Flash Chronopotentiometric Sensing of the Polyions Protamine and Heparin at Ion-Selective Membranes

Kebede L. Gemene[a] and Eric Bakker* [a,b]

Department of Chemistry, 560 Oval Drive, Purdue University, West Lafayette, IN 47907 and Nanochemistry Research Institute, Department of Applied Chemistry, Curtin University of Technology, Perth, WA 6845, Australia.

[a] Purdue University

[b] Curtin University of Technology

* To whom correspondence should be addressed. Email: bakker.eric@gmail.com

Abstract

We report here on a highly sensitive and rapid detection technique, multipulse flash chronopotentiometry, for the anticoagulant polyion heparin and its antidote protamine. The technique is based on a localized titration of the polyions at the surface of an appropriately formulated polymeric ion-selective membrane devoid of ion-exchange properties to prohibit spontaneous extraction processes. A defined ion flux from the sample side to the membrane is electrochemically induced by applying a current pulse of appropriate amplitude and sign. The resulting depletion of the measured ions at the membrane surface gives rise to a characteristic limiting current or transition time and is observed as an inflection point in the resulting chronopotentiogram. The limiting current and the square root of the transition time are linear functions of the concentration of the polyion and yield sensitive and rapid analytical information attractive for clinical diagnostics applications. The polyion protamine is detected in 10-fold diluted serum samples in a matter of seconds via a cathodic current pulse. The utility of the technique for monitoring heparin-protamine titrations in physiological saline solutions is demonstrated.

Introduction

Heparin, a highly sulfated polyanionic carbohydrate (average charge of –70), is widely used as anticoagulant in surgical procedures such as open-heart surgery. It is postsurgically neutralized with its antidote, protamine, a highly positively charged protein (charge of ca. +20) to avoid bleeding complications. Detection of heparin level in blood samples is very important and is commonly attempted in clinical laboratories by the so-called the activated clotting time measurement (ACT). However, this technique is indirect and nonspecific and the results can be affected by factors other than heparin [1, 2] The developments in polymeric membrane potentiometric heparin [3-6]- and protamine [7-8]-selective electrodes by the group of Meyerhoff have been major achievements for direct and titration-based measurements of heparin as well as direct measurement of protamine in clinical samples. In addition to selective recognition of the polyions, these sensors show unusually high sensitivities that cannot be explained on the basis of the Nernst equation. This has been attributed to a nonequilibrium diffusion process of the polyions from the sample to the membrane bulk, facilitated by the thermodynamically favored ion exchange of the polyions with the small ions from the membrane and the subsequent ion-pairing of the former with the lipophilic ion exchangers in the membrane [5]. While this irreversible extraction process allows for a higher sensitivity of the sensor compared to equilibrium extraction, it imposes limitations on the applications of the sensors due to unstable and irreproducible responses upon continued contact of the membrane with the polyions.

Recently, heparin and protamine sensors that employ cyclic voltammetry and amperometry at the interface between two immiscible electrolyte solutions (ITIES) have been developed by the groups of Amemiya and Samec [2, 9-11]. In contrast to the response mechanism of potentiometric polyion sensors (see above), the observed responses have here been ascribed to selective adsorption of the polyion at the polarized organic/aqueous interface [9-11]. This prevents coupled ion transfer and hence mixed ion-transfer potentials. In addition, the selective adsorption and desorption of the polyion-ionophore complex resulted in an enhanced sensitivity of the polyions through voltammetric stripping analysis [2]. Moreover, the current response in voltammetric/amperometric measurements, which is a direct function of the

