analytical chemistry

Letter

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Thin Layer Coulometry Based on Ion-Exchanger Membranes for Heparin Detection in Undiluted Human Blood

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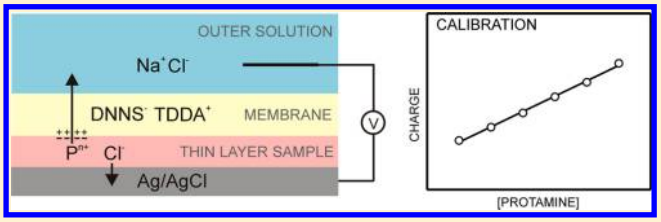
Supporting Information

ABSTRACT: We explore here for the first time a potentially calibration-free methodology for the detection of protamine (and, by titration, heparin) in undiluted human blood in the therapeutic concentration range from 20 to 120 mg L^{-1}. The use of a thin layer sample (5.8 μL) confined between a tubular protamine selective membrane (inner diameter, 600 μm) and a Ag/AgCl wire (diameter 400 μm) achieves an exhaustive depletion from the sample. Coulometry detection was chosen for the interrogation of the thin layer, employing a double pulse technique with 120 s for each pulse. Protamine calibration curves were recorded at physiological concentrations and in undiluted human blood. A linear relationship was obtained in both cases, but a diminished sensitivity was observed in contact with blood, which is explained with a partial passivation of the inner Ag/AgCl element. Heparin–protamine titrations were performed in undiluted human blood samples, mimicking the final application with patients undergoing critical care. The observed values correlate satisfactorily with those of an alternative technique, so-called flash-chronopotentiometry on planar membranes.



We demonstrate here for the first time the use of an exhaustive coulometric detection approach for the assessment of blood heparin based on the established protamine–heparin interaction.[1] Protamine is a polycation (ca. 20 charges per molecule) utilized to neutralize the polyanionic heparin, the anticoagulant of choice in the coagulation control of numerous surgical procedures. The typical therapeutic protamine concentration range is between 20 and 120 mg L^{-1}.[2] Established heparin assays are typically performed on plasma samples by a factor Xa assay, which is preferred oversimple activated clotting time (ACT) measurements.[3−5] However, the results of this method do not strictly depend on heparin concentration alone, since they reflect the physiology of the coagulation cascade. A drawback of the factor Xa assay is the long turnaround time, which increases the risk of improper heparin reversal that can be associated with bleeding complications after surgery, since heparin is continuously metabolized in the body.[6]

There has been significant progress over a period of two decades to develop electrochemical-sensing approaches for heparin. The early breakthroughs were by Meyerhoff and Yang, which used liquid PVC-based membranes doped with adequate sensing components for the direct detection of heparin and protamine in blood.[7,8] The strong protamine–heparin interaction makes it possible to use a protamine-selective electrode in heparin assays, which also minimizes possible biases arising from weaker nonspecific interactions of heparin with other blood components. Unfortunately, spontaneous extraction of the polyion into the membrane made these types of sensors operationally irreversible.

In subsequent years, a number of groups explored dynamic electrochemistry-based methods to render the heparin or protamine detection operationally reversible.[9−13] Our group used mainly controlled current techniques for this purpose. While protocols mainly useful as titration end points were successful early on,[14] linear calibration curves less dependent on the background electrolyte of the sample were achieved with chronopotentiometry.[15] An improved membrane formulation with a permselective membrane containing an excess of the protamine recognizing anion dinonylnaphthalene sulfonate (DNNS) was recently shown to exhibit the required sensitivity and selectivity as well as linear calibration curves for use in whole blood samples.[16,17]

Thin layer coulometry with ion-selective membranes has recently been explored in our group for the detection of a variety of ions.[18−20] While a thin layer allows one to work with just a few microliters of sample, coulometry is a very promising interrogation platform since the absolute counting approach reduces the need for frequent calibration. The present work aims to dramatically reduce the required sample volume and to introduce a heparin sensing principle that is potentially calibration free. This work aims to help establish the fundamental basis for the realization of a simple, yet accurate single-use heparin assay at the point-of-care.

