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Bioanalytical applications of polyion-sensitive electrodes

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Abstract

Recent developments in the design and bioanalytical applications of polyion-sensitive electrodes (PSEs) are reviewed. The general electrochemical principles governing the potentiometric response of such polymer membrane-based devices are summarized and new directions for the use of these novel sensors are detailed. These new directions include basic fundamental studies aimed at determining the thermodynamics of polyion extraction into ion exchanger-doped polymeric membranes, new methods to quantitate the anticoagulant drug heparin in whole blood via titrations with polycationic protamine, selective assays of protease activities (and inhibitors of such activities) using natural and synthetic polyionic peptides as substrates, and novel homogeneous immunoassay schemes based on potentiometric polyion detection.

Keywords: Polyion-sensitive electrode; Protease assays; Non-separation immunoassay; Heparin determination

1. Introduction

Recently, we have found that polymeric membranes (e.g. plasticized poly(vinyl chloride)) doped with appropriate lipophilic ion-exchangers can display significant non-equilibrium potentiometric response (EMF) to biologically important polyionic species (e.g. heparin, protamine, polyphosphates, DNA, etc.) [1–11]. When assembled in typical ion-selective membrane electrode configurations, the resulting sensors exhibit potentiometric responses to sub-μM levels of polyions in samples as complex as whole blood [2,8]. Such response has been attributed to a highly selective non-equilibrium extraction of the polyion into the membrane phase via a cooperative ion-pairing interaction with the lipophilic ion-exchangers [4,6]. This extraction process leads to a steady-state change in the phase boundary potential at the membrane/sample interface. For example, membranes formulated with tridodecylmethylammonium chloride (TDMAC), rapidly exchange chloride for certain polyanions, and thus exhibit a very large potentiometric response to such species (including porcine and beef lung heparins), with the magnitude of response dependent on both the chain length and charge density of the polyanion

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structure [6]. In contrast, polymer membranes containing tetraphenylborate derivatives or dinonylnaphthalene sulfonate sites (DNNS) respond potentiometrically to low levels of polycations, including the antidote of heparin, protamine, as well as synthetic peptides rich in arginine residues [7,10]. Such response is reversed when specific proteases cleave these polycationic peptides into smaller fragments that are not favorably extracted into the organic membrane phase, providing a convenient electrochemical method to detect protease activities [10].

Herein, we summarize results from our most recent fundamental and applied studies involving these new polyion sensitive electrodes (PSE). A brief discussion of the mechanism of potentiometric polyion response is provided and the roles of polyion molecular weight, lipophilicity and charge density on the magnitude of EMF response are assessed using different polyphosphate, poly (amino acid), and nucleic acid structures as test polyanions. Various methods to use polyion sensors for the determination of heparin levels in blood are also reviewed, including a new protamine titration method that functions effectively for monitoring the concentrations of both conventional and low molecular weight heparin preparations in whole blood (using a DNNS-based polymer membrane electrode as the end-point detector). Other novel bioanalytical applications of PSE detection are discussed, including the ability to monitor specific protease activities and inhibitors, and the design of novel non-separation electrochemical immunoassays for small hapten/drug molecules.

2. Fundamental studies

The potentiometric response of PSEs has been shown to originate from the favorable polyion extraction that takes place at the outer surface of the polymer membrane. The typical EMF response of a TDMAC-doped PVC membrane plasticized with bis(2-ethylhexyl)sebacate (DOS) toward porcine and beef lung heparin in a 0.12 M NaCl solution is shown in Fig. 1. The large response toward these polyions cannot be explained by the conventional Nernst equation (Eq. (1)), which describes the equilibrium response of classical ion-selective electrodes (ISE's):

$$\mathrm{EMF} = E^0 + \frac{R \cdot T}{z \cdot F} \ln a_i \quad (1)$$

Indeed, the Nernst equation would predict a response of < 1 mV per decade for polyions with charges (z) > 59 per molecule. However, as shown in Fig. 1, porcine heparin, which has an average charge of -70 per molecule, elicits a response of > -50 mV between 0.04 and 0.4 μM in the presence of 0.12 M NaCl (using an average MW of 15000 for a commercial heterogeneous product) for polymeric membrane electrodes doped with TDMAC. The total EMF response toward beef lung heparin is even larger (Fig. 1) owing to the higher charge density on this molecule.

As stated previously, the EMF changes observed toward polyions for membranes doped with TDMAC and other ion-exchangers are due to a non-equilibrium extraction process (resulting in tight ion pairs with the exchanger in the membrane phase) that occurs at the sample/membrane

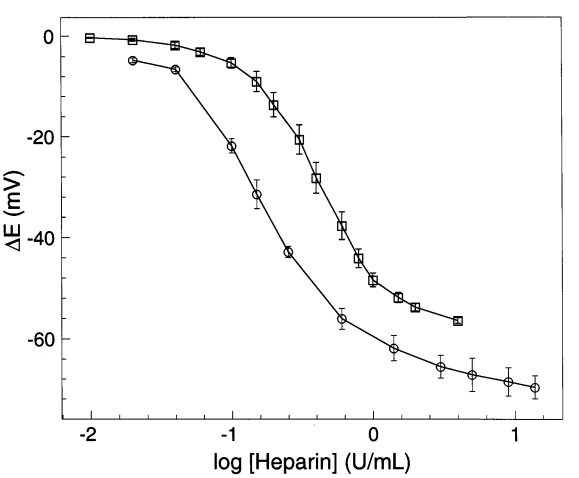

Fig. 1. Potentiometric response of polyanion-sensitive membrane electrodes toward beef lung heparin (○) and porcine heparin (□) in 120 mM NaCl. Membranes were made with 1.5 wt.% TDMAC, 66 wt.% PVC, and 32.5 wt.% DOS (bis(2-ethylhexyl)sebacate). Each curve represents the average response for three identical electrodes (see Fig. 2 for electrode configuration).

