

A moving-part-free protamine-sensitive polymeric membrane electrode for sensitive biomedical analyses

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ABSTRACT

Traditional potentiometric polyion-sensitive electrodes can only work effectively in samples with vigorous convection fulfilled by magnetic stirrer, electrode rotator, or other moving components. The dependence on complex moving parts prohibits the fabrication of compact, cost-effective, and energy-effective test devices from the commercial point of view. In this paper, a novel potentiometric sensing protocol without using any moving parts has been proposed for polycationic protamine. In contrast to traditional protamine-sensitive electrodes conditioned by discriminated ion (Na^+), the proposed electrode is conditioned with primary ion (protamine). Upon a medium exchange from the conditioning solution into an unstirred sample solution without protamine, protamine loaded in the membrane is stripped into the aqueous phase via ion exchange with aqueous sodium ion, thereby inducing a large potential drop. Interestingly, when the sample solution initially contains protamine, the ion-exchange process has been found to be sensitively inhibited by the sample protamine, and thus the potential drop is suppressed, which forms the basis of the moving-part-free potentiometric polyion sensing strategy. Utilizing the digestion ability of protease to protamine, the electrode was employed to determine the activity of trypsin with a detection limit at least one order of magnitude lower than traditional potentiometric methods. The trypsin inhibitor in both buffer and plasma samples was also sensitively detected with the moving-part-free protamine-sensitive electrode. Finally, the ability of the proposed electrode to detect polyanionic heparin was demonstrated.

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1. Introduction

Potentiometric polyion sensitive electrodes are a kind of ion exchanger-based polymeric membrane electrodes showing sensitive and reproducible potential responses toward polyionic macromolecules (Meyerhoff et al., 1996; Kang et al., 2011). One of the most commonly used polyion-sensitive electrodes is the electrode responsive to protamine, a small polycationic protein isolated from fish sperm. Beyond the direct detection of protamine, protamine-sensitive electrodes (PrSEs) have many applications in clinical diagnosis and pharmaceutical analysis (Dai et al., 1999; Kelly et al., 2010). Based on the specific proteolysis property of protease toward protamine, PrSEs have been used to determine the activities of proteases, which play crucial roles in a number of biological and physiological processes such as digestion, immune response, blood clotting and reproduction

(Hedstrom, 2002). On the other hand, using the electrostatic binding interaction of polycations and polyanions, various polyanions such as unfractionated heparin (Ramamurthy et al., 1998), low-molecular-weight heparin (Qin et al., 2003), carrageenan (Hassan et al., 2002), and nucleic acid (Durust and Meyerhoff, 2007; Ding et al., 2012) have been successfully sensed by PrSEs. Among them, the analysis of heparin, a widely used injectable anticoagulant in surgical procedures, is of special importance. The accurate monitoring of heparin levels in blood prevents patients from fatal heparin overdose effects such as bleeding during or after the open-heart surgery (Olson et al., 1992) and the discrimination of heparin with its analog, oversulfated chondroitin sulfate is an imperative task for the control of heparin purity (Wang et al., 2008; Kang et al., 2011).

Compared with other techniques such as nuclear magnetic resonance (Guerrini et al., 2008), high performance liquid chromatography (Keire et al., 2010), colorimetry (Guarise et al., 2006; Fu et al., 2012) and fluorometry (Pu and Liu., 2008; Li et al., 2011; Hu et al., 2012; Fan et al., 2012) for protease or heparin assay, the potentiometric method has advantages of portability, ease of use,

