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Solid-Contact Electrochemical Polyion Sensors for Monitoring Peptidase Activities

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We report here on improved solid-contact electrochemical polyion sensors for the detection of polyion protamine. The polymeric membrane sensors were fabricated with a conducting polymer as an ion−electron transduction layer. We observed that decreasing the magnitude of the applied current pulse caused a significant improvement of the sensor sensitivity to low protamine levels. The protamine sensors exhibited a stable and reversible response to protamine concentrations ranging from 0.05 to 30 mg L^{-1}. The sensors were used for monitoring peptidase activities utilizing galvanostatically controlled solid-contact membrane sensors. The polyion protamine was used as a substrate to detect the activity of the protease trypsin. The enzyme activity was continuously monitored by measuring the protamine concentration as it is cleaved by enzyme into smaller fragments to which the sensor is less sensitive. In the presence of a given level of protamine the initial rate of reaction can be linearly related to the trypsin activity within a 0−5 U mL^{-1} concentration range. The interference with the enzymatic reaction product arginine was specifically examined.

In the past decade the possibility of detecting polyionic macromolecules such as heparin and protamine created novel and exciting applications of ion-selective electrodes (ISEs).[1−3] Due to their response mechanism, the polyion-selective electrodes are not typically sensitive to small fragments of polyionic macromolecules. Thus, as an enzyme cleaves a polyionic molecule, these sensors can be used to monitor enzyme activity.

Electrochemical assays for monitoring peptidase activities have specific advantages over more traditional spectrophotometric and fluorescence techniques such as the ability to measure peptidase activities in turbid solutions, for example, whole blood, rapid and relatively simple test procedures, and improved sensitivity and selectivity.[4,5] A number of electrochemical assays based on polyion-selective electrodes have been developed for detecting specific peptidase activities and successfully tested.[3−7]

The main disadvantage of polyion-selective potentiometric electrodes lies in the intrinsic irreversibility of the underlying response mechanism. In the presence of detectable polyions in solution a strong potential drift is normally observed due to the instability of the ion concentration gradients.[8] After prolonged exposure to polyions in the sample, the polyions eventually displace the counterions in the membrane phase, and consequently, the sensor loses its response. Extracted polyions may be removed from the membrane phase, but the suggested methods require either prolonged reconditioning of the sensor in the stripping solution after each measurement[9] or disposal of the electrodes after a single measurement.[6]

A new detection technique was proposed utilizing electrochemically controlled, reversible ion extraction into polymeric membranes in an alternating galvanostatic/potentiostatic mode.[10] The solvent polymeric membrane of this novel class of sensors contained a highly lipophilic electrolyte and, therefore, did not possess ion-exchange properties in contrast to potentiometric polyion electrodes. Instead, the process of ion extraction was induced electrochemically by applying a constant current pulse. Polyion-selective sensors based on this principle were successfully employed for reversible, instrumentally controlled protamine detection.[11]

Recently we reported on the fabrication of solid-state pulsed galvanostatic sensors with solvent poly(vinyl chloride) (PVC) membranes in which the inner filling solution was replaced by a conducting polymer.[12] Conducting polymers were used to form a transduction layer between the membrane and electron-conductive substrate. Solid-contact membrane sensors with a conducting polymer (CP) ion−electron transduction layer represent a promising alternative to liquid-contact polyion sensors[10] in terms of both function and manufacturing. We developed and successfully tested a solid-state reversible membrane sensor based on the conducting polymer substrate poly(3,4-ethylendioxythiophene) doped with poly(styrene sulfonate) (PEDOT:PSS).

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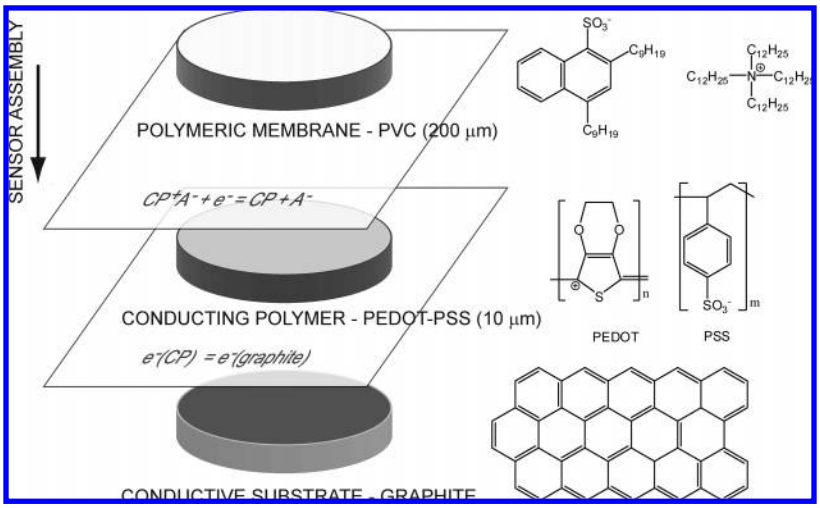

Figure 1. Fabrication process of protamine-selective membrane sensors. The electrochemical reactions at the interfaces are depicted on the planes between sensor structural layers. The structures of the sensor components in the membrane and transduction layer (TDDA−DNNS salt, PEDOT−PSS, graphite) are shown on the right.