EXPERIMENTAL SECTION

Materials and Chemicals. Tetradodecylammonium chloride (TDDA), 2-nitrophenyl octyl ether (o-NPOE), heparin sodium salt from porcine intestinal mucosa (H4784), prot-

Received: December 2, 2013
Accepted: January 8, 2014
Published: January 8, 2014

   dx.doi.org/10.1021/ac403902f | Anal. Chem. 2014, 86, 1357−1360

amine sulfate salt from herring (P4505), trizma hydrochloride (Tris·HCl), sodium chloride, sodium hydroxide (1 M), and tetrahydrofuran (THF) were purchased from Sigma−Aldrich. Dinonylnaphthalene sulfonate (DNNS acid form in 50% heptane) was a gift from King Industry. Heparin and protamine stocks solution (10 g L^{-1}) were freshly prepared before starting the experiments 100 mM NaCl. Aqueous solutions were prepared by dissolving the appropriate salts in Milli-Q-purified distilled water. PEEK tubing, 1/8 and 1/16 in. HPLC connectors, and all salts were purchased in the highest quality available from Sigma-Aldrich. Polypropylene (PP) hollow fiber (i.d., 600 μm) was obtained from Membrana.

Cell Assembly. Working and counter electrode were made of 640 μm silver wire using a commercially available jewelry drawplate and subsequently oxidized electrochemically in a solution of 1 M HCl for 3 h at 0.4 mA/cm^2. The working electrode was coiled with PVDF fish line (Ø 50 μm, pitch distance 225 μm) in order to avoid a direct contact between the electrode and polypropylene (PP) hollow fiber. Eleven centimeters of PP porous fiber was glued with epoxy to 1/8 in. diameter 35 mm long PEEK tubing and doped with the lipophilic cocktail, containing DNNS (11.8 mg), TDDA (8.82 mg, 2:1 molar ratio, respectively), and *o*-NPOE (180 mg). Subsequently, PP porous fiber was slid onto the working electrode fixed in the fluidic channel of the custom-built electrochemical cell. After that, the electrochemical cell was assembled and ion-selective membrane with the Ag/AgCl inner element was conditioned overnight in the solution of 100 mg L^{-1} protamine in 100 mM NaCl (for more details about coulometry cell, see the Shvarev et. al reference[19]). The resulting inner volume is 5.8 (±0.2) μL.

Electrochemical Measurements. The electrochemical measurements were performed with an μ-Autolab System (Metrohm Autolab, Utrecht, The Netherlands) controlled by a personal computer using Nova 1.8 (supplied by Autolab). After filling the hollow fiber membrane with the sample solution, the open circuit potential (OCP) was measured. Subsequently, a 120 s excitation pulse at OCP plus incremental multipliers of 30 mV for each cycle ranging from 30 to 300 mV were applied and the current was integrated over this time to give the charge (Figure 1S of the Supporting Information). The second background compensation pulse was applied for 120 s and again integrated. Before introducing a new sample, the system was regenerated for a period of 240 s, at the potential value that was determined as open circuit potential before the electrochemical excitation.

RESULTS AND DISCUSSION

The coulometry protamine detection concept was first evaluated with synthetic samples of 0.1 M NaCl containing different concentrations of protamine. The protamine membrane was interrogated with a double potentiostatic pulse protocol introduced earlier.[21] The applied potential was systematically varied between 30 to 300 mV with respect to the OCP value to establish the value at which protamine is exhaustively and selectively transferred from the thin layer to outer solution (see the schematic Figure 1).

Figure 2S of the Supporting Information shows the coulometric readout (integrated current) of the first and second pulse as a function of the applied potential for six protamine concentrations. During the first pulse, the protamine is depleted within the voltage range from 30 to 230 mV. At higher potentials, the background ion sodium is also transferred. During the second pulse, it is mainly the background ion that is depleted, since the current decays for the second pulse are independent of protamine concentration (Figure 2S of the Supporting Information). The subtraction of the observed charges for both pulses aims to minimize signal contributions unrelated to protamine (Figure 2), thus improving the linearity of the calibration curve.

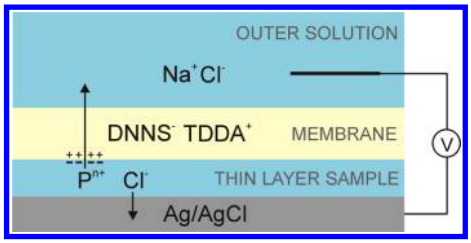


Figure 1. Schematic illustration of the thin layer coulometry ion selective electrode for determining protamine in the therapeutic range.