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simple and reliable instrument, and resistance to color and turbid interference. However, unlike classical ion-selective electrodes working under fully equilibrium conditions, the response of a polyion-sensitive electrode is a quasi-steady-state one highly dependent on the polyion flux from the sample bulk into the sample–membrane interface (Fu et al., 1994). Consequently, polyion-sensitive electrodes only work effectively in sample solutions with vigorous convection, which is routinely fulfilled by magnetic stirring or electrode rotating (Ye and Meyerhoff, 2001). Although usually unfocused in literatures for mechanism investigation, the necessity of moving parts actually highly disfavors the commercial application of polyion-sensitive electrodes, especially for the out-of-lab tests. The stirrer or rotator prohibits the fabrication of a compact, miniaturized polyion electrode-based analytical device and largely increases the cost and energy-consumption of the testing unit. Moreover, measurements using stir bars or rotating electrodes may require larger sample volumes, and suffer from larger noises owing to electromagnetic interference from rotating or stirring equipments and the undesirable vibration caused by unsmooth electrode rotating or stir-bar spinning (Davison and Harbinson, 1986; Qin et al., 2003). To solve these problems, an oscillating pressure source generated by a motor-driven syringe was used to move the sample solution in a cartridge back and forth across the protamine-sensitive polymeric membrane, thus ensuring effective sample mixing and the mass transport of protamine into the sensing membrane (Qin et al., 2010). More recently, in a microfluidic device for potentiometric polyion sensing, an electromagnet-driven actuator was designed to control a permanent magnet to squeeze and release sample solution on the sensing membrane, by which sample convection was fulfilled (Kang et al., 2011). However, these configurations, in essence, still rely on complex moving components and a potentiometric polyion-sensitive electrode truly free of moving parts is highly desirable to enable the fabrication of a simple, integrated, cost- and energy-effective polyion-based detection unit.

Recently, we developed a novel polyion sensing protocol using a primary ion-conditioned membrane instead of the conventionally used discriminated ion-conditioned one (Wang et al., 2012). In this method, protamine loaded in the membrane bulk suppressed the inward flux of target protamine from the sample–membrane interface and thus enhanced the sensitivity of PrSEs. Notably, to make the primary ion-conditioned polymeric membrane work, an activation procedure must be conducted before measurement, in which protamine in the membrane surface layer was stripped out by an aqueous solution of NaCl. Such an activation step is undesirable albeit necessary, since it renders the analytical procedure tedious and time-consuming. However, interestingly, we found that this stripping process is sensitively affected by protamine initially present in the sample solution. Accordingly, the potential change during this stripping process could be directly used to indicate the concentration of protamine in the sample solution. In contrast to traditional polyion responses based on convection-assisted inward polyion fluxes, the outward protamine stripping process was found to be spontaneous and it does not need any sample convection, thereby fully eliminating moving components in potentiometric polyion sensors. Herein, we will demonstrate the principle of the moving-part-free protamine sensing methodology as well as its applications in the sensitive detection of protease, protease inhibitor and polyanionic heparin.

2. Experimental

2.1. Reagents

Dinonylnaphthalene sulfonic acid (DNNS) as a 50% solution in heptane, 2-nitrophenyl octylether (*o*-NPOE), high molecular

weight poly(vinyl chloride) (PVC), protamine sulfate (from herring), heparin sodium salt (from bovine intestinal mucosa, 149 units/mg), trypsin (from bovine pancreas, 13816 BAEE units/mg), and Trizma base were purchased from Sigma-Aldrich. Sodium chloride, tetrahydrofuran (THF, redistilled before use), and hydrochloric acid were purchased from Sinopharm Group Co. Ltd. All chemicals and reagents were of Selectophore or analytical reagents grade. Aqueous solutions were prepared with freshly deionized water (18.2 M Ω cm specific resistance) obtained with a Pall Cascade laboratory water system.

2.2. Preparation of protamine-sensitive electrodes

Protamine-sensitive membranes (ca. 100 μ m in thickness) containing 49.5 wt% PVC, 49.5 wt% NPOE and 1.0 wt% DNNS were prepared by solvent-casting technique with THF as the casting solvent. Membranes were then glued to plasticized PVC tubes (i.d. 3 mm, o.d. 5 mm) to fabricate PrSEs. The PrSEs were conditioned in 100 μ g/mL protamine in 50 mM Tris–HCl buffer at pH 7.4 containing 0.12 M NaCl (Tris–NaCl buffer) overnight under ambient conditions (ca. 19 $^{\circ}$ C) and the same solution was used as the inner filling medium. Electrodes were stored at 4 $^{\circ}$ C in the conditioning solution when not in use.