Herein we describe the clinical utility of an improved method for the detection of specific peptidase activities using the solid-contact galvanostatic sensors.

Trypsin, a digestive enzyme secreted by the pancreas, is a prominent member in a family of digestive enzymes[13] and serves as the common activator of all pancreatic zymogens.[14] Elevated levels of trypsin are the most accurate indicators of acute alcoholic pancreatitis, but appropriate testing methods are not widely available.[15,16] Trypsin is a very specific peptidase and will only cleave the carboxyl side of arginine and lysine residues in polypeptide chains.[14]

The polycation protamine is rich with arginine and lysine residues that make it a suitable substrate for protease-sensitive electrochemical assays. Therefore, protamine represents a highly specific substrate for electrochemical assays of trypsin activity. Moreover, protamine is commonly administered after surgical procedures in order to counter the affects of the anticoagulant heparin. This sensor can be used as an end-point detector for the detection of the anticoagulant heparin via titration with protamine.[3]

EXPERIMENTAL SECTION

Reagents. High molecular weight PVC, 2-nitrophenyl octyl ether, tetradodecylammonium chloride (TDDA), tetrahydrofuran (THF), dinonylnaphthalenesulfonic acid (DNNS) as a 50% solution in heptane, poly(3,4-ethylenedioxythiophene) (PEDOT) doped with poly(styrene sulfonate), protamine sulfate (from herring), trypsin (from porcine pancreas), and L-arginine (from a nonanimal source) were purchased from Sigma-Aldrich (Milwaukee, WI). Tris-base was purchased from VWR (West Chester, PA). All aqueous solutions were prepared by dissolving the appropriate reagents in deionized water (18.2 MΩ cm).

Sensor Fabrication. The schematic representation of the sensor structure is shown in Figure 1. The conductive substrate was a graphite rod (6 mm in diameter, 99.9% spectroscopic grade), which was installed into the electrode housing (12 mm in diameter) fabricated from a PVC rod. The graphite surface was polished with 3 μm polishing alumina, washed with acetone, and then air-dried.

A transduction layer of PEDOT:PSS was deposited on the graphite substrate by drop casting 25 μL of PEDOT:PSS aqueous emulsion (1.8 wt % of PEDOT:PSS blend with a PEDOT:PSS mass ratio of 1:2.5) and letting it air-dry. After drying the conducting polymer films were conditioned in a 0.1 M NaCl solution for 24 h. Assuming the average density of PEDOT:PSS[17] of 1.5 g cm^{-1}, the calculated thickness of the polymeric film was 10 ($\pm$2) μm.

The lipophilic salt DNNS−TDDA was prepared according to the method outlined by Shvarev and Bakker.[11] Protamine-selective membranes contained PVC and 2-nitrophenyl octyl ether (1:2 by weight) in the membrane matrix as well as 45 mmol kg^{-1} of DNNS−TDDA (10% by weight).

The membranes were prepared via solvent casting using THF as a solvent. The ion-selective membranes were cut with a cork bore (6.6 mm in diameter) from the parent membrane, soaked in THF for a few seconds, pasted on top of the deposited conducting polymer layer, and left to air-dry for 24 h. The electrodes were conditioned overnight in 0.1 M NaCl.

Chronopotentiometric Measurements. Normal pulse chronopotentiometry was used to collect all data and is described elsewhere.[11] A conventional three-electrode cell was used for chronopotentiometric measurements. The sensor electrode was connected as a working electrode connected to a potentiostat/galvanostat. The reference electrode was a double-junction Ag/AgCl electrode with saturated KCl as the inner solution and 1 M LiOAc as a bridge electrolyte. A coiled platinum wire with a surface area of 0.1 cm^2 was used as a counter electrode.

A modified[18] AFCBP1 bipotentiostat (Pine Instruments, Grove City, MA) controlled by a PCI-6221 data acquisition board and LabView 7.1 software (National Instruments, Austin, TX) on a PC

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was used for measurements. The instrument was modified in order to switch rapidly between potentiostatic and galvanostatic modes.

A constant current pulse with fixed magnitude and duration of 1 s was applied to the sensor, and the potentials were recorded as a function of time during the pulse. Potential measurements were performed with the sampling rate of 1 kS s^{-1} and were averaged over 100 ms time intervals at the end of the current pulse.