interface [4,6]. At high concentrations of the polyion, the response levels off (Fig. 1) as the surface of the membrane achieves electrochemical equilibrium with the polyion in the sample phase (i.e. all the chloride of TDMAC is replaced by heparin). However, at lower sample concentrations of the polyion, a steady-state situation exists at the interface, where the flux of polyion to the surface is equal to the flux of the polyion-exchanger ion pairs into the bulk of the polymeric membrane. The change in EMF in this concentration regime is given by the following equation [4]:

$$\Delta\mathrm{EMF} = \frac{RT}{F} \ln\left(1 - \frac{zD_a\delta_m}{R_T D_m \delta_a} c_{\mathrm{polyion}}\right) \quad (2)$$

where ΔEMF is the change in membrane potential after addition of the polyion to the sample phase in which chloride ions (or sodium/potassium in the case of polycation PSEs) initially control the phase boundary potential at the membrane/sample interface; c_{polyion} is the concentration of polyion added to the test solution; z is the charge on the polyion; D and δ are the diffusion coefficients and diffusion layer thicknesses in the membrane (m) and aqueous sample (a) phases, respectively, and R_T is the concentration of ion-exchange sites in the organic membrane phase (i.e. $TDMA^+$ concentration in the case of polyanion sensing). In practice D_m is determined by the membrane composition (plasticizer/polymer ratio), δ_a is controlled by the stirring rate in the sample phase and R_T is dictated by the wt.% of lipophilic exchanger added to the membrane formulation. We have shown that these parameters can be changed to vary the concentration range of polyion that is detected via the non-equilibrium response [4]. In addition, the geometry of the sensor/sensing membrane also affects the mass transport of the polyion with the ability to detect even lower levels of polyions using cylindrical rather than planar electrode configurations [4]. Such a design, used to obtain much of the data presented herein, is illustrated in Fig. 2 along with the ion-pairing extraction chemistries that yield polyanion and polycation responses for membranes doped with DNNS and TDMAC, respectively.

Eq. (2) is only valid in a sensing regime where the response toward the polyion is kinetic in nature (i.e. non-equilibrium; there are still some chloride ions present in the outermost layer of the membrane in the case of polyanion sensing) and in a polyion sample concentration range where extraction of the polyion is favorable (dependent on structure of polyion and strength of ion-pairing with exchanger in membrane phase). At high concentrations of polyion, or after very long exposure time, all of the counter-ions of the exchanger are replaced by the polyion, and the interface is at true equilibrium with respect to polyion extraction (although the bulk of the membrane may not yet be at equilibrium). Hence, the EMF change resulting from the addition of a relatively high concentration of the polyion ($> 10^{-6}$ M in the case of heparin) provides information about the thermodynamics of polyion extraction into the ion-exchanger doped organic films.

Since the elucidation of the response mechanism of polymeric membranes doped with TDMAC or DNNS to polyanions and polycations, respectively, the response characteristics of these devices toward a select group of polyions have been investigated in detail. While the majority of studies have focused on heparin (and related sulfonated polysaccharides) [1–6], protamine [7], and polystyrenesulfonates as reported by Hattori and Kato [12], more recent work has involved studying the response toward other polyion structures including arginine rich synthetic peptides and highly phosphated polymers [10,11].

There are three key structural features of polyions that have been found to influence the EMF response of PSEs: molecular weight, lipophilicity, and charge density [6]. In the case of heparin, it has been found that the EMF response increases with an increase in the molecular weight of the polyanion [2]. This is presumably due to the enhanced cooperative interaction of many $TDMA^+$ ions with the polyanion (with nitrogen end of the $TDMA^+$ condensing along the polymer chain via electrostatic interactions with the sulfonate groups and the alkyl tails interacting with each other via hydrophobic effects). The same molecular weight dependence is

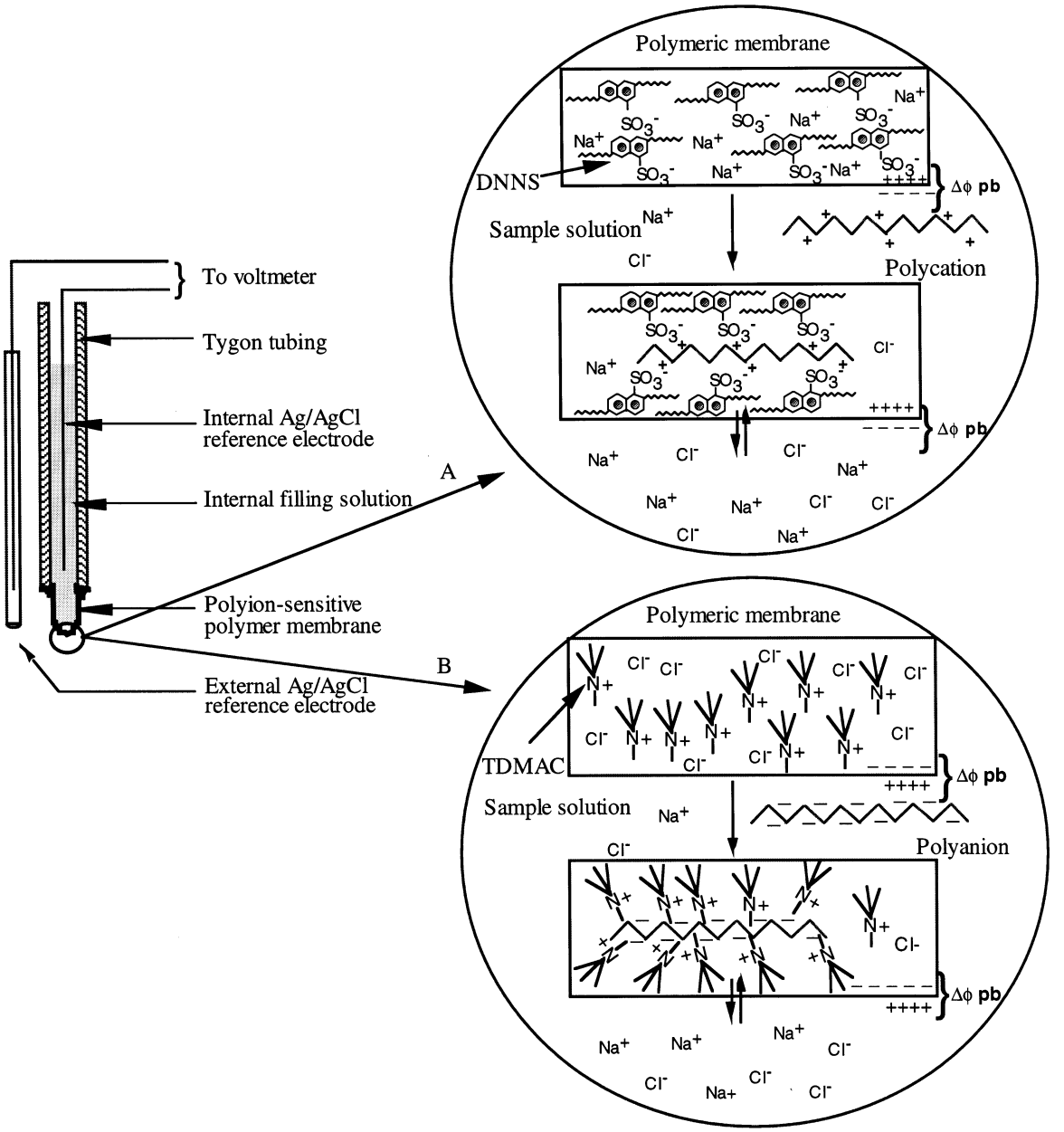

Fig. 2. Schematic of simple cylindrical PSE configuration, as well as an expanded view of the extraction and ion-pairing reactions of (A) polycations and (B) polyanions into and within the organic membrane phases of such devices.