2.3. EMF measurements

The potential was measured using a CHI 760D electrochemical workstation (Shanghai Chenhua Apparatus Corporation, China) in the galvanic cell: Ag, AgCl/3 M KCl/1 M LiOAc/sample solution/PrSE membrane/inner filling solution/Ag, AgCl. The external reference electrode was made as follows: a 100 μ l pipette tip with its narrower end obstructed by plastic foam was first filled with 3 M KCl; then an Ag/AgCl wire was inserted into this pipette tip and the other end of the pipette tip was sealed with Parafilm. All EMF measurements were conducted at room temperature and a Faraday cage was used to lower the noise.

2.4. Analytical procedures for protamine, protease, protease inhibitor and heparin

For all assays, the sensing membranes of protamine-conditioned PrSEs were thoroughly rinsed with distilled water before use. All tests were performed in 150 μ l of Tris–NaCl buffer in homemade microcells without stirring. The potential responses to protamine were recorded from 6 s to 600 s after immersing the protamine-conditioned PrSEs into stagnant Tris–NaCl buffer solutions containing different concentrations of protamine. For the determination of trypsin activity, buffer solutions with 20 μ g/mL protamine and varying amounts of trypsin were incubated for 5 min at room temperature. Then the protamine-conditioned electrode was placed in these solutions and potential values at 600 s were used for quantification. For aprotinin assay, a mixture of aprotinin and 2.5 U/mL trypsin was incubated for 1 min in buffer and then 20 μ g/mL protamine was added to undergo proteolysis for 5 min. Subsequently, potential traces were recorded at protamine-conditioned PrSEs in these assay mixtures. To detect aprotinin in plasma, a pretreated procedure with perchloric acid was firstly employed analogous to previously reported one for a traditional PrSE (Badr et al., 1997). Then 0.3 μ l of plasma with a given amount of aprotinin was mixed with 2.5 U/mL trypsin. After a 1-min incubation and a following 5-min proteolysis of 20 μ g/mL protamine, potential signals were measured. The concentration of aprotinin in plasma was finally calculated according to the dilution ratio. For heparin detection, 20 μ g/mL protamine was incubated with different concentrations

of heparin for 1 min and the unbound protamine was then detected by the moving-part-free PrSE.

3. Results and discussion

3.1. Operational principle of the moving-part-free PrSE

Unlike classical polyion-sensitive electrodes avoiding any contact of primary ion before measurement, the DNNS-doped polymeric membrane PrSE is here conditioned with primary ion, protamine. After conditioning, polycationic protamine could effectively occupy the anionic sites of the polymeric membrane via its ion-pairing interaction with anionic DNNS. However, when this membrane is removed from the conditioning solution with a high level of protamine into a measurement solution containing only high concentrations of discriminated ions (sodium ions), the membrane protamine cannot be held in the membrane and is prone to being stripped into the contacting aqueous phase owing to the limited selectivity of protamine against the sodium ion. Essentially, the stripping of protamine is an ion-exchange process of protamine cations in the membrane with sodium ions in the aqueous phase, which has been demonstrated by the subsequent potential responses to spiked protamine in the aqueous solution after stripping (Wang et al., 2012). The ion-exchange process would significantly increase the sodium ion concentration in the membrane surface layer, while that in the aqueous phase is relatively constant owing to the large sodium ion background (120 mM). Therefore, a potential drop would be induced according to the Nernst equation for the phase boundary potential (Bakker et al., 2004)

$$E_{PB} = E^0 + \frac{RT}{F} \ln \frac{C_{Na(a)}}{C_{Na(m)}}$$

where E_{PB} is the potential at the membrane–sample interface, $C_{Na(a)}$ and $C_{Na(b)}$ are the concentrations of sodium ions in the surface layers of aqueous phase and membrane phase,

respectively, and E^0 incorporates among other constant terms the free energy of transfer for sodium from water to the membrane phase.

Then we speculate that the potential drop is possible to be analytically utilized if the ion-exchange process (uptake of sodium ion and concomitant stripping of protamine) is affected by protamine initially present in the sample solution or residual protamine after proteolysis or electrostatic binding in the sample solution (Fig. 1). With high concentrations of protamine in the sample solution, the uptake of sodium ion into the membrane would be fully inhibited owing to the competition of more extraction-favorable protamine in the aqueous phase; i.e. ionic sites in the membrane should be still occupied by polycationic protamine rather than sodium ions from aqueous phase. Thus, the potential drop would be insignificant. On the other hand, when a lower concentration of protamine exists in the sample solution, a part suppression effect on the sodium ion uptake may be expected, which induces a moderate potential drop.