An uptake current pulse was followed by application of the stripping potential. Duration of the stripping cycle was usually 10−15 times longer than the corresponding current pulse. According to the procedure described in the previous work,[11] the value of the stripping potential was adjusted to match the open-circuit potential of the sensor in order to ensure effective expelling of extracted ions.

The open-circuit potentials were recorded prior to the experiment via a custom-built potentiometric station, which included several electrometric amplifiers (AD820, Analog Devices) connected to the 24-bit 8-channel data acquisition board NI-4351 controlled by the LabView software (National Instruments, Austin, TX).

The buffering solution used in all experiments was 50 mmol L^{-1} Tris−HCl at pH 7.4 containing 100 mmol L^{-1} NaCl. All measurements were conducted in well-stirred solutions at ambient temperatures (21 ± 2 °C). Unless otherwise noted, sensors were allowed to equilibrate within the sample for 2 min before each measurement.

RESULTS AND DISCUSSION

Protamine Sensor Response. In contrast to classical ISEs, which work under thermodynamic equilibrium at zero current, pulsed galvanostatic sensors[10−12,19] operate under periodic galvanostatic polarization. The measurement cycle of the protamine-selective sensor begins with a pulse of cathodic current i that induces a net flux J of cations in direction of the membrane phase.

Assuming for clarity that only sodium and protamine ions may be extracted into the membrane phase, the resulting electric current i is a sum of the fluxes of sodium, J_{Na}, and protamine, J_{PA}:

$$i = FS(J_{Na} + z_{PA}J_{PA}) \quad (1)$$

where F is the Faraday's constant, A is the membrane area, and z_{PA} is the charge of protamine (+20). Equations for the sensor response function can be derived on the basis of a simplified steady-state diffusion model.[11]

In the absence of protamine, the phase boundary potential at the sample/membrane interface is a function of sodium activity and magnitude and duration of the applied current:[11]

$$E = E^0 + \frac{RT}{F}\ln a_{Na} + \frac{RT}{F}\ln\left[\frac{F^2A^2D_m}{4i^2t}\right] + ir \quad (2)$$

where R, T, and E^0 are the universal gas constant, temperature, and standard cell potential, respectively, D_m is the diffusion coefficient in the membrane phase, r is the resistance of the membrane phase, a_{Na} is the activity of sodium ions in the sample, and t is the current pulse duration. If the magnitude and duration of the applied current are constant, the third and fourth terms in eq 2 are constant and the equation can be reduced to the Nernst equation. The latter is true for short current pulses for which no change in the bulk resistance of the membrane is anticipated.

If protamine is present in the sample it will compete with sodium in the extraction process (eq 1). Assuming that the applied current imposes a flux that is always larger than the flux that can be sustained by polycation diffusion alone and that migration is negligibly small, the sensor response function can be written as follows:[11]

$$E = E^0 + \frac{RT}{F}\ln a_{Na} - \ln\frac{RT}{F}\ln\left[\frac{\delta_m}{D_{m,Na}}\left(-\frac{i}{FA} - z_{PA}\frac{D_{aq,PA}}{\delta_{aq}}c_{PA,bulk}\right)\right] \quad (3)$$

$D_{aq,PA}$ is the diffusion coefficient of protamine in the aqueous phase, $D_{m,Na}$ is the diffusion coefficient of sodium ions in the membrane phase, and δ_m and δ_{aq} are the resulting diffusion layer thicknesses, respectively. If the activity of sodium ions is constant for a current pulse of fixed duration and magnitude, the phase boundary potential is a function of protamine concentration in the sample.

A baseline potential pulse followed each current pulse in order strip extracted ions from the membrane phase. Extracted ions leave the membrane in order to satisfy phase boundary conditions. This process regenerates the membrane before the next measurement cycle. Therefore, under identical conditions the concentration gradients and phase boundary activities of ions are reproducible from pulse to pulse.

It was shown that the duration of the stripping period has to be at least 10 times longer than the current pulse.[20] Moreover, the value of the baseline (stripping) potential must be equal the equilibrium open-circuit potential of the membrane electrode.[11] This open-circuit potential can be measured prior to the experiment with respect to the reference electrode.

Under galvanostatic control the periodic polarization of the membrane causes an analogous polarization process at the inner membrane interface. If the inner interface is formed by the conducting polymer, the periodic ion-extraction process is accompanied by appropriate periodic oxidation/reduction of the conducting polymer (Figure 1). Conducting polymers demonstrate a well-defined pathway of ion-to-electron transduction in combination with substantial redox capacitance, which contributes to the stability and reproducibility of the phase boundary potential.

In the case of a PEDOT-like conducting polymer, we may expect that the uptake of cations R^+ from the solution takes place, causing the concentration polarization of the lipophilic electrolyte R^+R^- in the membrane phase.[12] The ratio of activities of the R^+ cations at the inner membrane interface dictates the phase boundary potential.