also observed in the case of polyanions containing phosphate groups. As shown in Table 1, TDMAC-based PSEs elicit larger equilibrium potential changes to a 66-mer polyphosphate (-86.6 ± 1.1 mV) compared to a 5-mer polyphosphate (-62.2 ± 2.0 mV), and to a 12-mer DNA oligomer (-32.7 ± 3.9 mV) compared to an 8-mer DNA oligomer (-5.5 ± 2.2 mV). This trend has also been observed for peptide homopolymers containing lysine, aspartic acid,

and glutamic acid. By comparing the EMF responses of two different membranes, one doped with TDMAC and the other with the ion-exchanger tetradodecylammonium chloride (TDAC) (which does not ion-pair strongly with polyanions due to steric hindrance), the free energy of ion-pairing in the membrane phase can be estimated according to Eq. (3)[6].

$$\Delta g^0_{IP} = -\{\Delta EMF(TDAC) - \Delta EMF(TDMAC)\}F + RT\ln[R^-] \quad (3)$$

Table 1 reports that the apparent free energy of ion-pairing per charge increases from -7.23 ± 0.21 kJ mol^{-1} for the 8-mer DNA oligomer to -9.14 ± 0.39 kJ mol^{-1} for the 12-mer DNA oligomer. A schematic representation of the ion-pairing interaction of polyions with TDMAC and DNNS that occur in the sensing polymer membranes of PSEs is illustrated in Fig. 2.

Table 1
Equilibrium responses and ion-pairing free energies for several polyanions determined with poyanion-sensitive electrodes[a]

Polyanion	Response of TDMA-based electrodes (mV)	Δg^0_{IP} (kJ mol^{-1} z)
5-mer Polyphosphate	-62.2 ± 2.0	-8.5 ± 0.3
18-mer Polyphosphate	-66.2 ± 1.8	-9.2 ± 0.2
32-mer Polyphosphate	-83.7 ± 4.4	-10.2 ± 0.5
45-mer Polyphosphate	-93.6 ± 4.9	-13.6 ± 0.6
66-mer Polyphosphate	-86.6 ± 1.1	-14.4 ± 0.1
8-mer Polythymidylic acid	-5.5 ± 2.2	-7.2 ± 0.2
10-mer Polythymidylic acid	-21.9 ± 1.1	-8.1 ± 0.3
12 mer Polythymidylic acid	-32.7 ± 3.9	-9.1 ± 0.4

Refer to [11] for experimental details.

Charge density is another aspect of polyion structure that influences the magnitude of EMF response observed. It has been shown in the case of sulfonated polysaccharides that the EMF response toward heparins is much greater than that observed for dermatan sulfate and various carrageenans [6], species that are structurally similar but possess less total sulfonate groups per linear segment of carbohydrate polymer. For phosphate-based polyanions, three different polyanions that have the same number of charged groups, but different spacing between the charges, have also been examined (pentaphosphate, diadenosine pentaphosphate (a linear polyphosphate with two adenosine units at either end) and pentaadenylic acid). Pentaphosphate was found to elicit a non-equilibrium potential change of -16.8 ± 0.1 mV at 0.63 μg ml^{-1} in 10 mM NaCl, while 10 μg ml^{-1} of diadenosine polyphosphate was required to yield a comparable response (-15.6 ± 0.9 mV). No potential change occurred when the electrode was exposed to pentaadenylic acid, which possesses the lowest charge density. Thus, polyions with higher charge density will elicit larger EMF changes.

Lastly, the lipophilicity of the polyion structure also plays a significant role in the magnitude of the potentiometric response observed. For example, the EMF response of a TDMAC-based membrane to two DNA oligomers with the same charge density, but different lipophilicity, has recently been compared [11]. The 9-mer polyadenylic acid elicits a non-equilibrium potential change of -8.4 ± 1.3 mV at a concentration of 3.8 μg ml^{-1}, while no response is seen for the less lipophilic 9-mer polythymidylic acid at the same concentration. Similarly, earlier work with synthetic arginine rich synthetic peptides demonstrated that the total equilibrium EMF response of DNNS-based membranes toward these structures is enhanced when the peptides contain the lipophilic amino acids phenylalanine and leucine within their structure [10].

Knowing the effects of polyion structural characteristics on the magnitude of the EMF changes toward such species is extremely valuable in the development of new bioanalytical methods based on PSE detection. For example, as described be-

low (Section 3.2), the design of synthetic polypeptide substrates for new protease assays based on electrochemical polyion detection has been guided to a great extent by understanding the role of charge density, molecular weight, and lipophilicity on the response of PSEs toward targeted polyion structures.

3. Biomedical applications

There are two unique response characteristics that have contributed to the use of PSEs in biomedical applications. First, since PSEs respond to only free concentrations of polyion (during short measurement periods when the polyion participates in relatively strong binding reactions ($K_{eq} > 10^6$ M^{-1}); i.e. slow off rate constants), this makes them very useful detectors in potentiometric titrations to determine concentrations of polyionic drugs, including heparin and protamine (Section 3.1), and in the development of novel non-separation immunoassays (Section 3.3). Second, the molecular weight dependence of EMF response enables the use of PSEs as detectors for enzyme activity determinations, as well as for monitoring levels of specific enzyme inhibitors (drugs). Both of these bioanalytical applications will be described in Section 3.2.

3.1. Heparin determinations

One exciting application of PSEs is for the determination of heparin levels in whole blood samples from patients undergoing open heart surgery. Heparin is the most widely used anticoagulant in major surgical and extracorporeal procedures, such as coronary artery bypass surgery and hemodialysis. Heparin doses given to patients during bypass surgery are typically in the range of 2–8 U ml^{-1} (equivalent to 0.8–3.2 μM) [13]. However, it is essential to monitor the anticoagulant effect and, ideally, also the concentration of heparin during surgery, since higher doses of heparin are often associated with the risk of hemorrhage [14]. Currently, the most widely used clinical procedure to monitor anticoagulant activity is the activated clotting time (ACT), which is the time required for clot formation in whole blood after contact activation with agents such as kaolin. However, the ACT value is not necessarily a very accurate indicator of blood heparin levels since clotting time can also be affected by variations in temperature and hemodilution, which are commonly encountered during surgery [15]. Methods used to specifically determine the concentration of heparin in blood include a colorimetric anti-Xa assay (which measures heparin by its ability to inhibit Factor-Xa activity) [16] and a protamine titration-based clotting assay (Hepcon HMS, Medtronic Blood Management, Parker, CO). These assays are based on the detection of clot formation (Hepcon HMS) or a color change in the corresponding plasma sample (anti-Xa assay using chromogenic substrates) and, hence, can function poorly in samples that are highly colored (i.e. whole blood) or devoid of clotting factors (i.e. serum).