To demonstrate this idea, potential traces in sample solutions with different concentrations of protamine at the protamine-conditioned PrSEs were recorded (Fig. 2A). As can be seen, when the protamine concentration increases from 0 to 22 $\mu\text{g}/\text{mL}$ in the 50 mM Tris–HCl buffer solution containing 0.12 M NaCl, the potential drop was gradually suppressed and became insignificant for 22 $\mu\text{g}/\text{mL}$ protamine, which confirms the inhibition effect of aqueous protamine on the ion-exchange process. It should be noted that all these measurements were conducted in sample solutions without any stirring or other convection. In contrast, for a traditional PrSE conditioned by discriminated ions, even 22 $\mu\text{g}/\text{mL}$ protamine can only induce a fairly small potential response without stirring (Fig. S1).

As previously demonstrated (Wang et al., 2012), the electrode could be regenerated by reconditioning the membrane with protamine. With a 2 min-reconditioning step in stirred Tris–HCl buffer containing 5 $\mu\text{g}/\text{mL}$ protamine, the proposed electrode could be repeatedly used for at least five times with a standard deviation of 3.1 mV for 2 $\mu\text{g}/\text{mL}$ protamine. It should be noted that

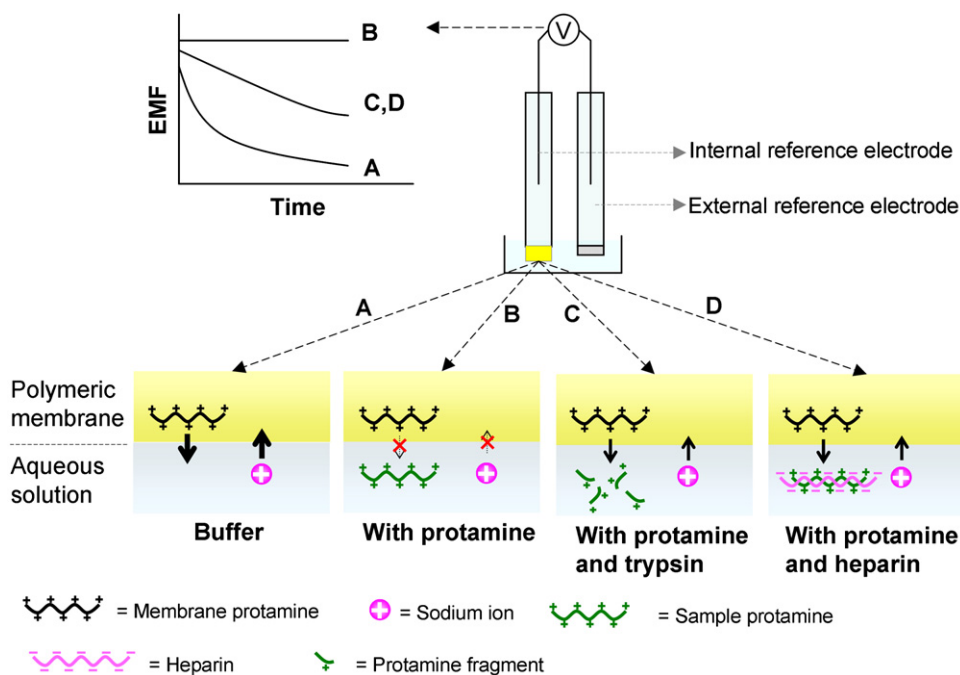


Fig. 1. Schematic representation of the biosensing strategy using a moving-part-free PrSE conditioned with protamine. Solid arrows across the membrane–sample interface indicate the ion exchanges of membrane protamine cations with aqueous sodium ions, which dictate the magnitude of potential drops.

