Membranes

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Reversible Sensing of the Anticoagulant Heparin with Protamine Permselective Membranes**

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A new approach to quantitatively sense heparin under physiological conditions is presented that uses the established protamine-heparin interaction.[1] This concept forms the scientific foundation for a continuous and convenient monitoring of heparin by protamine titration in blood samples desired in many types of surgeries and kidney dialysis. Protamine is a polycation (ca. 20 charges per molecule) that plays an important role in the blood coagulation process. It is post-surgically injected to neutralize heparin concentration (a polyanion with a charge of ca. −70) administrated during the procedure to control the blood clotting time. This neutralization is quantitative and rapid, allowing heparin levels to be detected by measuring excess protamine[2] if an adequate electrochemical measurement principle becomes available.

Different approaches have been proposed to determine heparin levels in blood samples in the required range (10–100 $mg\,L^{-1}$).[3] For instance, in clinical laboratories, heparin is measured by activated clotting time (ACT), which is indirect, nonspecific, and not very reproducible.[4] The group of Meyerhoff introduced a potentiometric protamine endpoint detector that successfully demonstrated the clinical determination of heparin in blood samples by protamine titration.[5] These membrane electrodes functioned on the basis of spontaneous ion-exchange processes with the negatively charged active membrane ingredient, dinonylnapthalene sulfonic acid (DNNS), which acts as a selective protamine recognition agent in the membrane. Unfortunately, the high polyion charge makes these sensors operationally irreversible, and the single-use nature of these sensors makes them difficult to be established in clinical practice.

Our group introduced a controlled-current chronopotentiometric principle, used earlier by Buck et al. in fundamental studies[6] to make these ion-selective electrodes operationally reversible.[4,7] These membranes were formulated to suppress spontaneous extraction of protamine into the membrane by using a carefully matched salt of the active ingredient dinonylnapthalene sulfonate and a tetradodecylammonium counterion. An applied current defined the flux of protamine from the sample into the membrane, while this flux was maintained at the back side of the membrane by the concomitant extraction of an ion of opposite charge. This method rendered the sensors operationally reversible, as the membrane was returned to its nonperturbed stated by potential control. It gave, in complete analogy to their potentiometric counterparts, a sigmoidal calibration curve and could be used as a reversible endpoint detector for heparin–protamine titration.

More recently, it was found that the same type of experiment may also be analyzed by chronopotentiometry.[8] The applied current imposes a constant cation flux in the direction of the membrane, which can only be maintained by protamine up to a critical time, after which local depletion occurs that results in a potential change. The square root of this transition time gives a linear dependence on the sample concentration, but the selectivity was not adequate for whole blood samples.

We have achieved a substantially improved membrane selectivity with permselective membranes that contain a molar excess of DNNS over the lipophilic quaternary ammonium counterion. Selectivity may be understood with the following ion-exchange reaction between protamine and the sodium ion (the most pre-dominant cation in blood) as follows:

$$P^{n+}(aq) + nDNNS^{-}(org) + nNa^{+}(org) \rightleftharpoons P(DNNS)_n(org) + nNa^{+}(aq) \quad (1)$$

where P is protamine with charge $n+$. Excess $DNNS^-$ in the membrane results in the spontaneous extraction of protamine to form a DNNS adduct. The DNNS concentration is given by the presence of anion exchanger, while the Na^+ concentration cannot substantially change owing to the large value of $n = 20$. According to the law of mass action, a thousand-fold increase of $Prot(DNNS)_n(org)$ concentration owing to a reduced concentration of anion exchanger results in an increase in $Na^+(org)$ of just 40%. Consequently, the extraction of sodium relative to protamine is significantly suppressed, resulting in improved selectivity. These expectations are confirmed experimentally below. In addition to improving operational selectivity, a cation permselective membrane (containing excess DNNS) can be made thinner and formulated to exhibit higher membrane mobilities, as recently shown with thin-layer coulometric ion-selective electrodes.[9]

The operational principle of the permselective membrane is shown in Figure 1. The optimal membrane contains a 100% molar excess of $DNNS^-$ over tetradodecylammonium ion

