bars represent one standard deviation for five measurements

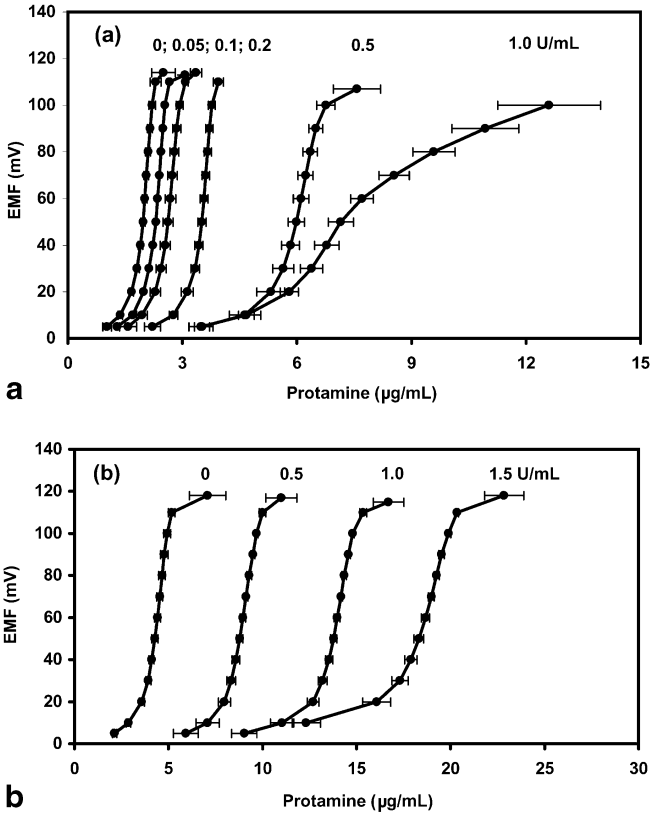

Fig. 8 Typical potentiometric titrations of varying amounts of Normiflo with protamine (1.0 mg mL^{-1}) at a low infusing rate of 5 µg min^{-1} (**a**) and at a high infusion rate of 50 µg min^{-1} (**b**), as monitored by rotating polycation-sensitive membrane electrodes (3000 rpm) in Tris buffer (6 mL). Error bars represent one standard deviation for four measurements

of 0.017 U mL^{-1} of Fragmin from the blank titration curve can be clearly observed. This is an approx. one order of magnitude lower detection limit than reported by Ramamurthy et al. using non-rotating polycation electrodes and manual titration of Fragmin (no syringe pump to infuse the protamine continuously) [14]. However, as also shown in Fig. 7, using a lower infusion rate limits the measurement speed. Hence, an appropriate balance needs to be achieved, depending on the desired speed of the titrimetric assay, vs. the sensitivity required for a given application.

The titration curves for another commercial LMWH product, Normiflo, are shown in Fig. 8. Again, a significant improvement in sensitivity is obtained by using the rotating electrode endpoint detector (compared to data reported in Ref. [14]), with a low protamine infusion rate. However, notably, as the concentration of Normiflo in the sample increases (e.g., 1.0 U mL^{-1}), considerable EMF response is observed even before the actual titration endpoint, thus resulting in a significant distortion of the titration curves, especially when titrating higher concentrations with the slower infusion rate (see Fig. 8a). Similar response was observed for the titration of higher concentrations of Lovenox (data not shown). This behavior is likely caused by the extraction of positively-charged protamine–LMWH complexes into the polymeric membrane, resulting from a charge imbalance between smaller polysaccharide fragments in the LMWH preparation and protamine [37]. Such a mechanism was proven by Ramamurthy [37] using fluorescein- and tritium-labeled heparin species, and determining the uptake of the labeled heparins into the polycation sensing membrane in the presence of less than stoichiometric amounts of protamine added to the heparin solution. These positively charged complexes represent a small fraction of the total complexes formed in solution during the titration, and therefore their concentration increases as the total level of LMWH species increases. The formation of such complexes is highly dependent on the average molecular weight and the molecular weight distribution of the given LMWH preparation [37]. Indeed, compared to Fragmin, such behavior was much more pronounced for Lovenox and Normiflo, since they are known to have higher polydispersity and a lower average molecular weight [38]. Fortunately, it is possible to decrease the response toward these positively charged extractable polycation/polyanion complexes by increasing the infusion rate of protamine into the sample solution. As shown in Fig. 8b, the distortion of the titration curves for higher concentrations of Normiflo can be nearly eliminated when the infusion rate of protamine titrant delivery is increased by 10-fold. This apparently is due to the rapid speed of the titrations, wherein the electrodes do not yield significant EMF response to the low concentrations of positively charged LMWH–protamine complexes within the duration of the titrations [37].