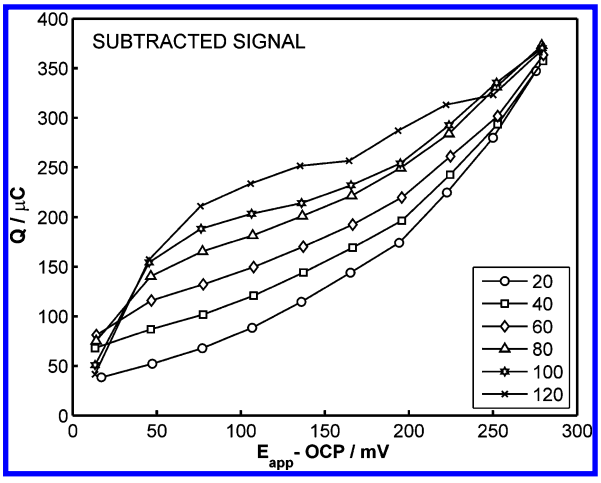


Figure 2. Ion transfer charge as a function of the applied potential vs Ag/AgCl wire for different concentrations of protamine (subtracted signal). Note that the inset legend corresponds to protamine concentration in milligram per liter units.

We selected an applied potential of 120 mV (relative to the open circuit) as a suitable potential for performing all the following experiments, giving a suitable compromise between sensitivity and selectivity. Figure 3a displays the calibration curve for five replicates (charge = 1.31 μC L mg^{-1} + 54.74 μC, RSD, 1%). From Faraday's law, the calculated slope for this cell geometry should be 2.18 μC L mg^{-1}, assuming 100% electrolysis.

Ten cells were fabricated and characterized at the same overpotential of 120 mV. All of them behave very similarly (<5% RSD), which makes the concept promising as a basis for a simple assay not requiring prior calibration. The main uncertainty in the homemade cell design comes from the limited reproducibility of the inner volume of nominally 5.8 μL and the associated thin layer spacing. Microfabrication will help improve this aspect further.

Protamine detection using flat sheet membranes and controlled with chronopotentiometry (see Figure 3S of the Supporting Information) was used for comparison purposes. Identical solutions were measured with both methods. Figure 3b shows the correlation plot between both methodologies. In addition, Figure 4S of the Supporting Information shows the

dx.doi.org/10.1021/ac403902f | *Anal. Chem.* 2014, 86, 1357−1360

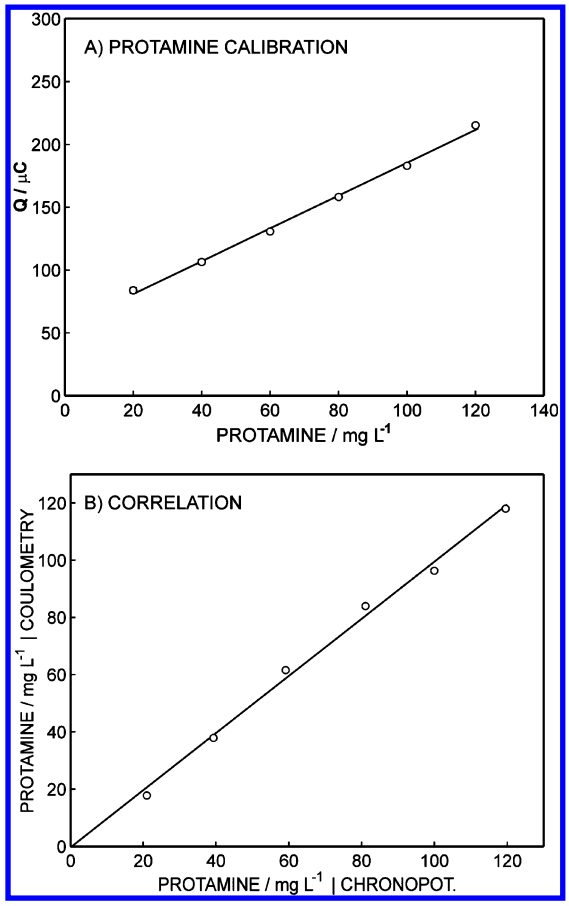


Figure 3. (a) Protamine calibration curve recorded at 120 mV. The sample solution contains from 20 to 120 mg L^{-1} + 100 mM NaCl. The outer solution contains 100 mg L^{-1} of protamine + 100 mM NaCl. % RSD = 1%. (b) Correlation between coulometric and chronopotentiometric method. Note that the obtained readout signal for coulometry and chronopotentiometry was normalized by the sensitivity of each method.

respective joint confidence hyperbole, which at 95% confidence reveals no statistical differences between the methods.

The protamine sensor was successfully tested to detect heparin under physiological conditions by adding aliquots of protamine. Figure 4 shows the obtained calibration curve for two conditions: (1) successive additions of protamine to a physiological background solution and (2) successive additions of protamine to physiological background solution containing a known amount of heparin (80 mg L^{-1}). At the beginning, three calibration curves were performed in the absence of heparin (y_1 = 1.47 μC L mg^{-1} + 54.32 μC, RSD 2%).