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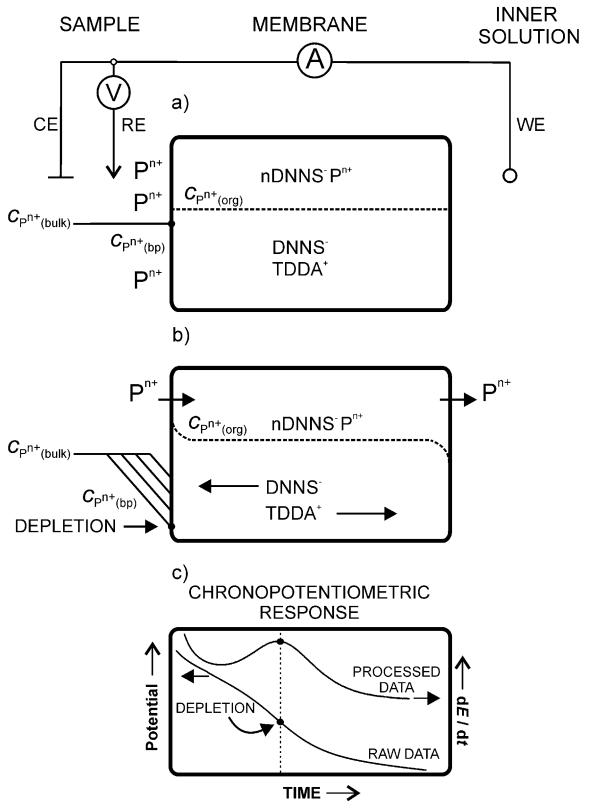

Figure 1. Illustration of the proposed protamine sensing mechanism. a) The polypropylene-based sensing membrane contains TDDA and protamine (P^{n+}) bound to excess DNNS. The protamine concentration in the membrane before electrochemical perturbation is denoted with a dotted line. The protamine concentration in the aqueous phase (C_{Pn+}(bulk)) is close to that in the phase boundary (C_{Pn+} (bp)). b) An applied current provokes a defined protamine flux across the permselective membrane and is sustained up to a transition time. The accumulation of protamine at the left side of the membrane during the pulse is stabilized by ion-pair formation with $DNNS^-$ from the added salt DNNS-TDDA, while the liberated $TDDA^+$ migrates to the right side of the membrane to counterbalance the excess $DNNS^-$ generated from protamine release. c) Observed raw and processed chronopotentiometric response data of the protamine depletion step.

(TDDA) as anion exchanger. Both compounds are dissolved as their respective acid, and chloride salt forms in the solvent *o*-NPOE, which is used to impregnate the pores of a 25 μm porous polypropylene membrane. The membrane is mounted into a commercial electrode body (see the Supporting Information for details). Such membranes were chosen because they allow an efficient equilibration with the sample solution in a matter of minutes and exhibit attractive ion mobilities[10] that translates into a low membrane resistance. Also, the rate limiting step more easily remains the depletion in the aqueous phase, not the membrane phase. Consequently, higher limits of detection can be obtained of up to 100 $mg\,L^{-1}$.

Upon first exposure to a protamine containing solution, the hydrogen-ion counterion of DNNS may quantitatively exchange with protamine, while excess HCl from the DNNS-TDDA electrolyte are similarly expelled. Membranes based on plasticized poly(vinyl chloride) with otherwise the same membrane ingredients were also tested, but they turned opaque, presumably because the expelled HCl promoted the formation of water droplets within the membrane.[11]

An applied constant-current pulse imposes the transport of protamine from the sample across the membrane into the inner solution with a defined flux. This results in the accumulation of protamine at the sample side of the membrane, facilitated by the dissociation of DNNS-TDDA and the migration of TDDA to the other membrane side (Figure 1; Supporting Information, Figure S1). Indeed, membranes containing only DNNS as active ingredient did not give operational responses with the method discussed here (Supporting Information, Figure S2). The transition time τ is found as the inflection of the chronopotentiometric response (Supporting Information, Figure S3) and signals the local depletion of protamine at the membrane surface (Figure 1b).

After this transition time, a background cation, such as sodium, is co-extracted along with protamine to maintain the imposed ion flux, which results in a decreased membrane potential. The potential change is a direct function of the membrane selectivity, which is associated with the membrane affinity to each ion. In agreement with the discussion above, membranes with increasing molar ratio of DNNS to anion exchanger give clearly improved selectivity in a 0.1M NaCl background (Supporting Information, Figure S4). The transition is visualized as the maximum of the time derivative of the potential (Figure 2a,b) for different protamine concentrations. After each chronopotentiometric protamine determination, a potentiostatic pulse is applied for 30 s at open-circuit potential (determined before the current pulse) to return the membrane concentration gradients to a state close to the unperturbed situation (Supporting Information, Figure S5). Figure 2 demonstrates the quantitation of protamine in buffered 0.1M NaCl samples (Tris pH 7.4) at an applied cathodic current density of 21 $\mu A\,cm^{-2}$.