concentration and charge of the target ion, is more sensitive than the potential response of equilibrium potentiometric measurements, which varies with the logarithm of concentration and the inverse of the charge of the analyte ion [12-14]. Very recently, Rodgers et al. studied the electrochemically controlled molecular recognition of heparin using the synthetic heparin mimetic Arixtra to obtain a more thorough understanding of interfacial heparin recognition. Their results supported their original notion of a sensor response dominated by an interfacial adsorption step [12]. The researchers were successful in detecting heparin in whole blood [2]. Despite the undeniable progress in the field, an intuitive theoretical explanation is still needed to clarify the discrepancy between the adsorption-based response mechanism of the proposed voltammetric sensors [10, 11] and the established extraction-based response of potentiometric sensors, since both classes of sensors are based on related membrane materials. In particular, it has not been clearly explained what processes may hinder the facilitated diffusion of the polyions into the bulk organic phase when unsupported liquid/liquid interfaces such as water/1,2-dichloroethane [15] or water/nitrobenzene [12] are used. Earlier work [16, 17] based on the voltammetric/amperometric transduction of polymeric membrane ion-selective electrodes explained the response mechanism of such sensors for simple ions on the basis of extraction principles. Accordingly, it was suggested that cyclic voltammetry may not be an adequate method for such applications since the electrochemically extracted ions may not be fully expelled from the membrane during the reverse scan and thus advanced pulse techniques were recommended for this purpose [19].

Reversible pulsed chronopotentiometric polyion sensors were recently introduced by Shvarev and Bakker [1, 18]. These sensors are interrogated under well formulated galvanostatic/potentiostatic pulse sequences to ensure stable and reproducible responses. A controlled current pulse applied across the polyion-selective membrane devoid of ion-exchanger [19] causes the extraction of the polyions from the sample into the membrane while the potential is monitored at the same membrane. This is followed by a baseline potential pulse to expel the ions extracted in the previous pulse, which effectively renews the membrane and assures reproducible and stable responses. These sensors have been applied recently to measure heparin concentration in whole blood in a stable and reproducible fashion via titration with protamine. In addition, a protamine-selective

pulsed chronopotentiometric sensor was recently utilized for monitoring enzymatic activity [20, 21]. It must be noted, however, that this sensor protocol gives rise to undesirable non-linear calibration curves.

Pulsed chronopotentiometric flash titration at ion-selective membranes, where an ion depletion process at the membrane surface is chronopotentiometrically monitored in the course of the experiment, was recently introduced by Gemene and Bakker [22, 23]. Here, a controlled current pulse is applied across the ion-selective polymeric membrane to induce a strong defined flux of ions from the sample side to the membrane while the potential is measured at the same membrane. This causes the depletion of the analyte ion at the membrane surface at a limiting current or transition time, either of which may be related to the ion concentration. The ion depletion is depicted as an inflection point of the resulting chronopotentiograms, in analogy to conventional potentiometric titrations. The most important advantages of this technique are its speed (titration times in seconds), high sensitivity compared to the Nernst equation, and linear calibration curves. This method was introduced for the detection of total acidity [22] and total calcium in physiological samples [23]. We report here for the first time the application of this technique to the detection of the important biological polyions heparin and protamine.

Experimental

Reagents. High molecular weight poly(vinyl chloride) (PVC), o-nitrophenyl octyl ether (o-NPOE), tetradodecylammonium tetrakis(4-chlorophenyl) borate (ETH 500), tetradodecylammonium chloride (TDDACl), dinonylnaphthalenesulfonic acid (DNNS), tetrahydrofuran (THF) and all salts were purchased from Fluka (Milwaukee, WI). Sheep whole blood stabilized with trisodium citrate was purchased from Hemostat Laboratories (Dixon, CA). Tris base, protamine sulfate (from herring) and heparin sodium salt (from bovine intestinal mucosa) were purchased from Sigma (St. Louis, MO). The inert salt tetradodecylammonium dinonylnaphthalene sulfonate (TDDA-DNNS) was prepared as described elsewhere [1]. Aqueous solutions were prepared by dissolving the appropriate compounds in Nanopure-deionized water (18.2 MOhm cm).

Membrane Preparation. Protamine-selective membrane (~200 μm thick) for the

chronopotentiometric sensor was prepared by solvent casting with THF as a solvent, a membrane cocktail containing 10 wt% of the inert lipophilic salt TDDA-DNNS and PVC and o-NPOE 1:2 by weight.

