Spectrophotometric bioanalytical flow-injection system for control of hemodialysis treatment

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A spectrophotometric flow-injection analysis (FIA) system for monitoring clinical hemodialysis is demonstrated. The role of a dialysate urea detector incorporated in this bioanalytical system is played by an optical flow-through biosensor based on Prussian Blue film with chemically linked urease forming a monomolecular layer of the enzyme. This pH-enzyme optode-FIA system is useful for the selective determination of post-dialysate urea in the range of concentration corresponding to its level in real clinical samples (2–16 mmol l⁻¹). This bioanalytical system allows the analysis of about 15 samples of spent dialysate per hour. The operational and storage stabilities of the applied biosensor are longer than 2 weeks and 2 months, respectively. Clinical evaluation of the bioanalytical system was performed.

Introduction

Chronic hemodialysis is a common life-saving therapy for more than one million patients worldwide.¹ ² Patients affected by renal disorders undergo hemodialysis treatment 2–4 times every week. This therapy should be strictly controlled, as patient morbidity and mortality depend directly on its adequacy.³ The main goal of this clinical treatment is the removal of toxic metabolites from a patient’s body. As urea is the main product of protein metabolism, monitoring of its level in spent dialysate is a recommended approach for the control of the hemodialysis process.¹ ³ For such purposes, bioanalytical systems with urea enzyme electrodes⁴ ⁵ or urease reactors (columns or cartridges with the immobilized enzyme⁶ ⁷ have been developed. These are flow analytical systems based on potentiometric⁵ ⁷ or conductometric⁴ ⁸ detectors. The use of the bioanalytical systems permits individual prescription, administration and assessment of hemodialysis therapy.

In contrast to all these electrochemical devices, here an optical flow-through biosensor for post-dialysate urea detection is described. This absorbance biosensor based on a pH-sensitive film of Prussian Blue with covalently immobilized urease molecules⁹ works in a single-pass transmission mode of optical measurement. Its utility as a monitor of hemodialysis and the clinical evaluation of the developed bioanalytical system are demonstrated.

Experimental

Materials

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride and urease (EC 3.5.1.5, type IX, from jack bean) were obtained from Sigma (USA). 4-(Pyrol-1-yl)benzoic acid was purchased from Aldrich (Germany). A reagent kit for the reference method of urea determination was obtained from Hoffman–La Roche (France). All organic and inorganic reagents were of analytical-reagent grade and were used without further purification. Experiments were carried out using solutions prepared with doubly distilled water. All solutions were prepared immediately prior to use.

The composition of the standard liquid for hemodialysis was 0.140 mol l⁻¹ sodium, 0.002 mol l⁻¹ potassium, 0.001 mol l⁻¹ magnesium, 0.003 mol l⁻¹ calcium, 0.029 mol l⁻¹ total acetate, 0.035 mol l⁻¹ total carbonate and 0.111 mol l⁻¹ chloride. The liquid was produced on-line by a hemodialysis machine. Aseptic de-ionised water and two concentrates for preparation of the fluid were obtained from Karima (Poland).

Instrumentation and measurements

The preparation of urea-sensitive Prussian Blue-based biofilm has been reported previously.⁹ For optical measurements a polyester foil with the enzymatically modified pH-sensitive film was mounted so that it formed one wall of the flow-through cell detector. Both the bioanalytical FIA system and the flow-through biosensor are shown schematically in Fig. 1. Measurements were performed using a double-channel flow-injection manifold consisting of a Minipuls 3 peristaltic pump (Gilson Medical Electronics, France), a rotary injection valve and a flow-through optical cell (both laboratory-made). A rectangular 12 × 12 × 40 mm (external dimensions) cell made of transparent polystyrene was used for measurements with the urea biosensor. The total effective volume of the flow-through cell was 0.15 ml. Optical measurements with the urea biosensor were carried out at a wavelength of 720 nm (i.e., at the absorption maximum of Prussian Blue) using a Model 2401/PC spectrophotometer (Shimadzu, Japan). No reference cell was used. The flow rate and injection volume used were 1.85 ml min⁻¹ and 0.50 ml, respectively.

The bioanalytical system demonstrated in this paper was tested on hemodialysators models Secura, Dialog and Fresenius (Germany). Blood flow ranged from 200 to 250 ml min⁻¹. The flow rate of the dialysate fluid was 500 ml min⁻¹.

Reference determinations of urea in post-dialysate fluid samples were performed using the spectrophotometric urease–glutamate dehydrogenase coupled enzymatic method.¹⁰ Changes in absorbance resulting from enzymatic oxidation of NADH to NAD were measured at a wavelength of 340 nm. The rate of decrease in NADH concentration is directly proportional to urea concentration.