Protamine Sensor Calibration. Accurate monitoring of substrate concentrations in an electrochemical assay requires a calibration procedure. The automation of an electrochemical assay based on polyion-selective electrode requires an appropriate fitting of the calibration plot using a well-defined response function.

A classical ISE in the absence of interfering ions has a response function that obeys the well-known Nernst equation, which

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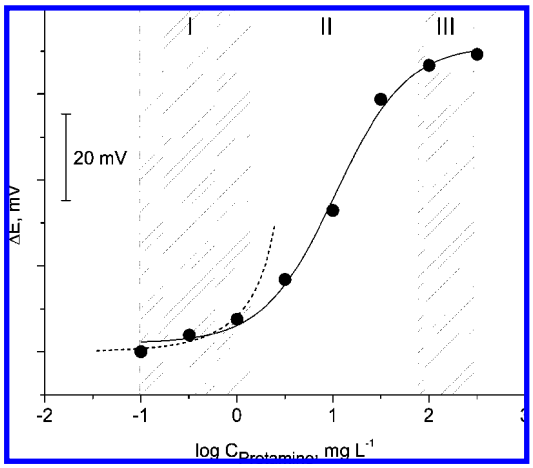

Figure 2. Typical protamine sensor response (scattered points) in a solution of 0.1 M NaCl and 50 mmol Tris−HCl with the pH of 7.40. The current pulse density is 18 μA/cm^2, and the pulse duration is 1 s. The dashed line represents a theoretical response curve computed according eq 3. The solid line is the sensor response that was fit according to the Boltzmann function.

predicts a linear relationship between electrode potential and the logarithm of activity of the primary ions in the sample. In the presence of a constant interfering background, the Nikolski−Eisenmann equation with appropriate selectivity coefficients can be employed. Better formalism developed by Nagele et al.[21] yields more accurate results if primary and interfering ions have different charges. Thus, plotting potential versus activity in semilogarithmic scale ideally yields a straight line, and a simple least-squares linear fit can be applied.

In contrast to ion-selective sensors, both potentiometric and galvanostatically controlled polyion-selective sensors exhibit nonlinear response. The typical experimental response of a solid-contact protamine sensor is shown in Figure 2 (scattered points). Similar to its potentiometric counterpart,[8] a typical pulsed galvanostatic protamine sensor has an S-shaped or sigmoidal response curve in a semilogarithmic scale.

It seems logical to apply eq 3 in order to predict the protamine sensor response. The following parameters were used: $D_{aq,PA} = 1 \times 10^{-10}$ m^2 s^{-1}, $D_{m,Na\,PA} = 1 \times 10^{-12}$ m^2 s^{-1}, $\delta_{m,Na}$ and $\delta_{aq,PA}$ equal to 1.4×10^{-6} m and 6×10^{-7} m, respectively, protamine charge was assumed of +20, membrane area of 25 mm^2, and applied current of -5 μA. The standard cell potential was of 0.1 V. The diffusion layer thicknesses were calculated according the simplified steady-state diffusion model.[11]

The theoretical response function of the sensor is shown in Figure 2 as a dotted line. A reasonably good agreement with the experimental data was achieved only if the protamine concentration did not exceeded 1 mg L^{-1}. As the protamine concentration increases the deviation of the theoretical and experimental response function increased drastically. Interestingly, in the beginning of the calibration curve at very low protamine concentrations, eq 3 is apparently valid and the potential versus concentration function is practically linear. The same deviation of theoretical and experimental responses was observed for the potentiometric polyion sensors.[8] Obviously, eq 3 cannot be used for fitting purposes.

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Detailed investigation of the steady-state diffusion model employed here allows one to reveal an intrinsic limitation of the model, which is disguised in eq 2. The initial assumption was that the phase boundary potential is governed by the ratio of activities of sodium ions in the sample and membrane phases. Apparently, this assumption is valid at low protamine concentrations (Figure 2, area I). The second assumption was that the flux, which was imposed by the applied current, is always larger than the flux that can be sustained by polycation diffusion alone. However, as the protamine concentration increases the protamine depletion at the interface is insufficient to reduce the concentration at the interface to zero.

Therefore, we may expect the following: first, at lower protamine concentrations, protamine ions are present at both sides of the interface during the uptake current pulse. At intermediate protamine concentrations, the phase boundary potential is dictated not only by sodium ions but by protamine ions as well (Figure 2, area II). As we approach the higher detection limit, it is apparent that the protamine completely displaces sodium ions in the membrane during the uptake pulse and the resulting phase boundary potential is dictated by protamine ions alone. In complete analogy with eq 2 we can write:

$$E = E^0 + \frac{RT}{z_{PA}F}\ln C_{PA} + \frac{RT}{z_{PA}F}\ln\left[\frac{F^2A^2D_m}{4i^2t}\right] + ir \qquad (4)$$

The high charge of protamine ions makes the slope of the response function ($RT/z_{PA}F$) negligibly small (<3 mV), and the resulting potential is practically constant regardless of protamine concentration (Figure 2, area III). Thus, the potential value corresponds to the right horizontal asymptote.

