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Flash chronopotentiometric sensing of the polyions protamine and heparin at ion-selective membranes

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

[a] Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47907, USA
[b] Nanochemistry Research Institute, Department of Applied Chemistry, Curtin University of Technology, Perth, WA 6845, Australia

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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 induced electrochemically 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 blood 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.

Heparin, a highly sulfated polyanionic carbohydrate (average charge of −70), is widely used as an anticoagulant in surgical procedures such as open heart surgery. It is postsurgically neutralized with its antidote protamine, a highly positively charged protein (charge of ~+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 activated clotting time (ACT)[1] measurement. 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 Meyerhoff group 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]. Although this irreversible extraction process allows for a higher sensitivity of the sensor compared with equilibrium extraction, it imposes limitations on the applications of the sensors due to unstable and irreproducible responses on 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 Amemiya and Samec groups [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

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* Corresponding author. Address: Nanochemistry Research Institute, Department of Applied Chemistry, Curtin University of Technology, GPO U1987, Perth, WA 6845, Australia.
E-mail address: bakker.eric@gmail.com (E. Bakker).

[1] *Abbreviations used:* ACT, activated clotting time; ITIES, interface between two immiscible electrolyte solutions; PVC, poly(vinyl chloride); *o*-NPOE, *o*-nitrophenyl octyl ether; ETH 500, tetradodecylammonium tetrakis(4-chlorophenyl) borate; TDDACl, tetradodecylammonium chloride; DNNS, dinonylnaphthalenesulfonic acid; THF, tetrahydrofuran; TDDA–DNNS, tetradodecylammonium dinonylnaphthalene sulfonate.

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measurements, which varies with the logarithm of concentration and the inverse of the charge of the analyte ion [12–14]. Recently, Rodgers and coworkers 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 given that 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] and 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 might not be an adequate method for such applications because the electrochemically extracted ions might not be fully expelled from the membrane during the reverse scan; thus, advanced pulse techniques were recommended for this purpose [18].

Reversible pulsed chronopotentiometric polyion sensors were introduced recently by Shvarev and Bakker [1,19]. 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 an ion exchanger [18] 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 that effectively renews the membrane and ensures 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 used recently for monitoring enzymatic activity [20,21]. It must be noted, however, that this sensor protocol gives rise to undesirable nonlinear calibration curves.

Pulsed chronopotentiometric flash titration at ion-selective membranes, where an ion depletion process at the membrane surface is monitored chronopotentiometrically in the course of the experiment, was introduced recently 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 with 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.

Materials and methods

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, USA). Sheep whole blood stabilized with trisodium citrate was purchased from Hemostat Laboratories (Dixon, CA, USA). Tris base, protamine sulfate (from herring), and heparin sodium salt (from bovine intestinal mucosa) were purchased from Sigma (St. Louis, MO, USA). 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 ($\sim$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/*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 (cat. no. 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 h prior to experiments and were kept in the conditioning solution when experiments were not under way. 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, USA) controlled by a PCI-MIO-16E4 interface board and LabVIEW 5.0 data acquisition software (National Instruments, Austin, TX, USA) 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 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 a potentiostatic/galvanostatic control switching system, see Ref. [18]. All experiments were conducted at room temperature (21–22 °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