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Results and discussion

Performance of the bioanalytical system

The pH-sensitive sensor membrane utilized in the biosensor is composed of Prussian Blue doped with poly(N-pyrrollylbenzoic acid). The optical pH sensitivity of the film is associated with pH-dependent equilibria between fully isocyanico hexacoordinated and partially hydrated high-spin Fe(III) ions in Prussian Blue. These pH-dependent processes can be optically monitored at a wavelength of 720 nm, i.e., at the absorption maximum of Prussian Blue. As an optical pH indicator Prussian Blue works reproducibly and reversibly up to pH 8. In more alkaline solutions irreversible decomposition of the compound by hydroxyl ions is observed (Fig. 2). Ferricyanide anions are removed from the material and a yellow film composed of hydrated ferric oxide is formed. This effect is well observed at pH > 9.

The organic polymer plays a double role in the composite material. It is a binding agent for inorganic compounds leading to high mechanical stability of the film. Moreover, carboxylic groups of the organic component allow chemical binding of biomolecules to the film surface using a simple one-step carbodiimide method. The monomolecular layer of enzyme molecules linked covalently directly to the surface is completely transparent and does not change the optical properties of the composite film. This biomodified film was used for the construction of the flow-through optical urea biosensor applied in the demonstrated bioanalytical system (Fig. 1).

The specificity of both the enzyme and pH sensing with Prussian Blue leads to high selectivity of the urea biosensing. Unfortunately, the main drawback of this urea detector, typical of all kinds of pH-based biosensors, is the strong cross-sensitivity to the buffer capacity of the samples. Moreover, the samples should have nearly the same pH. The pH should be >5.5 because in more acidic solutions the biocatalytic activity of urease decreases drastically. On the other hand, the pH cannot be >8.0 as in more alkaline solutions decomposition of Prussian Blue is observed (Fig. 2).

The pH of dialysate fluid and post-dialysate samples varies from 7.4 to 8.3. Taking into account the composition of the dialysate (see Materials in the Experimental section), it can be concluded that the predominant protolytic form existing in this low buffer capacity as determined by the CO$_3^{2-}$/HCO$_3^-$ equilibrium (pK$_A$ = 6.4) rather than by HCO$_3^-$/CO$_2^+$ equilibrium (pK$_A$ = 10.4). This CO$_2$/HCO$_3^-$ buffer system is not stable. Differences in the pH of samples are caused mainly by the release of carbon dioxide and additionally by solutes removed from blood to the dialysate (hemodialysis is a convenient clinical way for suppressing of metabolic acidosis (hyperoxemia)). In the case of measurements with dialysis fluid used as a carrier buffer, a slow, continuous decay of absorbance of the sensing film was observed. Under such conditions, urea sensing caused a further decrease in the absorbance as the alkaline products of the enzymatic hydrolysis hastened the decomposition of Prussian Blue [Fig. 3(A)]. To prevent this destructive effect of pH, the second phosphate buffer line was incorporated into the FIA manifold (Fig. 1). It was found that 10 mM phosphate buffer composed of equal amounts of NaH$_2$PO$_4$ and Na$_2$HPO$_4$ is sufficient for suppressing differences in the pH of the samples. In such a case the baseline was stable, injections of urea samples did not cause the destruction of Prussian Blue film and the resulting analytical signals were highly reproducible [Fig. 3(B)]. The absorbance of the pH-sensing film without urease layer was not affected by injected post-dialysate samples. Both the buffer capacity and pH of the samples were stable, constant and well defined by the H$_2$PO$_4^-$/HPO$_4^{2-}$ buffer system. Under such conditions the biosensing system exhibits excellent operational stability and also higher sensitivity owing to the higher biocatalytic activity of the immobilized enzyme (the optimum pH for urease is near 7).

Clinical evaluation of the bioanalytical system

Once the buffer capacity and pH are well established and controlled, the responses of the urea biosensor are reproducible [Fig. 3(B)] and highly selective and not affected by alkaline cations, commonly existing anions and other substances transferred from patients’ blood to dialysate. Sometimes the standard liquid contained a lower level of calcium (0.002 mol l$^{-1}$). In the case of hemodialysis treatment prescribed for hyperkalemic patients, low-potassium (0.001 mol l$^{-1}$) dialysate fluid is often used. On the other hand, it is known that patients accumulate potassium during periods between dialyses and potassium removal is a major function of chronic hemodialysis. In spite of these changes in sample composition, in all cases the results of post-dialysate urea determinations with the biosensor are fully comparable to those obtained using the reference method recommended for the analysis of complex clinical samples. The correlation equation for the analysis of 90 various real post-dialysate samples (Fig. 4) was $y = 0.997 (± 0.007)x -0.073 (± 0.056)$ with regression coefficient $r = 0.998$ and standard deviation $s = 0.20$ mM.

The demonstrated biosensor–FIA system exhibits good operational stability. No decay of sensitivity was observed in the course of a measurement cycle consisting of calibration and monitoring of hemodialysis (Fig. 5). After monitoring of six successive hemodialysis treatments (one 4 h session every day, with over 60 injections/urea determinations for each), the same biosensing film was still useful as only a 5–10% decrease in the initial sensitivity of the system (evaluated for 10 mM urea standard) was observed.