Electrodes. The membranes were cut with cork borer (6 mm diameter) from the parent membrane and incorporated onto a Philips electrode body (IS-561, Glasblaserei Moller, Zurich, Switzerland). The outer membrane area was calculated from its geometry as 20 mm^2. The inner solution was in contact with an internal Ag/AgCl electrode. The external reference electrode was a double-junction Ag/AgCl electrode with saturated KCl as inner solution and a 1 M LiOAc bridge electrolyte. A high surface area coiled Pt-wire was used as a counter electrode in contact with the sample. The working electrodes were conditioned for at least 12 hours prior to experiments and kept in the conditioning solution when experiments were not underway. The inner filling and conditioning solution was 10 mM NaCl.

Experimental setup. A conventional three-electrode setup was used for the chronopotentiometric measurements where an internal Ag/AgCl electrode acted as the working electrode and the external reference electrode and counter electrode were immersed into the sample solution. The galvanostatic measurements were conducted with an AFCBI bipotentiostat (Pine Instruments, Grove City, PA) controlled by a PCI-MIO-16E4 interface board and LabVIEW 5.0 data acquisition software (National Instruments, Austin, TX) on a Macintosh computer. The potentials were sampled at 2 ms intervals. For fixed time experiments, the potential was calculated during the last 10% of the cathodic current pulse time, with an uptake time of 1 s and a stripping time of 15 s were used throughout the experiment unless specified otherwise. A baseline potential pulse of 0 V versus Ag/AgCl was applied as a stripping potential. For the details of potentiostatic/galvanostatic control switching system, see reference [19]. All experiments were conducted at room temperature (21 - 22 ^{0}C).

Results and Discussion

The detection of the polyion protamine was explored with pulsed chronopotentiometric flash titration at a polyion-selective membrane. Successive current pulses of increasing

amplitude were applied across a polymeric membrane protamine selective electrode containing the inert salt TDDA-DNNS to cause a defined flux of the polyions from the sample in direction of the membrane while the monitoring the potential at the same membrane. As explained in previous work with hydrogen ion-selective membranes as model system, a localized analyte depletion may occur at a characteristic limiting current or transition time that is observed chronopotentiometrically [22]. Figure 1A shows the observed potential responses as a function of the applied current in sample of varying protamine concentrations in a background of 10 mM NaCl buffered at pH 7.4 with 10 mM tris-HCl. While the potentials were not corrected for the so-called *iR* drop, inflection points are clearly visible as a function of the applied current. Accordingly, the first derivatives of the resulting potential responses presented in Figure 1A show sensitive and well-defined peak-shaped response curves (see Figure 1B) that signal the flash titration endpoints. These peaks shift to higher current amplitude with increasing protamine concentration. The limiting current is expected to be a linear function of the concentration for a fixed pulse time [22], which is confirmed in Figure 2A, where the limiting current obtained from Figure 1B is plotted as a function of the polyion concentration.

In clinical situations, protamine is used to monitor the heparin level in blood by titration, using clotting time or more accurately a protamine-selective electrode as an endpoint indicator. The capability of the method proposed here to detect the protamine-heparin binding event was evaluated by monitoring the potential response as heparin was added to a protamine solution. Figure 2B shows the response curve for the successive addition of heparin to the protamine containing solution from the previous calibration step (see Figures 1 and 2A). A continuous shift in the peak position towards lower current amplitude was observed with increasing heparin levels, indicating the consumption of protamine. A linear relationship was observed between the added concentration of heparin and the limiting current. The remaining level of protamine was calculated using the literature binding ratio between protamine and heparin of 1.5:1 (weight: weight) and plotted as a function of limiting current in Figure 2C, corresponding well to the protamine calibration curve. The best correspondence between curves A and C for the experimental conditions used here was found with a binding ratio of 1.37:1 .

Note that this method was chiefly established [22, 23] for the measurement of total concentrations by facilitating the dissociation equilibrium of weakly bound ions through electrochemical means to result in the depletion of all labile species at the membrane surface. However, in this work, the strong and specific protamine-heparin interaction renders the complex too inert for the method and hence only unbound protamine is detected. It is worth mentioning that the technique proposed here may also detect heparin directly via an anodic current pulse, but this was not explored in greater detail (data not shown).