PSEs provide a relatively simple means to determine heparin levels accurately in whole blood. Such heparin determinations are readily performed via classical potentiometric titrations using protamine, a polycationic protein rich in arginine, as the titrant. Protamine binds stoichiometrically, via electrostatic interactions, with heparin (with affinity constant $> 10^7$ M^{-1}) [3]. Since PSEs respond only to free polyion and not the complexed form, they can serve as end-point detectors for such titrations.

In our earliest work, TDMAC-doped PSEs were used to determine heparin levels in whole blood samples via pseudo-titrations with protamine [8]. A direct titration could not be performed in this case due to the irreversibility of the membrane electrode response after exposure to a sample containing heparin. Such pseudo-titrations were carried out by adding small volumes (250 μl) of blood to a series of tubes containing known, increasing amounts of protamine. The potentiometric response of the polyanion-sensitive electrode was noted upon sequentially switching the electrode (and appropriate reference electrode) from the tube containing the highest to the least amount of protamine. A decrease in potential was observed in those tubes where the heparin present in the blood was in excess of the protamine in

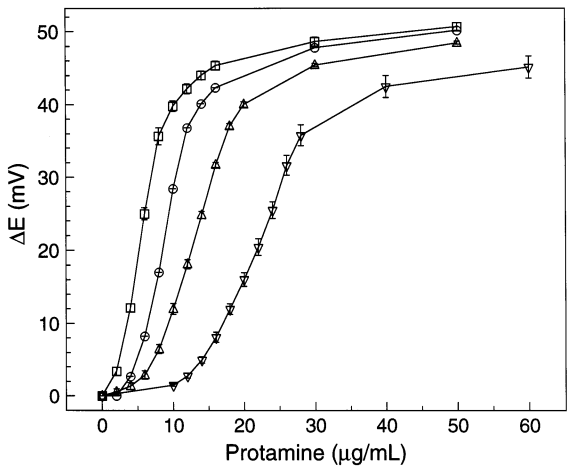

Fig. 3. Typical potentiometric titration of 0.0 (□), 0.5 (○), 1.0 (△) and 2.0 (▽) U ml^{-1} of porcine heparin with protamine as monitored by polycation-sensitive membrane electrodes containing 1 wt.% DNNS, 49.5 wt.% NPOE and 49.5 wt.% polyurethane-M48 in undiluted human plasma. The average response of three identical electrodes is shown.

that tube. The end-point of the titration was then determined by extrapolating the two linear regions of the titration curve. Heparin levels thus determined showed good correlation to the Hepcon HMS assay [8,17]. However, the rather cumbersome titration procedure made its routine application quite difficult.

More recently, we found that an improved polycation-sensitive membrane electrode utilizing DNNS as the membrane active component [10] can be employed to monitor heparin levels in whole blood from patients undergoing bypass surgery [18]. The advantage of using the polycation-sensitive electrode arises from the ability to perform direct potentiometric titrations, via the addition of small aliquots of protamine to a heparinized sample. Consequently, the titrations are much more convenient to perform when compared with the earlier pseudo-titrations using the polyanion-sensitive electrode. Fig. 3 shows the typical titration curves recorded for varying levels of porcine heparin in undiluted human plasma. The end-point of these titrations can be then determined by applying the Kolthoff method [19]. Heparin levels in whole blood samples ($N \geq 157$) determined using this method showed good correlations with the Hepcon HMS assay ($r = 0.934$) and the previously mentioned polyanion-sensitive electrode method ($r = 0.973$). In addition, reasonably good correlation ($r = 0.895$) was also found to a colorimetric anti-Xa assay (Coatest; Chromogenix) after correction for blood hematocrit levels (since the anti-Xa assay functions only in plasma samples), thus demonstrating the analytical utility of these electrodes for clinical heparin determinations.

Efforts have also been made to devise a more user friendly heparin assay method by fully automating these heparin titrations. This has been achieved by using a syringe pump to provide a continuous infusion of protamine into a heparinized blood sample (e.g. 2 ml). The resulting change in potential over time is monitored by a polycation-sensitive electrode. The presence of heparin in the sample causes a shift in the time required for the electrode to reach the end-point (Fig. 4). Consequently, the heparin concentration can be determined from the time taken to reach the end-point by knowing the infusion rate and the protamine concentration in the titrant solution. Such automated titrations can be performed

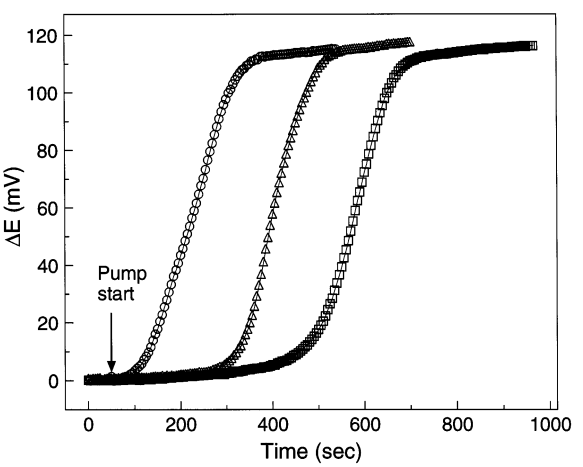

Fig. 4. Titration of 0.0 (○), 1.0 (△) and 2.0 (□) U ml^{-1} of porcine heparin performed by continuous infusion (25 μl min^{-1}) of a protamine solution (1 mg ml^{-1}) in 10 ml of buffer (50 mM Tris–HCl, pH 7.4, 120 mM NaCl) as monitored by polycation-sensitive membrane electrodes containing 1 wt.% DNNS, 49.5 wt.% NPOE and 49.5 wt.% M48; (adapted from [18] with permission).

in a relatively short period of time (< 10 min). Multiple automated titrations performed on samples spiked with varying levels of heparin (1.0 and 2.0 U ml^{-1}) show good accuracy and precision (1.00 ± 0.17 and 1.97 ± 0.25 U ml^{-1}), thus providing an even more rapid means to determine heparin in whole blood samples using PSEs.