In other words, polyion sensor response mimics the so-called logistic function or a logistic curve, which models the growth of a population in which members compete for the same critical resource. The initial stage of growth is approximately exponential. As saturation begins, the growth slows, and eventually stops.

The sigmoidal protamine calibration curves were fit to a Boltzmann function, which is a member of a logistic functions family and can be represented by the following equation:

$$y(x) = \frac{A_1 - A_2}{1 + \exp[(x - x_0)/dx]} + A_2 \qquad (5)$$

in which y and x are the ΔE and log $C_{Protamine}$, respectively. The function parameters are the following: A_1 is the initial value (left horizontal asymptote), A_2 is the final value (right horizontal asymptote), x_0 represents the center (point of inflection), and dx is a width (the change in x corresponding to the most significant change in y values). In principle, the calibration curve can be plotted with linear concentration readings on the x-axis instead of logarithmic units. In this case a slightly different logistic function can be used for a nonlinear curve fit.

The resulting fit to the Boltzmann function is plotted as a solid line in Figure 2. The nonlinear fit showed very good agreement with the experimental sensor response. The calculated correlation coefficient was 0.999, and relative standard deviation of potentials did not exceed 1%. It is noteworthy to report that the parameters

A_1 and A_2 have clear physical meaning. The parameter A_1 (left horizontal asymptote) is the phase boundary potential in the absence of protamine calculated according eq 2. Analogously, A_2 (right horizontal asymptote) directly corresponds to the potential at high concentrations of protamine given by eq 4.

The development of a theoretical model that describes the entire response function of a polyion-selective sensor represents an interesting and challenging problem. On one hand, the apparent simplicity of the Boltzmann function is, perhaps, an indication that the resulting response equation might be very simple. On the other hand, in order to develop appropriate formalism one must consider a formation of mixed phase boundary potential dictated by the activity ratio of the protamine and sodium ions.

Optimization of a Solid-State Protamine Sensor. The validity of a simple steady-state diffusion model is apparently limited to low polyion concentrations (Figure 2). Nonetheless, this fact allows one to model important parameters such as the magnitude and duration of the applied current pulse, which apparently influence the selectivity and low detection limit of protamine sensor.

Considering a membrane that is in contact with the aqueous sample, we may expect that, with increasing magnitude and duration of the applied current, polarization in the aqueous phase takes place. As demonstrated earlier, with respect to sodium ion activity a near-Nernstian response slope (eq 2) is expected as long as concentration polarizations at the sample side are negligible. At low concentrations, however, a significant concentration polarization is observed forming an apparent super-Nernstian response.[18] The equation that predicts the critical bulk concentration of the extracted ion was proposed on the basis of a simple steady-state diffusion model. The equation shows that such a polarization is dependent on the magnitude and duration of the applied current pulse.

We can rearrange this equation in order to calculate the limiting diffusion current for a given polyion concentration as follows:

$$i = -\frac{z_{PA}FS}{2C_{PA}}\sqrt{\frac{D_{aq,PA}}{t}} \qquad (6)$$

With respect to polyion-selective galvanostatic sensors, application of this equation allows us to calculate a limiting diffusion current for a given bulk concentration of a polyion at which the concentration in the aqueous phase at the interface is equal to zero and complete depletion is observed.

In other words, if the limiting current is achieved, any further current increase will not affect the amount of polyions extracted into the membrane and, therefore, leads to reducing the ratio of protamine/sodium ions in the membrane phase, thus, increasing the corresponding low detection limit.

Assuming a protamine charge of +20, a pulse duration of 1 s, a protamine diffusion coefficient in water of 10^{-6} cm^2 s^{-1}, and the membrane area of 0.25 cm^2, we calculated the limiting currents for different heparin concentrations. The results are shown in Table 1.

If the applied current is higher than the limiting value, an uptake of sodium ions takes place which is a necessary condition in the formation of the sensor response. However, as seen in the Table 1, if the current is set at 10 μA and the protamine concentration is 0.1 μM L^{-1} (0.5 mg L^{-1}), for example, the fraction of protamine ions in the total amount of ions extracted is less than 10%. The resulting potential change calculated according eq 3 is less than 5 mV. Indeed, eq 6, a simple linear relationship, predicts that we need to reduce the current 10-fold in order to improve the low detection limit by an order of magnitude.