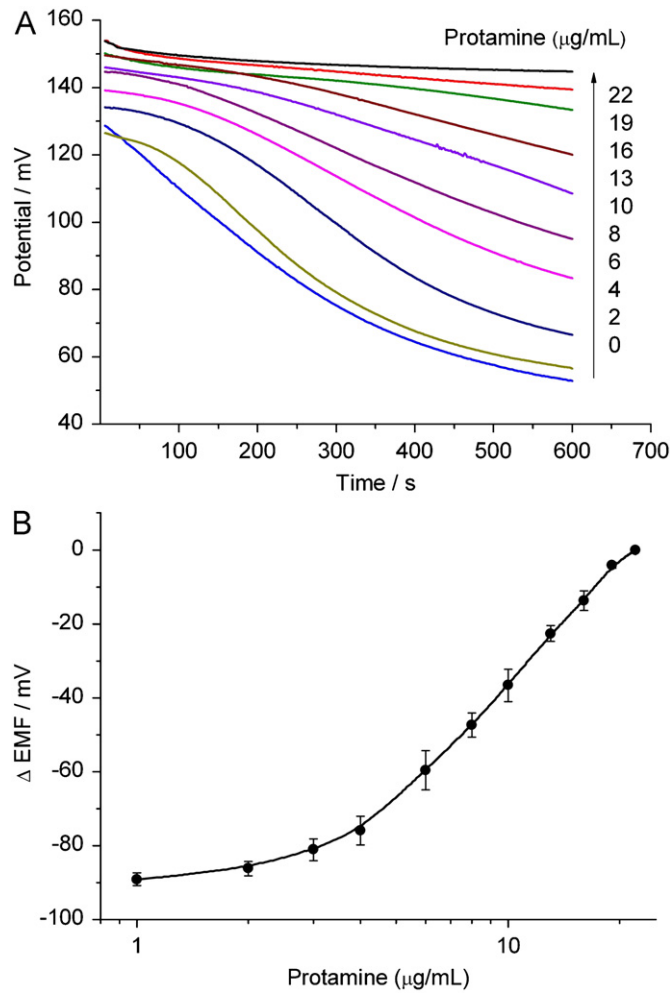


Fig. 2. (A) Potential-time traces of protamine-conditioned PrSEs in unstirred Tris-NaCl buffer solutions containing various concentrations of protamine from 6 s to 600 s. (B) Protamine calibration curve as obtained by the moving-part-free PrSE. Each error bar represents the standard deviation of three measurements.

DNNS-based electrodes, without using specific ionophore, are quite cheap and easily prepared. Therefore, to avoid the tedious regeneration step, all electrodes were for single-use in the subsequent experiment.

Like the quantification method for other non-equilibrium potential responses (Nevins Buchanan et al., 2004; Wang and Qin, 2012), potential values after a fixed time fragment were utilized here for quantification. Using the potential value for 22 µg/mL protamine at 10 min as a reference, the differences between potential values in other samples measured at 10 min and this reference value were used to plot the calibration curves throughout this study. The calibration curve for protamine is shown in Fig. 2B with a log-linear range from 4 to 19 µg/mL. Such a potential-protamine relationship forms the basis of the assays of protease activities and polyanions.

3.2. Assays of protease and its inhibitor using the moving-part-free PrSE

As a low-molecular-weight protein rich in arginine, protamine is a suitable substrate for trypsin-like serine protease cleaving polypeptide mainly at the carboxyl side of the amino acids lysine or arginine (Hedstrom, 2002). In the presence of trypsin, protamine can be digested into fragments with less charge and smaller molecular weight. It has been shown that these fragments have