Low molecular weight heparins (LMWHs) have acquired increasing significance for the prophylaxis of deep venous thromboembolisms (DVT) in patients undergoing elective surgical procedures such as hip-replacement surgery. Almost 50% of the patients undergoing such surgical procedures may develop DVT and, hence, heparin therapy is necessary. LMWHs are preferred to unfractionated heparin (UFH) in these procedures since they have much higher bio-availability (about 95%) and longer elimination half-lives. This makes their administration much simpler than UFH, which has rather long onset times (≈ 1 h) when administered subcutaneously. Further, there is a low incidence of hemorrhage for patients on LMWH therapy and this makes the use of this product even more attractive. However, monitoring levels of LMWH in blood can be quite difficult because broadly used clotting type-based assays, such as the ACT or aPTT, are not affected significantly by the presence of LMWHs. Thus, the only useful approach to measure the concentration of these drugs is to employ colorimetric anti-Xa assays which, of course, cannot be performed in whole blood samples (only on diluted plasma).

The DNNS-based polycation-sensitive electrode, however, can also be used to monitor the titrations of some LMWH preparations using protamine as the titrant. Potentiometric titrations performed on in vitro blood samples show that Fragmin (Pharmacia) can be determined at concentrations that are typically encountered in clinical practice (0–2 U ml^{-1}), with titration curves quite similar to that observed for unfractionated heparins (Fig. 3). However, some other preparations (Lovenox, Rhone-Poulenc) are not as easily titrated, with titration end-points less pronounced. The difference probably arises from the varying methods adopted in the preparation of these LMWHs and the precise molecular weight distribution of the final products. Indeed, it is known that Lovenox has a lower average MW compared to Fragmin, and shorter sulfated polysaccharides present in such a preparation likely bind protamine with much lower affinity, making it more difficult to achieve sharp breakpoints using the titrimetric approach. However, it may be possible to synthesize positively charged peptides (rich in arginine residues) that may bind more tightly to these LMWHs than protamine, thus enabling this methodology to be more broadly applied for measuring a wide range of commercial LMWHs in whole blood samples. Efforts in this direction are currently underway in this laboratory.

3.2. Determination of enzyme activities and enzyme inhibitors

Polyion-sensitive electrodes are also potentially useful detectors in the design of novel electrochemical enzyme assays. Using PSEs, it is possible to determine the activity of given enzymes that cleave polyionic substrate molecules into smaller fragments of less charge and lower molecular weight. Since the EMF response of PSEs is dependent on charge and molecular weight, the activity of the enzymes can be directly monitored by observing the reversal in potentiometric response of PSEs to specific polyionic substrates.

The first assay based on this novel detection method was designed for trypsin, which cleaves arginine rich peptides, such as protamine, into smaller fragments [7]. A reversal of the EMF response was recorded in real time after the enzyme was added to a protamine solution. The rate of potential decrease was shown to be directly proportional to the amount of trypsin activity present in the assay mixture.

This same potentiometric format has also been successfully employed for the assay of other protease activities, including chymotrypsin and renin [10]. Chymotrypsin and renin cleave only at specific sites of a polypeptide chain, the carboxylic side of lipophilic amino acids and between sequential Leu-Leu residues, respectively. Using the synthetic peptide P4 (Phe-Arg-Arg-Arg-Phe-Val-Arg-Arg-Phe-NH_2), which includes the enzymatic cleavage site between Phe-Val, DNNS-based PSEs

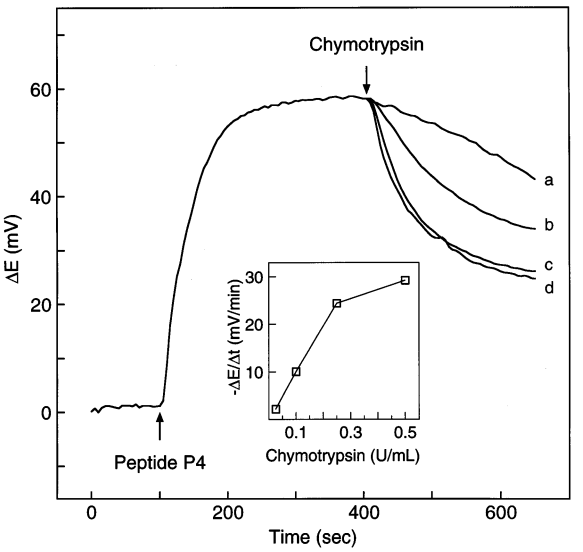

Fig. 5. Potentiometric assay for chymotrypsin. Shown is the electrode response of polycation-sensitive electrodes (average of two) in 50 mM Tris–HCl, pH 7.4, 120 mM NaCl toward 5 μg synthetic peptide P4 and its reversal after the addition of different amounts of chymotrypsin to this solution: (a) 0.025; (b) 0.10; (c) 0.25; and (d) 0.50 U ml^{-1}. Inset: shown is the rate of potential decrease ($-\Delta E/\Delta t$) immediately after addition of chymotrypsin versus the activity of the protease (adapted from [10] with permission).

exhibit an EMF reversal after chymotrypsin is added to a buffered solution containing the synthetic peptide. Fig. 5 shows the typical time dependent response obtained by Han et al. [10] for the assay of chymotrypsin using the P4 polycationic substrate. From the inset it can be seen that the rate of the potential reversal is linearly related to the chymotrypsin activity present in the range of 0.025 and 0.25 U ml^{-1}. It is important to note that no such EMF reversal was observed when testing other synthetic substrates that did not contain a specific cleavage site for chymotrypsin.

Similarly, this measurement scheme can also be employed to devise an assay for monitoring renin activity using the synthetic peptide P5 (Arg-Arg-Arg-Leu-Leu-Arg-Arg-Leu-Leu-Arg-Arg-Arg). Since lower concentrations of renin were used compared to other proteases, it was necessary to incubate the substrate with the enzyme for 4 h at 37°C before immersing a DNNS-based PSE into this sample solution. Fig. 6 shows the resulting EMF responses toward the incubated solutions, which were found to be proportional to the renin activity present in each tube.

Currently, new synthetic substrates are being designed for use in devising PSE-based assays for monitoring clinically relevant serine proteases, including factor Xa and thrombin. A new method to facilitate the detection of such activities by PSEs is under investigation. To increase the assay sensitivity, we believe that it would be more prudent to measure the appearance of polycations or polyanions (via an EMF change) in a test solution, rather than a decrease in the level of a given synthetic polyionic substrate. This can be accomplished by using synthetic solid-phase polyionic substrates in which enzymatic activity will cleave a polyion segment from the solid phase, enabling detection of these intact larger fragments in the solution phase by PSE detection.