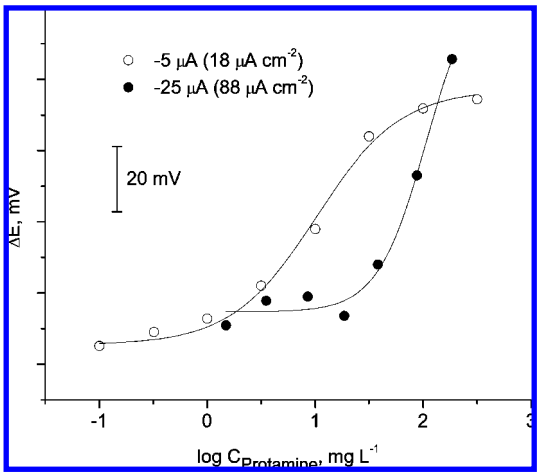

Figure 3. Protamine sensor response at different cathodic currents and corresponding current densities. The negative sign of the cathodic current is omitted. The sample solution contains 0.1 M NaCl and 50 mmol Tris−HCl with the pH of 7.40.

Table 1. Limiting Diffusion Current for Different Concentration of Protamine Calculated According Eq 6

protamine concn, μmol L^{-1}/mg L^{-1}	limiting diffusion current, μA/cm^2
10/50	243
1.0/5.0	24.3
0.1/0.5	2.5

Two different current densities were used to calibrate solid-state protamine sensors. The results are shown in Figure 3. The low detection limit of 40 mg L^{-1} at a current of −25 μA and the corresponding current density of −88 μA cm^{-2} is in good agreement with the low detection limit calculated according the model (Table 1). Interestingly, reducing the current density resulted in improvement of the low detection limit, which was reported earlier.[12] The lowest detection limit at a current of −5 μA and the corresponding current density of −18 μA cm^{-2} was 0.05 mg L^{-1}, which is also with a relatively good agreement with the model prediction.

The improvement of the low detection limit of the protamine sensor allows one to use the protamine sensor as a more sensitive end-point indicator in the heparin−protamine titration. The published results[10,11] concerning the detection of the anticoagulant heparin via heparin−protamine titration suggest this low detection limit of 0.05 mg L^{-1} is sufficient to achieve heparin sensitivity better than 0.02 U mL^{-1}. Considering that the target therapeutic heparin concentrations are 0.2−0.4 U mL^{-1}, this detection limit in undiluted whole blood is sufficient for very accurate heparin monitoring via electrochemical sensors.

The recorded calibration curve and time traces for the improved protamine sensor are presented in Figure 4. The sensor exhibited a stable and reversible response to protamine within

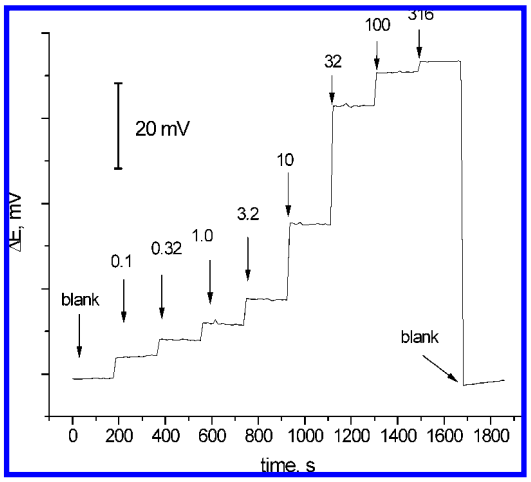

Figure 4. Resulting calibration curve of the sensor. The numbers above the curve represent protamine concentration in mg L^{-1}. The current pulse density is 18 μA/cm^2. The electrode was renewed in the potentiostatic mode at a stripping potential of 44 mV for 10 s before the next current pulse.

the concentration range of 0.1−316 mg L^{-1} in a solution of 0.1 M NaCl and 50 mmol Tris−HCl with the pH of 7.40. The observed potential drift did not exceed 0.5 mV min^{-1} (Figure 4). The sensor response function was acceptably reproducible (within ±2 mV) after the sensor was stored in 0.1 M NaCl solution for 14 days.

Electrochemical Assay for Monitoring Peptidase Activities. The remarkable sensitivity and selectivity of protamine-selective sensors allowed us to utilize these devices when designing an electrochemical assay for the monitoring of peptidase activities.[5] As peptidase trypsin selectively cleaves the arginine bonds in protamine, we monitored the decrease of protamine in the solution and, thus, related the decrease in protamine to the activity of trypsin.

The experiments determining trypsin's activity were performed by adding an aliquot of protamine to a solution of 0.1 M NaCl and 50 mmol Tris−HCl with the pH of 7.40. The enzyme activity plots were constructed by plotting the change in potential (ΔE) when compared to the initial background potential reading versus time. The resulting potentials as a function of time are shown in Figure 5A.

The sensor response was recorded in a blank saline solution, followed by addition of an aliquot of protamine resulting in a final protamine concentration of 10 mg L^{-1}. The stable and reproducible potential change was observed. Then, after approximately 200 s, varying amounts of trypsin were added to the solution containing protamine. The resultant decrease in the sensor response corresponded to the enzymatic digestion monitored over time. It can be seen that increasing concentration of enzyme caused a faster reversal of the sensor response.