The protamine sensor was successfully tested to detect heparin under physiological conditions by adding aliquots of protamine. Figure 2a shows the concentration dependent potential changes that are used to find the transition time in the absence of heparin. Figure 2c shows a plot of the square root of these transition times as a function of protamine concentration, demonstrating a linear calibration curve expected from the Sand equation (Supporting Information, Eq. (1)).[12] In principle, a change in current density results in a variation of the transition time and can thus be used to fine-tune the available measuring range. At that current density, the observed detection limit is 6 $mg\,L^{-1}$ for protamine and 8.4 $mg\,L^{-1}$ for heparin. The slope of the calibration curve (0.0133 $s^{1/2}\,L\,mg^{-1}$; see Figure 2c), along with a charge for protamine of +21 and the geometrical membrane area of 0.237 cm^2, gives a diffusion coefficient for protamine of 6.20×10^{-6} $cm^2\,s^{-1}$.

Figure 2b,d demonstrates the detection of heparin with the proposed electrochemical sensor. The transition times are shown to decrease with increasing added concentration of heparin to the sample containing 90 $mg\,L^{-1}$ of protamine (0.1M NaCl at pH 7.4). A linear dosage response is observed in Figure 2d, which suggests a quantitative protamine–heparin interaction with a well-defined stoichiometry

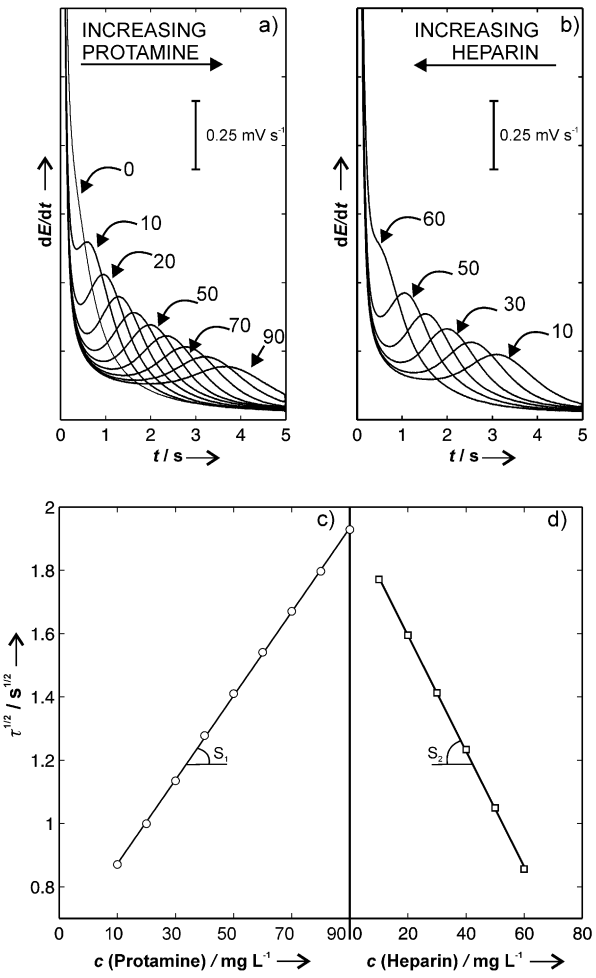

Figure 2. a) Observed time derivatives of the chronopotentiometric responses on successively increasing the final protamine concentration from 0 to 90 mg L^{-1} under physiological conditions (0.1 M NaCl, Tris pH 7.4). b) Time derivative potential response to subsequent heparin additions (0–60 mg L^{-1} final concentrations) to 90 mg L^{-1} protamine in 0.1 M NaCl pH 7.4). c,d) Observed linear calibration curve of the square root of the transition time τ as a function for the data shown in (a) and (b), respectively. The ratio of the two slopes gives a protamine-heparin binding stoichiometry of 1.4:1 in units of mg L^{-1} of protamine.