It should be stressed that the use of PSEs is not limited to the determination of protease activities alone. Enzyme assays based on polyanion-sensi-

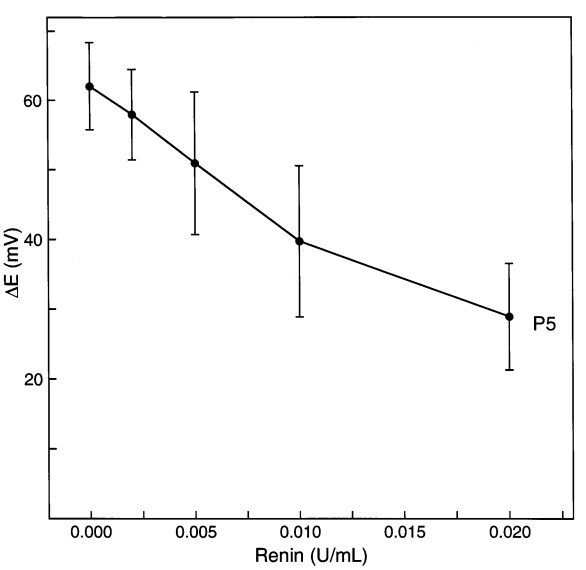

Fig. 6. Potentiometric assay for renin. Shown is the DNNS-based electrode response (average of three) toward synthetic peptide P5 (5 μg ml^{-1}) after 3 min. The peptide was incubated in 5 ml 50 mM Tris–HCl, pH 7.4, 120 mM NaCl with different amounts of renin for 4 h at 37°C before the measurement (adapted from [10] with permission)

tive electrodes have also been developed for the detection of acid phosphatase and ribonuclease A activities [11]. Here the potential reversal after cleavage of polyanionic substrates (polyphosphates and pyrimidine polynucleotides) is measured. The linear ranges of the present assays are not yet optimized; however, sensitivity in the mU ml^{-1} concentration range of these enzymes makes such PSE-based assays already comparable to conventional spectrophotometric methods in terms of sensitivity.

Beyond the direct measurement of enzyme activities, it is also possible to use PSE detection as a means to monitor specific enzyme inhibitors. Indeed, Badr et al. have recently shown that it is possible to monitor trypsin-like protease inhibitors (α_1-antiproteinase inhibitor, α_2-macroglobulin, aprotinin and soybean inhibitor) via PSE detection of trypsin activity using protamine as the substrate [20]. The hydrolysis of protamine upon the addition of proteinase-inhibitor test solutions was monitored in real time. Fig. 7 shows the resulting electrode EMF response for this assay scheme. It can be seen that the rate of reversal in the EMF response toward protamine is dependent on the amount of the α_1-antiproteinase inhibitor present in the solution (in the presence of a fixed amount of trypsin and protamine). The inset shows the linearity of the assay to be between 0 and 16 μg ml^{-1} of the inhibitor. In analogous assays for the other trypsin-like inhibitors, linear ranges in the sub μg ml^{-1} and mg ml^{-1} ranges were obtained. Badr et al. also showed that the efficacy of inhibition by a given inhibitor (expressed as I_{50} values) correlates well with the association constant of the proteinase–antiproteinase complexes (k_{assoc}) published in the literature.

An assay for aprotinin, a serine protease inhibitor often used to prevent post-operative bleeding in bypass surgery patients, was studied in further detail. It was found that the choice of protease employed to monitor the inhibitor influenced the assay sensitivity. Additionally, when this inhibitor assay was carried out in pretreated plasma (using trypsin as the indicator enzyme), approximately the same sensitivity and detection

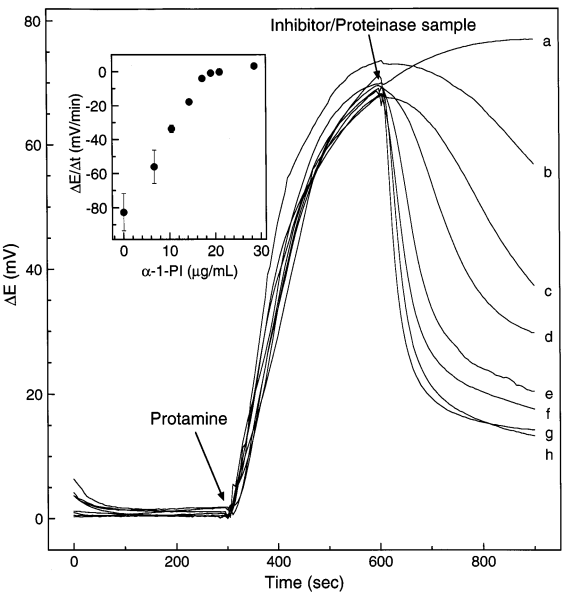

Fig. 7. Potentiometric assay for α_1-antiproteinase inhibitor. Shown is the electrode response of polycation PSE (average of five) in 50 mM Tris–HCl, pH 7.4, 120 mM NaCl containing 25 μg ml^{-1} protamine and its reversal after the addition of 0.50 ml aliquots of solutions containing 50 U ml^{-1} trypsin and different concentrations of α_1-antiproteinase inhibitor: (a) 28.6; (b) 21.0; (c) 19.1; (d) 17.1; (e) 14.3; (f) 10.5; (g) 7.0 and (h) 0.0 μg ml^{-1}. Inset: shown is the rate of potential decrease ($-\Delta E/\Delta t$) immediately after addition of the trypsin/α_1-antiproteinase inhibitor mixture versus the concentration of the inhibitor (adapted from [20] with permission).

limits were achieved as in an assay performed in buffered samples. Recovery measurements of aprotinin in spiked treated plasma yielded recovery rates of 97–105% with a mean standard deviation of 2.8% for blood samples containing 0.19 to 0.48 μg ml^{-1} aprotinin.

The use of the potentiometric protease and inhibitor assays for routine clinical analysis may have significant practical utility because they are less time-consuming and simpler than the current methods for the determination of protease inhibitors; e.g. HPLC, chromogenic and immunological assays [21–23]. In addition, there is the potential to implement such assays using undiluted whole blood samples, and this would allow the measurement of such activities/inhibitor drugs at or near the patient's bedside using appropriately designed portable instrumentation.

3.3. Non-separation immunoassays using polyion-sensitive membrane electrode detection

In addition to employing PSEs to monitor enzyme and enzyme inhibitor activities, these electrodes may also have applications as detectors in novel non-separation competitive binding immunoassay designs for monitoring levels of small organic molecules (e.g. therapeutic drugs, illicit drugs, etc.) in undiluted blood samples. Competitive immunoassay formats favor detectors that exhibit good sensitivity toward the labeled reagents (labeled form of analyte), since immunoassay detection limits generally decrease as the amount of required reagents decreases. Because PSEs can detect certain polyions at levels as low as 10^{-8} M in physiological samples, the use of these electrodes in rapid immunoassays is an intriguing concept. There are two non-separation competitive binding assay formats using PSE detection that are currently under investigation: homogeneous binding assays and enzyme multiplied immunoassays.