In contrast to previous work,[6] the experiment shown in Figure 5A was performed with a single sensor without any reconditioning procedure. During this experiment shown in Figure 5, a total sequence of ca. 250 current pulses was applied to the membrane for 1 h. Low drift and excellent reversibility of the galvanostatically controlled sensor allowed us to use the protamine probe for the continuous monitoring of enzyme activity.

In order to calculate the concentration of protamine during the digest, the sensor calibration curve shown in Figure 4 was fit

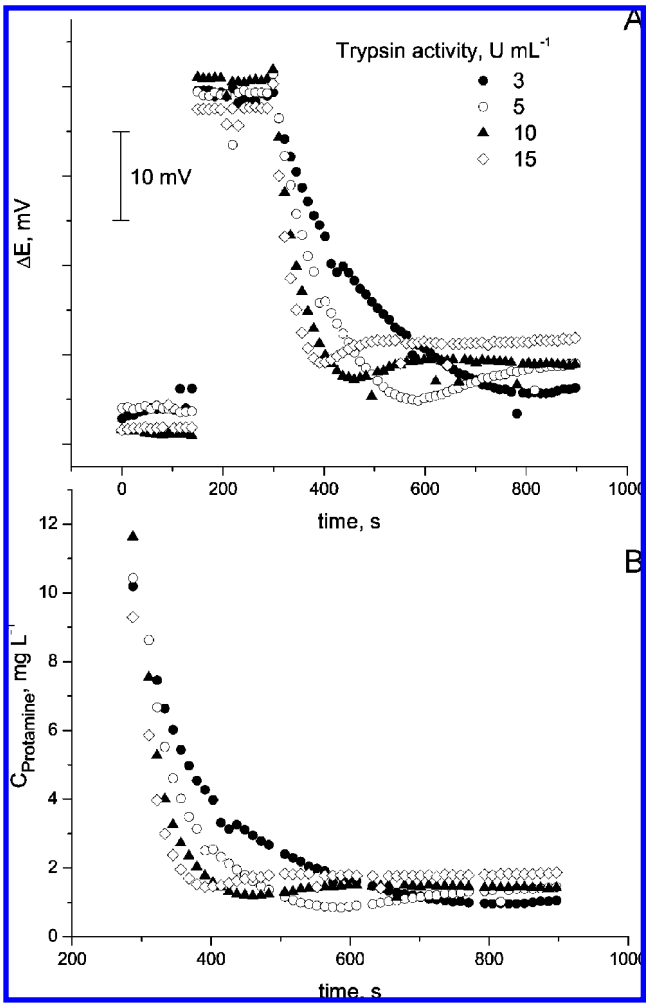

Figure 5. (A) Monitoring trypsin activity via recording the potential of the protamine sensor in a solution containing an initial protamine concentration of 10 mg L^{-1} by varying concentrations of trypsin. (B) The actual protamine concentration monitored after trypsin addition as a function of time calculated using the Boltzmann function fit.

to the Boltzmann with the following parameters: $A_1 = 7.2$, $A_2 = 76.6$, $x_0 = 1.02$, and $dx = 0.37$. The actual protamine concentration was calculated according to the rearranged eq 5 for each potential reading. The rate of protamine concentration decrease is shown in Figure 5B immediately after addition of trypsin.

The initial rate of trypsin activity, as measured by the rate of protamine concentration decrease, is shown in Figure 6. At a substrate concentration of 10 mg L^{-1}, a linear response region corresponded to the trypsin activity from 0 to 5 U mL^{-1}. The calculated confidence interval computed at the 95% level was ±0.4 U mL^{-1}. Higher enzyme activity leveled the assay response. In principle, the assay detection range can be increased up to 100 U mL^{-1} by increasing the substrate concentration and, thus, sacrificing the sensitivity, as demonstrated using protamine ISEs.[6]

Interestingly, at the end of the curve shown in Figure 5A an apparent reversal of the sensor response was observed corresponding to a small increase of protamine concentration (Figure 5B). Higher activities of enzyme made this effect more pronounced.