Time-resolved flash chronopotentiograms upon titration of protamine with heparin and the corresponding first derivatives are presented in Figures 3A and 3B, respectively. Here, a constant current was applied and the phase boundary polyion depletion resulted in a transition time τ, which is clearly discernable from the chronopotentiograms. The square root of the transition time, which can be obtained from the peaks of the first derivative curves or more clearly from the zeros of the second derivative curves of the chronopotentiometric responses is expected to be a linear function of the bulk concentration [22]. Figure 4A shows the calibration curve obtained for varying concentration of protamine added from a 1 g/l stock solution to 10 mM NaCl (pH 7.4 with 10 mM Tris-HCl) while Figure 4B shows the linear dependence of the square root of transition time on the concentration of the unbound protamine upon subsequent titration of the resulting solution with heparin from a 1.5 g/l stock solution (see Figure 4). The results are in agreement with the data shown in Figure 2, and suggest that localized protamine titrations may indeed be performed in a matter of seconds without perturbing the bulk sample composition.

The effect of ionic strength on the measurement of protamine was evaluated (see Figure 5). No considerable shift in the limiting current (peak position) was observed when the background NaCl concentration was varied from 1 to 160 mM. The molar background electrolyte concentration, which was successively increased by addition from a 1-M NaCl stock solution to a 0.032 g/l protamine solution in 10 mM tris-HCl buffer, pH 7.4, are indicated in the figure (units not shown) and correspond to the top to bottom curves.

The key relevant application of detecting the polyions heparin and protamine is for clinical analysis. The detection of these important ions in 10-fold diluted blood was

explored. The differential chronopotentiometric responses shifted predictably to higher current upon addition of protamine to a 10-fold diluted blood sample (Figure 6A) and subsequently back to lower currents with increasing heparin additions (Figure 6B). The arrows indicate the direction in which each calibration was performed. The linear dependences of the limiting currents on protamine concentrations are presented in Figure 7, confirming the ability of the technique to monitor heparin concentration in 10-fold diluted blood.

This technique may also be utilized as an endpoint indicator to determine the concentration of analytes that are not directly accessible, but may be measured by titration with the polyion to which the sensor is sensitive. Chondroitin sulfate, a polyanion that was recently found in tainted heparin [24], is one example. The concentration of chondroitin sulfate could not be assessed directly with the TDDA-DNNS-based membrane electrode. However, the concentration of chondroitin sulfate, in its pure form or as a mixture with heparin, could be successfully monitored by this technique through its titration with protamine (data not shown).

Conclusions

Pulsed chronopotentiometric flash titration at ion-selective membranes may be of significant interest for physiological, clinical and environmental applications: It is selective and sensitive, rapid, simple, tunable, miniaturizable, nondestructive to the sample, inexpensive, and robust. This paper shows that the technique is promising for the detection of polyions in buffers and physiological samples, although more work needs to be done to demonstrate its use in routine clinical diagnostics.

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Comment [Eb1]: Correct the journal names!

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Figure Captions

Figure 1 (A) Pulsed chronopotentiometric responses to protamine from a 1 g/l stock solution added to a background of 10 mM NaCl buffered at pH 7.4 with 10 mM tris-HCl and (B) first derivatives of the potential responses shown in (A). Numbers show concentrations of protamine in the sample in mg/l.

Figure 2 Observed linear relationship between the limiting current and (A) protamine concentration upon successive addition (from the data in Figure 1), (B) added heparin concentration and (C) remaining protamine concentration as calculated from the titration data using a 1.5:1 protamine to heparin binding stoichiometry

Figure 3 (A) Observed time-resolved chronopotentiograms and (B) first derivative curves of the data in (A) upon titration of protamine with heparin.

Figure 4 Observed linear relationship between the square root of transition time and protamine concentration (A) as added from a 1 g/l stock solution to the background under Figure 1 and (B) remaining (unbound) protamine upon addition of heparin as calculated from the stoichiometric binding data.

Figure 5 Dependence of the pulsed chronopotentiometric transition time upon the background electrolyte concentration. The numbers show molar background electrolyte concentrations obtained by successive addition from a 1-M NaCl stock solution.

Figure 6 Observed first derivative curves (A) upon successively increasing protamine concentration and (B) upon titration of the resulting solution from (A) with heparin in a background of 10 times diluted blood. All other conditions are as stated under Figure 1.

Figure 7 Observed linear relationships between the limiting current and the protamine concentrations from the data in Figure 6.