Fig. 8 schematically illustrates how polyion detection can be employed to detect hapten type molecules without discrete separation or washing steps by using polyions as labels. Since PSEs respond essentially only to free polyions in solution, this assay scheme involves use of a polyion labeled-analyte reagent that is capable of being bound by antibodies/binding proteins directed toward the analyte that are either free in solution or immobilized on a solid support. If the antibodies are immobilized on a support, such as agarose beads, polyion-labeled analyte molecules will bind to this surface. The amount of free polyion labeled-analyte in solution will depend inversely on the concentration of free analyte molecules added to the sample solution. Free analyte molecules will then compete with the polycation-labeled analyte for a limited amount of immobilized antibodies and therefore, a larger EMF change will be observed in the presence of increasing concentrations of analyte.

To test the feasibility of this assay concept, the biotin-avidin binding pair has been chosen as a model system, with polylysine-labeled biotin as the signal generator. The biotin–avidin system was selected due to the extremely high binding affinity of avidin for biotin, while polylysine (MW = 9600) was chosen as the label due to the significant response of a DNNS-based PSE toward this polycationic species and the ease in which conjugation with *N*-hydroxysuccinimide biotin can be carried out with this structure (reaction with primary amine groups). To demonstrate the assay principle, polylysine was first biotinylated using a 5:1 molar conjugation ratio of biotin to polylysine by reaction with the *N*-hydroxysuccinimide derivative of biotin. The resulting biotinylated polylysine yields almost an identical EMF response as unmodified polylysine ($+65$ mV at 10 μg ml^{-1} biotinylated polylysine in Tris buffer, pH 7.4, with 12 mM NaCl).

Using immobilized avidin on agarose beads (0.5 units), it is possible to achieve an ED_{50} value of 3×10^{-7} M biotin (1 unit binds 1 μg biotin) using 4 μg of polylysine labeled biotin in the assay mixture (Fig. 9). It is important to note that the detection limit of this novel binding assay is highly dependent on the lower limits of detection exhibited

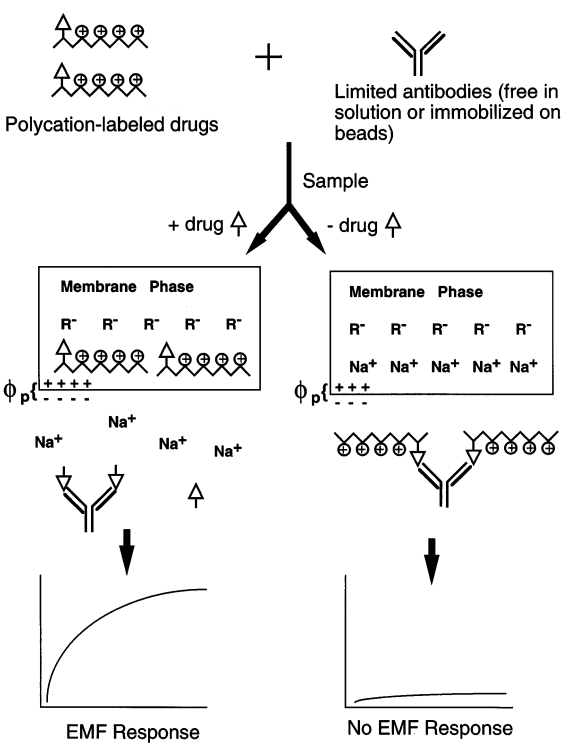

Fig. 8. Schematic representation of homogeneous binding/immunoassay system using polycations as labels.

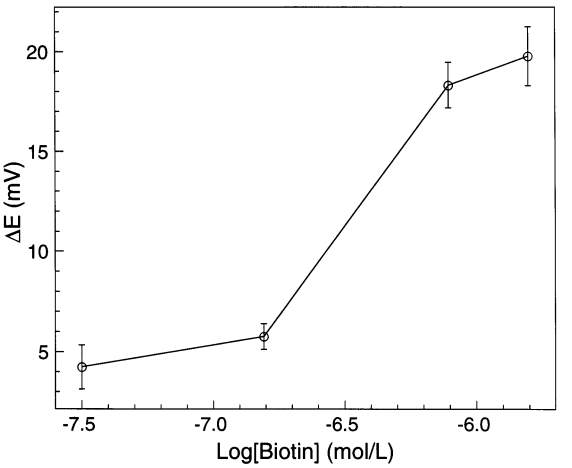

Fig. 9. Biotin dose response curve for binding assay using biotin conjugated polylysine and immobilized avidin on agarose in 5 mM Tris–HCl buffer, pH 7.4, 12 mM NaCl as monitored with DNNS-based polycation PSE (data points are average of three experiments).

by the PSE toward the polyion label. Therefore, using synthetic peptides with higher lipophilicities or charge densities (compared to polylysine), as discussed previously, may aid in obtaining significantly lower detection limits. Ongoing studies are now focused on using such synthetic peptides as labels to devise a competitive binding assay for the bronchial dilator theophylline using soluble anti-theophylline antibodies.

Another route to devising homogeneous immunoassays using PSE detection involves adapting the well know EMIT technology (enzyme multiplied immunoassay technique) to this type of electrochemical detection scheme. In the area of separation-free immunoassays, the EMIT system, pioneered by Ullman et al. [24], has been one of the most successful methodologies. This method is based on the homogeneous inhibition of the catalytic activity of an enzyme–analyte conjugate by selective antibodies. As demonstrated above (Section 3.2), DNNS-based PSEs can be used effectively to monitor enzyme activities with various polycationic polypeptides serving as enzyme substrates. Thus, as shown schematically in Fig. 10, it should be possible to devise a PSE-based EMIT type assay by using a selective protease as a label (e.g. trypsin). If an enzyme–analyte conjugate can be prepared without significantly affecting the enzymatic activity of the protease, this conjugate can then serve as the signal generating reagent in the proposed competitive binding assay. In the presence of an appropriate anti-analyte antibody or binding protein in solution, binding of the conjugate should yield a significant decrease in the catalytic activity of the protease label (as measured by monitoring the rate of polyion consumption with the PSE). In the presence of increasing amounts of free analyte, these molecules will compete with the conjugate for antibody binding sites, and the activity of the enzyme–analyte conjugate will increase in an amount directly proportional to the number of analyte molecules present in the sample solution.