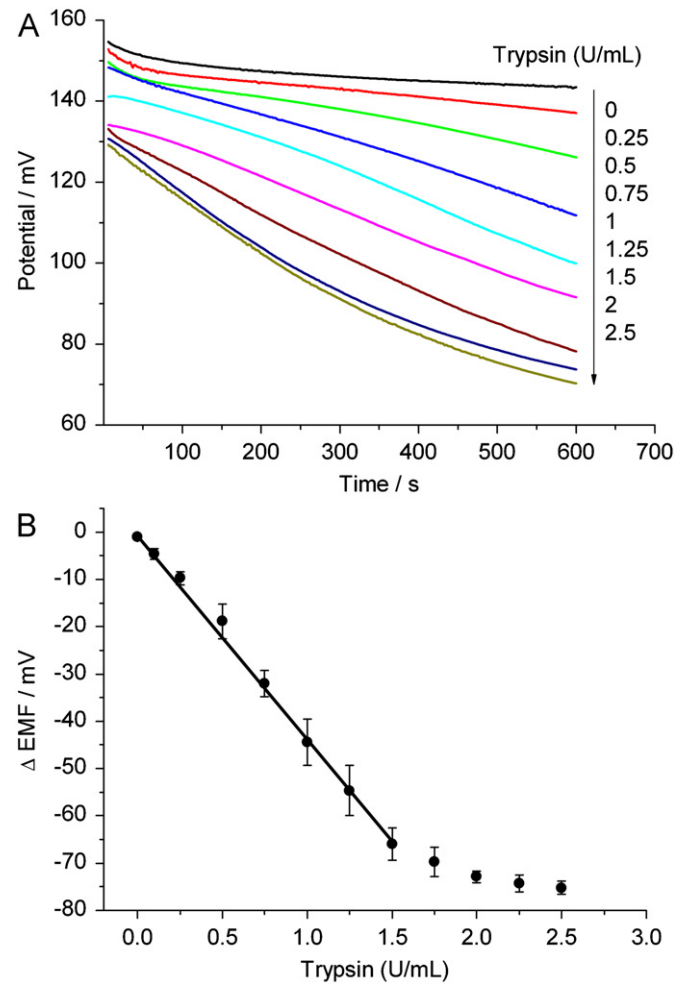


Fig. 3. (A) Potential-time traces of protamine-conditioned PrSEs in unstirred Tris-NaCl buffer solutions containing 20 µg/mL protamine and trypsin of various activities from 6 s to 600 s. (B) Trypsin calibration curve obtained by the moving-part-free PrSE. Each error bar represents the standard deviation of three measurements.

poorer affinity toward protamine-sensitive polymeric membrane as compared to the intact protamine. Therefore, PrSEs are capable of potentiometrically transducing the proteolytic process of protamine and indicating the concentration of trypsin. Similarly, for the protamine-conditioned moving-part-free PrSE, the small protamine fragments should not act as effectively as intact protamine in competing with sodium ion to occupy anionic sites in the membrane also owing to the poorer membrane affinity of protamine fragments. Thus, after enzymatic digestion, the uptake of sodium ion becomes more favorable as compared to before digestion. The higher the activities of trypsin, the less intact protamine could be left and the suppression effect on sodium uptake becomes weaker, thereby inducing a larger potential drop. As shown in Fig. 3A, while only a negligible potential drop could be obtained in the solution with 20 µg/mL protamine, the potential drop indeed became increasingly larger with increasing activities of trypsin in the solution. Based on the calibration curve in Fig. 3B, the detection limit to trypsin was calculated to be 0.034 U/mL or 2.5 ng/mL (3σ) and the linear range is 0 to 1.5 U/mL. This detection limit is lower by at least one order of magnitude than chronopotentiometric (about 1 U/mL) (Fordyce and Shvarev, 2008) and traditional potentiometric (5 U/mL) (Yun et al., 1995; Abd-Rabboh et al., 2003) methods. In addition, compared to these protocols, the moving-part-free electrode

requires much smaller sample volume. In traditional polyion sensing schemes using magnetic stirrer or rotating electrode (Ye and Meyerhoff, 2001; Nevins Buchanan et al., 2004), sample solutions of 3 mL or more were used. Rather, a sample volume of 150 μL was found to be competent for the moving-part-free PrSE.

The specificity of the moving-part-free PrSE toward trypsin was examined. When trypsin was substituted by other enzymes that cannot specifically cleave protamine, such as chymotrypsin, pepsin, and glucose oxidase, no potential signals discriminated from the blank experiment were obtained (Fig. 4). However, on the other hand, the proposed polyion sensing principle is anticipated to be extended to the detection of other protease using electrodes sensitive to other polycationic proteins or peptides. For example, synthetic polycationic peptide containing a hydrophobic phenylalanine–valine sequence is a suitable substrate for chymotrypsin, and the corresponding proteolysis reaction can be indicated by the DNNS-doped polycation-sensitive polymeric membrane electrode (Han et al., 1996).