The behavior observed above for Normiflo was also found when titrating Levenox with protamine (data not shown), using the rotating polycation sensor as the endpoint detector. As shown in Fig. 9, the endpoints of the titrations for all three species, defined as the amount of protamine required to achieve 1/2 the total EMF response – (minus) the amount of protamine required to reach 1/2 the total EMF response for a blank titration (no LMWH present) yield a nearly linear response to the respective

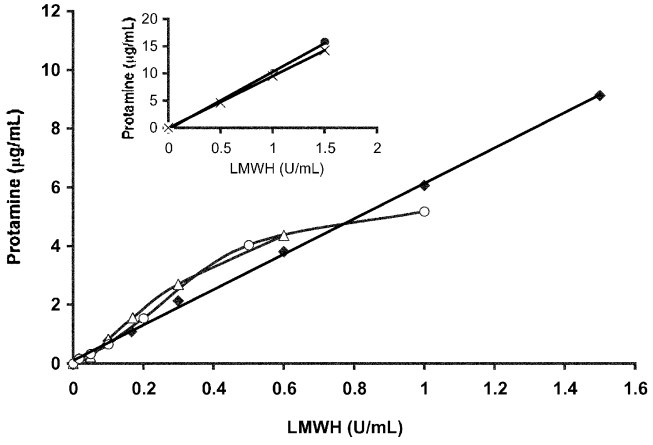

Fig. 9 Calibration curves for Fragmin (*diamonds*), Normiflo (*open circles*) and Lovenox (*triangles*) via titrations with protamine (1.0 mg mL^{-1}) at a low infusion rate of 5 μg min^{-1} as monitored by rotating polycation-sensitive membrane electrodes (3000 rpm) in Tris buffer (6 mL). Amount of protamine required to reach endpoint was calculated based on the value at $EMF_{1/2,\ max}$. The inset denotes to the calibration curves obtained at a high infusion rate of 50 μg min^{-1} for Lovenox (*filled circles*) and Normiflo (*crosses*), respectively

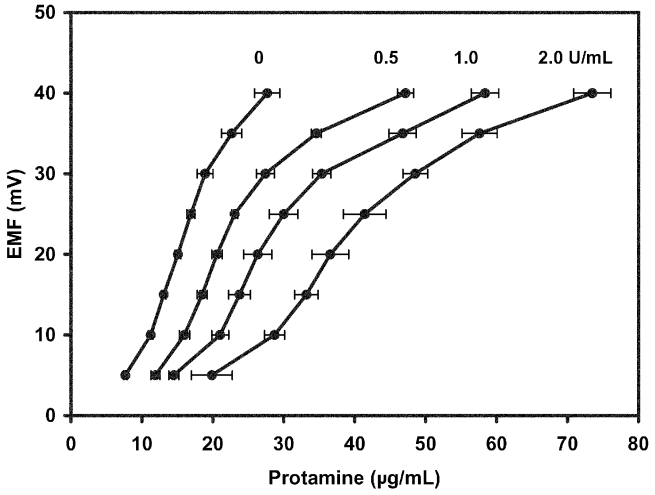

Fig. 10 Typical potentiometric titrations of varying amounts of Fragmin in undiluted plasma (2.5 mL) as monitored by rotating polycation-sensitive membrane electrodes (3000 rpm). Protamine (1.0 mg mL^{-1}) was infused at a rate of 50 μg min^{-1}. Error bars represent one standard deviation for four measurements