A typical measurement cycle is shown in Fig. 5. The analytical responses for standards and samples are fast and reproducible. The corresponding calibration graph is shown in inset (A). The system offers a response in the urea concentration range from 1 to 16 mM (linear range up to 10 mM urea). This range fully covers urea levels found in the real clinical post-

Fig. 1 Scheme of FIA manifold: dialysate fluid (D), phosphate buffer (B), peristaltic pump (PP), injection valve (IV) for post-dialysate sample (PS), spectrophotometer (S) with flow-through cell (FTC; details in the inset) data storage/processing personal computer (PC) and waste (W).
dialysate samples (Fig. 4). The presented bioanalytical system can be used for the on-line evaluation of time profiles of urea levels in spent dialysate [Fig. 5, inset (B)].

These profiles are useful for the prediction and calculation of various biomedical parameters of hemodialysis described by the so-called urea kinetic model, such as $K/V$ and $KT/V$ (where $K$, $V$, and $T$ are the dialysator clearance, body water volume and total time of hemodialysis session, respectively) and urea reduction ratio. Additionally, urea removal and total urea removal can be simply evaluated by multiplying the area under the urea level–time curve by the flow rate of the effluent dialysate. If successive hemodialysis sessions are monitored, the results of such analyses are useful for the estimation of the urea generation rate and protein catabolic rate. In our previous work devoted to potentiometric biomonitoring of hemodialysis, we demonstrated how to calculate all these biomedical parameters. The parameters are necessary for the quantitative clinical description of both intradialytic and interdialytic intervals of the therapy. The spectrophotometric bioanalytical system presented here could be used for the control and modeling of hemodialysis in the same way. For example, the

![Fig. 2](image-url) Optical pH sensitivity of Prussian Blue film. pH values of universal buffer used in the experiment are given. The fast decomposition of the Prussian Blue film after exposure to an alkaline environment (pH 10) is readily observed. The corresponding calibration graph is shown in the inset. The range of pH evaluated according to the indicating (reversible) scheme of sensing is marked with a solid line.

![Fig. 3](image-url) Stability of the analytical signal for series of injections of urea standards prepared in dialysate fluid; 4.0 and 10.0 mM urea standards were injected in turn. The carrier was dialysate fluid only (A) or was composed of equal volumes of phosphate buffer (5 mM Na$_2$HPO$_4$ + 5 mM Na$_2$HPO$_4$ + 1 mM Na$_2$EDTA + 140 mM NaCl) and dialysate fluid (B).

![Fig. 4](image-url) Correlation of the results of post-dialysate urea determination obtained using the biosensor–FIA system and the reference method.

![Fig. 5](image-url) Run-time data trace of urea post-dialysate determinations in the course of a hemodialysis session. The standard dialysate fluid was used. The recording includes calibration of the system (the corresponding calibration graph is shown in inset (A)). Each urea standard and each sample of post-dialysate fluid were injected twice. Spent dialysate fluid was sampled every 15 min. Changes in the post-dialysate urea level measured using the biosensor (solid points) and the reference method (open points) are shown in inset (B) (line a). Line b in inset (B) represents urea removal in the course of the monitored hemodialysis session.
values of $K/V$, $KT/V$, percentage removal of urea and total urea removal for a hemodialysis session illustrated in Fig. 5 are 0.22 h$^{-1}$, 0.88 (dimensionless factor), 58.5% and 55.6 g of urea, respectively. Such control of hemodialysis using the much more expensive and more time- and work-consuming reference method recommended for clinical analysis\textsuperscript{10} leads to similar results (Figs. 4 and 5).

Conclusions

To the best of our knowledge, this is the first report on the application on an optical biosensor for the control of hemodialysis therapy. We would stress main advantages of the developed biosensor and bioanalytical system as follows. The pH-sensitive film is inexpensive, very stable and robust (no changes to the film were observed after 3 years of storage). The one-step enzyme immobilization procedure is inexpensive and very simple. The lifetime of the resulting biofilm is satisfactory (after 2 months of storage the activity of the immobilized enzyme under ambient conditions had not decreased). The shape of the flow-through cell detector is fully compatible with typical spectrophotometers. A reference cell is not necessary because analytical signal changes of the film absorbance (not absolute values) are measured. The (bio)sensing film is optically compatible with economic near-infrared light-emitting diodes. The demonstrated FIA system is very simple and only small amounts of cheap reagents are consumed in the course of analysis. Although, from the instrumental point of view, the demonstrated analytical system is simpler than these reported in the literature\textsuperscript{3–8} (*i.e.*, without any additional reference detectors, separators, reactors, thermostating units, *etc.*), it is fully acceptable for analytical needs connected with hemodialysis therapy.

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References