Given the important role of protease inhibitors in many physiological and pathophysiological processes (Cheronis, 1993), the utility of the moving-part-free PrSE in protease inhibitor assay was also studied. Aprotinin is a bovine pancreatic trypsin inhibitor used as a drug in complex surgery such as heart and liver surgery (Mangano et al., 2006). After 2.5 U/mL trypsin was mixed with different concentrations of aprotinin, the digestion ability of trypsin toward a given amount of protamine was inhibited, thereby resulting in smaller potential drops as compared to samples with only trypsin and protamine. Using 2.5 U/mL trypsin and 20 $\mu\text{g}/\text{mL}$ protamine, the detection limit of moving-part-free PrSEs toward aprotinin is 12 ng/mL (3σ) and the linear range is 0–150 ng/mL (Fig. 5). Such a detection limit is one order of magnitude lower than traditional potentiometric methods (Badr et al., 1997). If a lower concentration of trypsin was used, the detection limit could be further improved (e.g. 9 ng/mL using 1.5 U/mL trypsin) although the total EMF change and the dynamic range would be lowered. Furthermore, plasma samples containing aprotinin whose concentrations lie in those employed in cardiac surgery (Davis and Whittington, 1995) were successfully detected by the proposed sensing protocol and recovery rates ranging from 95% to 106% were obtained (Table S1). For applications in whole blood samples, the sensitivity of the electrode

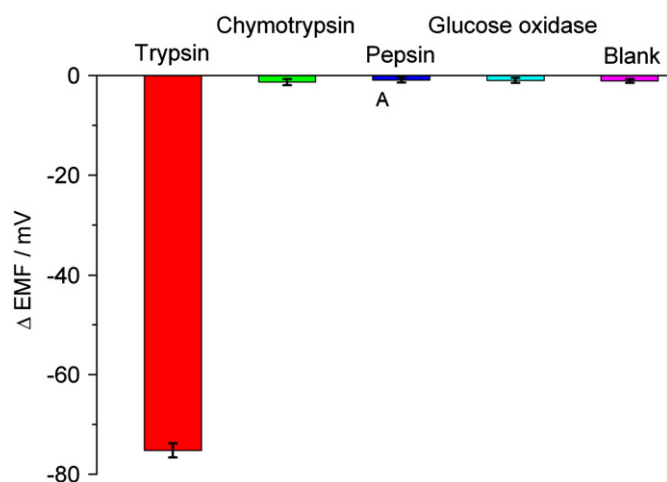


Fig. 4. Potential signals (differences between potential values in each samples measured at 10 min and the reference value obtained with 22 $\mu\text{g}/\text{mL}$ protamine) induced by 0.2 $\mu\text{g}/\text{mL}$ trypsin, chymotrypsin, pepsin, and glucose oxidase in unstirred Tris–NaCl buffer solutions containing 20 $\mu\text{g}/\text{mL}$ protamine. The blank experiment was conducted with only 20 $\mu\text{g}/\text{mL}$ protamine. Each error bar represents the standard deviation of three measurements.

would be reduced by the suppressed protamine transport in aqueous phase owing to the high sample viscosity and the adsorption of blood proteins (Qin et al., 2003). Efforts to improve the performance in blood will be made on optimizing the conditioning methods and the membrane components (e.g. using polyurethane M48 with better biocompatibility to reduce non-specific protein adsorption (Ramamurthy et al., 1998)).

3.3. Detection of heparin using the moving-part-free PrSE

With twenty positive charges, protamine can form complexes with a number of polyanions such as heparin and nucleic acid via the electrostatic interaction. Utilizing the poorer affinities of protamine-sensitive membrane toward these complexes as compared to free protamine, PrSEs have been employed to detect the concentration of polyanion (Qin et al., 2003), analyze the impurity of heparin (Kang et al., 2011), and investigate the binding constants of the protamine–polyanion systems (Durust and