LMWH species at low concentrations, with an enhanced linearity at higher concentrations observed in the case of Fragmin, owing to its higher average molecular weight and lower polydispersity. As stated above, linearity to even higher levels of Lovenox and Normiflo could be achieved by using higher infusion rates of the protamine titrant (see inset), but this decreases the ability to resolve low levels of these species from the blank titration curves.

Since the rotating protamine-sensitive membrane electrode yields clear endpoints for the titration of LMWHs at much lower concentrations in buffered saline solution, the rotating electrode design should also be applicable for detecting lower levels of LMWHs in complex samples such as whole blood or undiluted plasma. However, the high viscosity of blood can dramatically decrease the diffusion coefficient of the protamine in such a matrix and thus lower the mass transport of protamine to the membrane surface. Indeed, a higher detection limit toward protamine was observed previously using a static polycation sensor in viscous plasma samples compared to buffer solution [13, 14]. This higher detection limit for the indicator electrode would make the titration of LMWHs in plasma or whole blood rather time-consuming. For rapid titrations, it is desirable to use the new rotating electrode design to decrease the thickness of diffusion layer and lower the protamine detection limit. In addition, the analysis time can be further reduced by increasing the titration speed (infusion rate of protamine).

To demonstrate the potential application of this new LMWH assay method to a real clinical sample matrix, 1.0 mg mL^{-1} protamine was infused into 2.5 mL of plasma samples spiked with varying levels of Fragmin (using a high infusion rate of 50 μg min^{-1}). The titration was followed continuously with a rotating polycation sensor (at 3000 rpm). With this high infusion rate, analysis of samples containing up to 2.0 U mL^{-1} Fragmin can be completed within 4 min. Fig. 10 illustrates the titration curves obtained for these undiluted plasma samples spiked with different amounts of Fragmin. Similar titration curves were also obtained for Lovenox or Normiflo spiked into plasma (data not shown). The titration curves shift to higher concentrations of protamine compared with those shown in Figs. 6 and 8, relating to the higher detection limit toward the titrant protamine in the undiluted plasma matrix. The precision obtained by the rotating electrode in the undiluted plasma samples is somewhat less than that observed in buffer, probably due to the slower rate of protamine/LMWH complex formation in such a viscous matrix. Hence, the precise location of the distal tip of the tubing that carries the protamine titrant into the sample could influence the apparent endpoint observed. Nonetheless, the precision observed for these very preliminary experiments is adequate to enable the determination of LMWHs at concentrations as low as 0.2 U mL^{-1} (distinguishable from blank titration).

Another notable difference in the potentiometric titration curves in plasma samples compared to buffer, is that the polycation electrode exhibits a much reduced total EMF response towards protamine in this matrix (see Figs. 10 vs. 6). It has been found that proteins existing in plasma, such as albumin and globulin, adsorb onto the surface of the electrode membrane and modulate the selectivity coefficient of the membrane interface towards smaller cations like Na^+ and K^+. This results in an increase in the absolute starting potential of the electrodes that leads to a reduced total EMF response to given concentrations of protamine [37].

Conclusion

A novel rotating electrode-based potentiometric titration method for detecting low levels of newer LMWH anticoagulants has been demonstrated. The method offers high precision and excellent detection limits for the potential quality control of LMWH pharmaceutical preparations. In addition, it may be applicable for measuring therapeutic levels of LMWHs in undiluted plasma or whole blood samples. As demonstrated previously in a clinical study for measurement of UFH in human blood [13], the potentiometric titration methodology enables rapid assays in samples that use either EDTA or citrate as anticoagulants. Further efforts to evaluate the clinical utility of static and rotating electrode configurations for measuring LMWHs in human blood samples are currently in progress in this laboratory.

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