The trypsin-catalyzed reaction is believed to produce small fragments of protamine molecules. The calculations according to eq 3 clearly showed that as the charge z_{PA} of polyion molecules

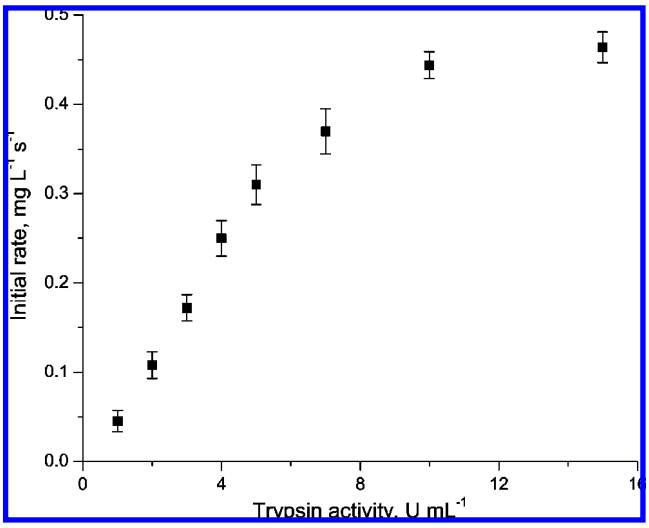

Figure 6. Assay response to trypsin activity as calculated using initial reaction rates from the data shown in Figure 5.

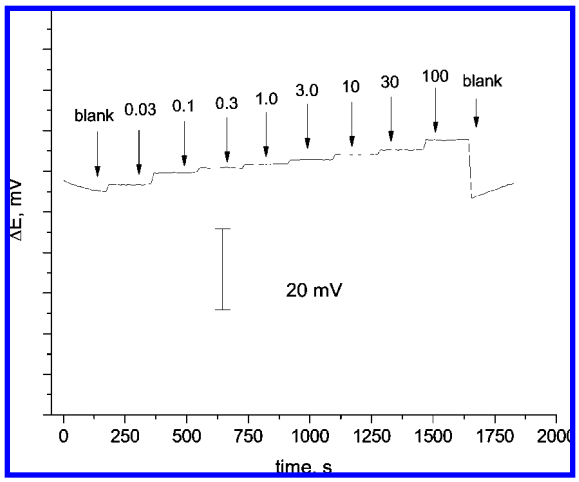

Figure 7. Sensor response to varying concentrations of arginine. Arginine concentrations (mg L^{-1}) are indicated in the figure.

decreases the corresponding sensor response decreases dramatically, thus making the sensor apparently insensitive to small protamine fragments. Previously, experiments with protamine-selective ISEs[6] demonstrated that small molecules such as lysine monomer, trimer, and pentamer produce negligibly small potentiometric response in comparison to protamine.

To prove that the apparent increase of protamine concentration was caused by a sensor response to reaction products we examined the influence of L-arginine concentration on the sensor potential. The corresponding calibration curve and time traces are shown in Figure 7. The magnitude of the resulting response at an arginine concentration of 10 mg L^{-1} was very close to 10 mV, which is in very good agreement with the observed potential reversal at the end of the reaction. The lower selectivity of the galvanostatically controlled sensors toward small molecules compared to polyion-selective ISEs can be explained by much higher ion fluxes induced by the applied current.

Unfortunately, lowering the magnitude of the applied current causes the leveling of ionic concentration gradients in the membrane due to the relatively fast diffusion transport into the membrane bulk. Higher current can sustain a substantially steeper gradient, which forms well-defined phase boundary conditions, and the resulting potential is stable. In order to form a steep gradient at low currents, we need a membrane with lower diffusion coefficients. In this case, low current can form a steep concentration gradient in the membrane phase. Obviously, increasing membrane resistance represents a complex experimental problem.

In the future we plan to improve the sensor selectivity and low detection limits by reducing current density and utilize the membranes with much lower diffusion rates than used before. This can be achieved using PVC membranes with much higher polymeric content, similar to that used for the heparin-selective potentiometric electrode.[8]

CONCLUSION

The solid-contact protamine electrodes represent a novel and valuable tool for the monitoring of enzyme activities using polyions as substrates. Excellent reversibility, selectivity, and low sensitivity to small polyion fragments make the sensors useful for accurate monitoring of enzyme-catalyzed reactions over long periods of time. The main advantages of the electrochemical assay based on polyion sensors are its high sensitivity and a possibility to detect enzyme activities in turbid samples. Fully solid-state designs based on polymeric membrane and conducting polymer as the transduction layer allow one to fabricate the sensor in a portable format using screen-printing technology, thus making the assay suitable for point-of-care applications.

The simple steady-state diffusion model describing the galvanostatically controlled polyion-selective sensors is of limited applicability and cannot be used to predict the sensor response within the entire detection range. The sensor calibration curve can be fit to the Boltzmann function, which describes the sensor calibration curve satisfactorily. The existing theoretical model predicted a reduction of the low detection limit, which was successfully demonstrated. The sensor exhibited a stable and reversible response to protamine within the concentration range of 0.05−30 mg L^{-1} in the presence of 0.1 M NaCl.

An interesting and exciting application of this technology is the ability to recognize and selectively detect activities of different proteases using synthetic substrates which have specific cleavage sites. Moreover, the specificity and sensitivity of these electrochemical assays can be used in the future in the development of novel electrochemical immunobinding assays, in which both proteases and artificially designed polyion substrates function as labels.

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