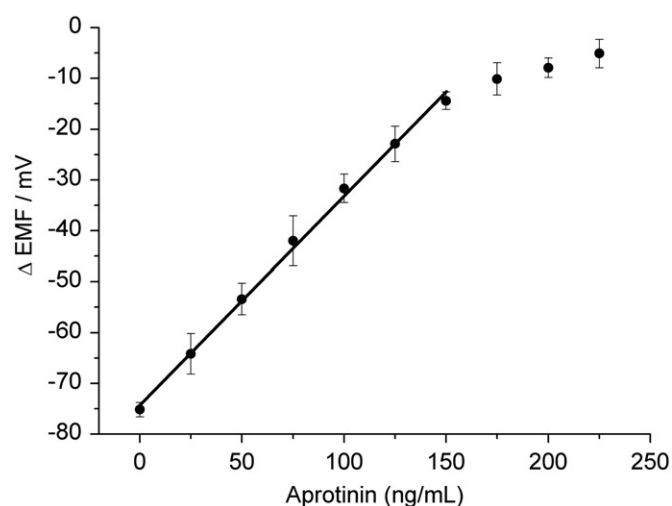


Fig. 5. Calibration curve for aprotinin detection in unstirred Tris–NaCl buffer solutions. A mixture of aprotinin and 2.5 U/mL trypsin was allowed to digest 20 $\mu\text{g}/\text{mL}$ protamine for 5 min, and potential signals were measured in the assay mixture. Each error bar represents the standard deviation of three measurements.

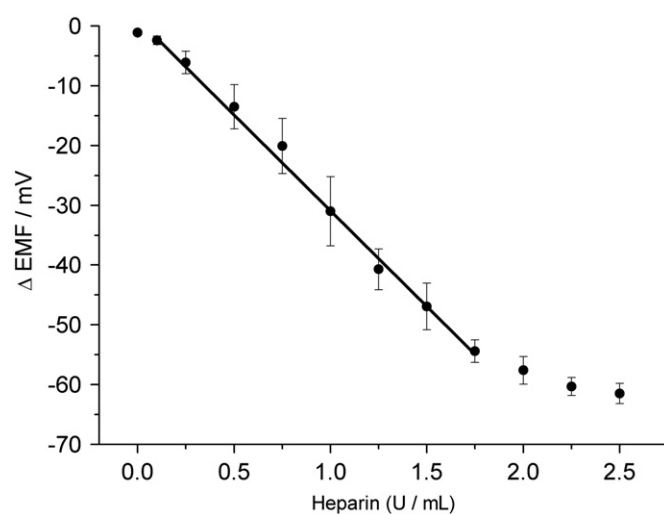


Fig. 6. Calibration curve for heparin detection in unstirred Tris–NaCl buffer solutions. 20 $\mu\text{g}/\text{mL}$ protamine was used to interact with different concentrations of heparin. Each error bar represents the standard deviation of three measurements.

Meyerhoff, 2007). The poor affinities are also expected to render these polycation–polyanion complexes incompetent in suppressing sodium uptake into the protamine-conditioned PrSE. Hence, when a measurement solution with a high concentration of protamine, inducing a negligible potential drop, is spiked with polyanions, potential drops can be observed again at the protamine-conditioned electrode. As a preliminary application of this mechanism, the moving-part-free electrode was used to analyze heparin which possesses an average charge of -70 . As shown in Fig. 6, 0.1 U/mL heparin could be clearly identified and the linear range for heparin detection is 0.1–1.75 U/mL.

4. Conclusions

A novel polyion response principle based on a protamine stripping process from the cation exchanger-doped polymeric membrane into the sample solution has been explored. By using this mechanism, potentiometric polyion sensing protocols free of any moving parts were developed. This protocol fulfilled the determination of trypsin activities with a much lower detection limit than that obtained by traditional polyion-sensitive electrodes, as well as the sensitive detection of aprotinin in both buffer and plasma samples. Furthermore, the capability of the proposed PrSE in indicating polycation–polyanion interaction was demonstrated by taking heparin as an example. Since PrSEs are also powerful tools in other biosensing applications such as label- and substrate-free aptasensing (Ding et al., 2012) and nonseparation immunoassay (Dai and Meyerhoff, 2001), the moving-part-free PrSE may be utilized to develop more polyion-involved biosensing protocols with potential advantages of simplicity, low cost, low energy consumption, small sample volumes, and ease of miniaturization and integration.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.05.020>.

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