Preliminary results for such an assay scheme using the biotin–avidin system again as a model have shown that biotinylation of trypsin does not affect its catalytic activity toward protamine. However, as shown in Fig. 11(A), in the presence of 5 μg ml^{-1} of avidin, the trypsin-labeled biotin

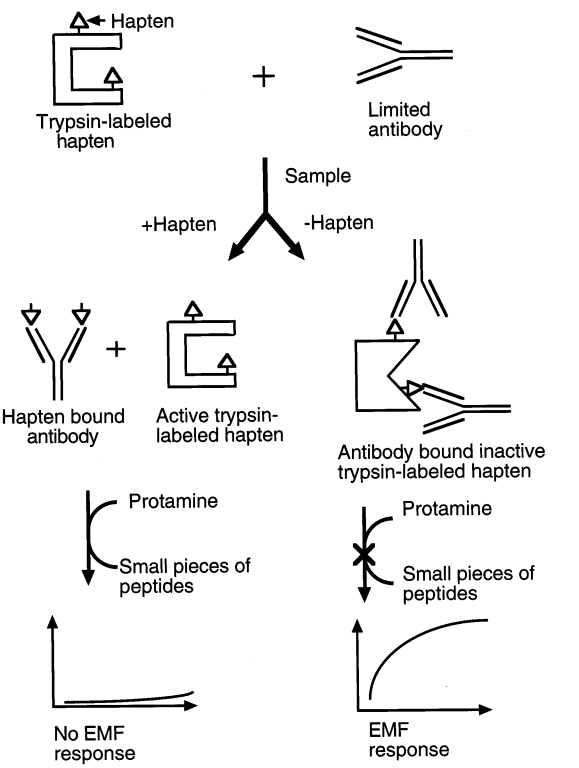

Fig. 10. Schematic representation of homogeneous enzyme multiplied immunoassay using polycation sensitive electrode detection of enzyme activity.

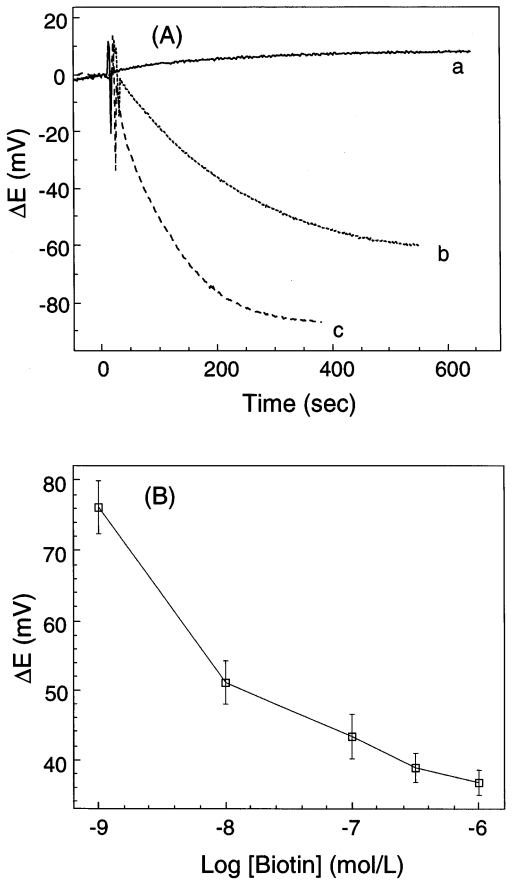

Fig. 11. (A) Trypsin assay monitored by polycation sensitive electrode: (a) 1 μg ml^{-1} trypsin-labeled biotin incubated with 5 μg ml^{-1} avidin; (b) 1 μg ml^{-1} unlabeled trypsin incubated with 5 μg ml^{-1} avidin; and (c) 1 μg ml^{-1} biotin labeled trypsin only. (B) Dose-response toward biotin using new homogeneous EIA method based on PSE detection. EMF values are changes from baseline signal after addition of protamine (at 2 μg ml^{-1}) into assay mixture after pre-set incubation period. Avidin: 2 μg ml^{-1}; trypsin-labeled biotin: 1 μg ml^{-1}; protamine: 2 μg ml^{-1}; incubation time: 13 min; membrane composition: 1 wt.% DNNS, 49.5 wt.% PVC, 49.5 wt.% NPOE (data points are average of three measurements).

is almost completely inhibited. This inhibition is reversed in the presence of free biotin, with an ED_{50} value of 1.0×10^{-8} M biotin when using potentiometric detection of trypsin activity (with 2 μg ml^{-1} protamine in the assay mixture; Fig. 11B). Such an assay method is currently being developed for the measurement of theophylline in blood using theophylline-8-butyric acid lactam conjugated to trypsin and anti-theophylline antibodies as the binding reagent. Through the use of PSE detection, it is envisioned that such an EMIT type of assay will enable detection of the drug in whole blood samples with little or no dilution of the sample.

4. Conclusions

As summarized above, PSEs are a completely new class of electrochemical devices that function via a unique non-equilibrium potentiometric response mechanism. This mechanism, however, limits the re-use of such sensors and mandates employment of single-use disposable devices for routine measurements (such as the sensor illustrated in Fig. 2). It has been shown that one highly successful biomedical application of this technology lies in the area of quantitating heparin levels in whole blood samples. This is most conveniently carried out via a simple potentiometric titration of the heparinized blood sample with protamine and monitoring the appearance of free protamine at the titration endpoint via a DNNS-based protamine sensitive membrane electrode. It has also been shown that the PSE technology may be advantageously employed to monitor specific enzyme activities that cleave larger polyionic substrates into smaller fragments, including specific proteases, phosphatases, RNase, etc. The ability to use synthetic polyion peptides to create 'designer' substrates that have desired cleavage sites to detect the activity of a given protease in the presence of others represents an exciting future direction for this technology. Further, the capability of PSEs to detect sub-μM levels of polyions in samples as complex as whole blood holds promise that these devices may also have a future role in the development of novel non-separation immuno/binding assay arrangements, in which protease enzymes and/or synthetic polyionic species will serve as labels. The preliminary results reported here for these two approaches (using biotin/avidin system as a model system) are encouraging and point to the likelihood of success in the future. Finally, it should be noted that the principles of polyion detection used here can also

be employed to design thin polymeric films that respond optically to heparin, protamine, and other polyions [25,26] through the addition of a lipophilic proton chromoionophore into the polymeric films. It is envisioned that such optical polyion sensor designs will also find use in heparin measurements, enzymatic assays, and immunoassays, especially when employed in a reflectance meter-type spectrophotometric measurement configuration.

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