Subsequently, three calibration curves in the presence of heparin were carried out (y_2 = 1.68 μC L mg^{-1} − 126.28 μC, RSD 2%). As expected, the difference between the two curves indicates the bound protamine concentration (112 mg L^{-1}), which assuming a binding stoichiometry of 1.4,[16] indeed corresponds to 80 mg L^{-1} of heparin.

Protamine−heparin appears to be reversibly adsorbed on the Ag/AgCl wire, deteriorating the analytical life of the sensor. This effect is important, because after three calibration runs (18 measurements) in the presence of heparin, the electrochemical cell (membrane and wire) became unusable (sensitivity is highly deteriorated). The same heparin titration was simultaneously monitored with chronopotentiometry without observing such effect, suggesting that the membrane is not affected by the measurement. While more work would need to elucidate this further, one may argue that the Ag/AgCl wire is passivated by a polyelectrolyte heparin−protamine coating. Fortunately, this may be inconsequential if the device is used for a single measurement.

The sensor was tested in undiluted human blood (see Figure 5). Ten μL of human blood with different levels of added

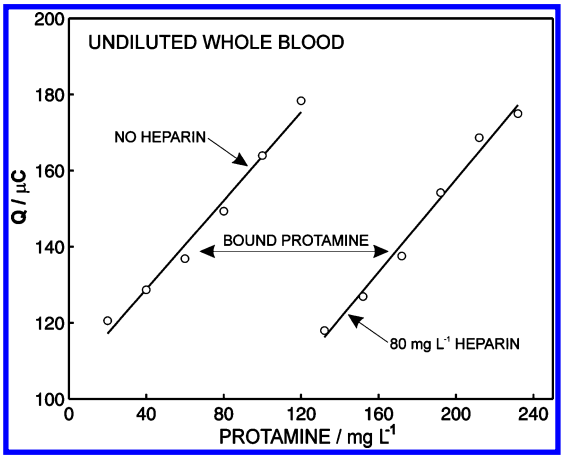


Figure 4. Heparin−protamine titration in NaCl 0.1 M at 120 mV.

Figure 5. Coulometric heparin−protamine titration in undiluted human blood.

protamine were drawn into the coulometric cell. A calibration curve between 20 and 120 mg L^{-1} was obtained as y_3 = 0.58 μC L mg^{-1} + 105.54 μC, RSD 4%. Consequently, a titration of 80 mg L^{-1} heparin with protamine was also performed (y_4 = 0.61 μC L mg^{-1} + 35.71 μC, RSD 5%). As mentioned before, the difference between both curves is assigned to the bound protamine to heparin (132 mg L^{-1}). While the results are promising, a blood matrix effect is observed, since the sensitivity is reduced 2.5 times compared to the artificial sample (see above). However, the linearity is maintained, and the device is still capable of heparin quantification.

In order to characterize the robustness of the sensor, a single standard solution (60 mg L^{-1} of protamine added to human blood) was measured 15 consecutive times. After 6 measurements, the signal decreases gradually down to 60 μC (Figure 5S of the Supporting Information, around 15% less signal). Additionally, before and after blood exposure several calibration

dx.doi.org/10.1021/ac403902f | Anal. Chem. 2014, 86, 1357−1360

runs were performed (see Figure 6S of the Supporting Information). After blood exposure, the cell was washed for 30 min with a protamine solution and subsequently with NaCl 0.1 M. The sensitivity was found to decrease by a factor of 1.4 after blood contact. After that, the membranes were disassembled and visibly checked for contamination. Figure 7S of the Supporting Information shows clearly an accumulation of blood on the silver wire surface. This adsorbate is very likely responsible for a reduction of electrochemical surface area of the wire and, hence, a reduction in coulometry efficiency.

In conclusion, we demonstrate here for the first time that protamine ion-selective membranes can be controlled by a thin layer coulometric approach. Heparin, in the therapeutic range, can be quantified in artificial samples (establishing selectivity) and in undiluted human blood (characterizing sensitivity). These preliminary results suggest that the development of robust, simple, and recalibration-free sensing systems for heparin may eventually become a reality. We believe that further progress should involve (1) an improvement of the biocompatibility of the inner electrode element in contact with blood, (2) the microfabrication of a precise and accuracy thin layer compartment accompanied with a tuning of the electrochemical protocol to reduce the intercept of the calibration curve as much as possible, and (3) replacing the inner liquid solution by a solid ion-to-electron transducer. Further, such progress may result in a point-of-care analysis system for heparin that will be as simple to use as an off-the-shelf glucose test strip.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the Swiss National Science Foundation for supporting this research.

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