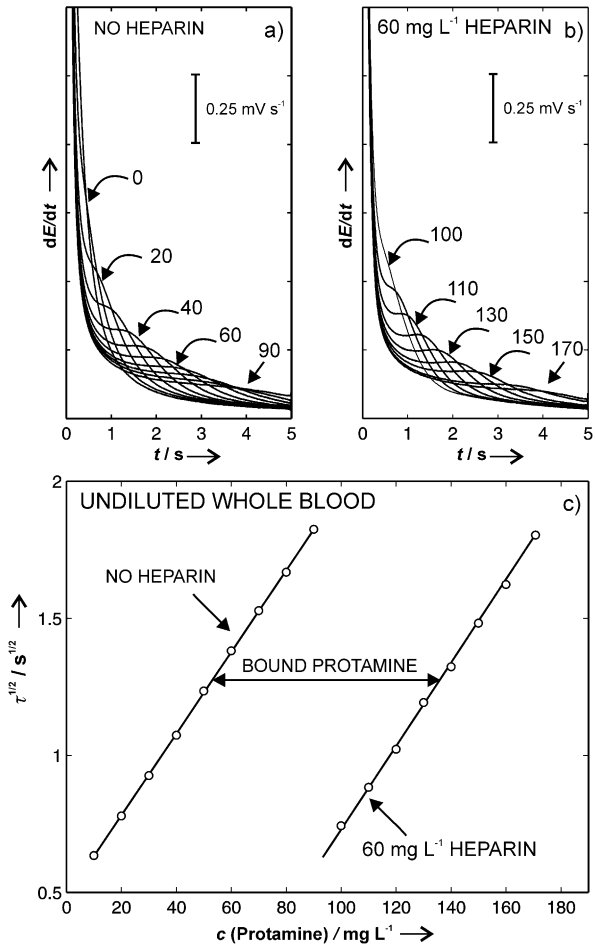

Figure 3. Observed potential time derivatives for in undiluted whole blood samples (human blood bag) upon successively increasing the final protamine concentration at the indicated levels a) in the absence of heparin and b) in the presence of 60 mg L^{-1} heparin. c) Corresponding linear response of square root of transition times vs. protamine concentration for (a) and (b), respectively. The bound protamine level, and therefore the heparin concentration, is quantitatively obtained from the horizontal distance between the two dose response curves (85 mg L^{-1}).

(65 mg L^{-1} heparin for 90 mg L^{-1} protamine concentration. This is in agreement with earlier findings where the experimental binding ratio was 1.4 to 1.[5f,8b] Unlike previous reports to develop polyion-responsive sensors, the principle reported herein yields linear calibration curves. In practice, this may mean that an added excess of protamine can be accurately quantified in a single, rapid measurement.

The principle was evaluated in preliminary work in undiluted human whole blood (citrated blood bag from the university hospital of Geneva, HUG). Figure 3a,b demonstrates that the potential transients yield transition times in complete analogy to that shown in Figure 2 for electrolyte solutions. The square root of τ versus concentration plot (Figure 3c) is linear and is described by $0.0149\,c_{\text{Protamine}}/[\text{mg L}^{-1}] + 0.482$, giving a diffusion coefficient of protamine of 7.83×10^{-6} cm^2 s^{-1}. In a separate experiment, the blood sample was spiked with 60 mg L^{-1} (final concentration) of heparin. Protamine additions give visible transition times at above 85–90 mg L^{-1} of added protamine (Figure 3c), which is consistent with the binding stoichiometry discussed above. The corresponding dose response shown in Figure 3c is again linear and described with $0.0153\,c_{\text{Protamine}}/[\text{mg L}^{-1}] - 0.806$. The dose response curves exhibit nearly the same slopes but are offset by the amount of protamine bound by the heparin in the sample. The offset corresponds to 85 mg L^{-1}, as expected. The reproducibility between three different freshly prepared membranes was found to be acceptable, with an RSD of 3.2% and 5.6% for electrolyte solution and blood experiments, respectively. Depending on the desired precision, a calibration of the sensor will be required for practical use. The reproducibility from data using a single membrane displays a RSD of 1% (Supporting Information, Figure S6).

In summary, the chemical approach introduced here uses permselective membrane electrodes that allow supported

liquid membranes to be employed that exhibit higher mobilities and rapidly equilibrate with the contacting samples. The square root of observed transition times correlate linearly with protamine concentration. Experiments in 0.1M NaCl electrolyte backgrounds and in undiluted whole blood suggest negligible interference by the sample matrix, making it a promising approach for the continuous monitoring of heparin in clinical settings.

Experimental Section

Tetradodecylammonium chloride (TDDA), 2-nitrophenyl octyl ether (o-NPOE), Heparin sodium salt from porcine intestinal mucosa (H4784), Protamine sulfate salt from herring (P4505), Trizma hydrochloride (Tris·HCl), sodium chloride, sodium hydroxide (1M), 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 in Tris buffer (10 mM buffer at pH 7.4 + 100 mM NaCl). DNNS stock solution was prepared in THF (dry DNNS (112 mg) in THF (1 mL)) and used to prepare the membrane cocktail labeled as MC1 composed of DNNS (11.8 mg), TDDA (8.82 mg, 2:1 molar ratio respectively), *o*-NPOE (180 mg), and THF (1 mL). The solvent was allowed to evaporate overnight from the cocktail. Porous polypropylene membranes (Celgard, 0.237 cm^2 surface area) were used as supporting material, and the cocktail solution (3 μL) was deposited on it. Afterwards, the membrane was conditioned in the buffer solution for 20 min. Finally, the membrane was mounted in the electrode body. Both inner and outer compartments were composed of the same background solution and concentration before starting the experiment. Protamine or heparin stock solutions were always successively added to the outer compartment.

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