Development of Multi-parameter Analyser based on Electrochemical Urea Biosensors and Electrolyte Electrodes for Monitoring of Hemodialysis Patients

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Abstract: The idea of developing multi-parameter urea analyser comprising urea, Na^+ and K^+ selective electrodes has been considered. For this purpose the urea biosensors based on urease and recombinant urease working in amperometric and potentiometric way were developed. The working parameters of both urea biosensors were studied and optimized. Possibilities of real samples analysis using the developed biosensors were shown by measuring urea concentrations in blood dialysate taken from patients with renal failure. Both the potentiometric and the amperometric biosensors demonstrated high degree of signal reproducibility (the relative standard deviation of responses did not exceed 5 %). Change of sodium and potassium concentrations during blood hemodialysis is dangerous life-threatening condition and their monitoring is an important feature of point-of-care analyser. For this purpose high integrity commercial Na^+ and K^+ selective electrodes were analysed and our own signal amplification and processing system proposed.

1 INTRODUCTION

Urea is the final product of protein metabolism and it is synthesized in the liver as a result of amino acid deamination (Kuralay et al., 2005). Excessive urea in organism is excreted by renal system during blood filtration, and elevated levels of urea concentration in blood or serum usually indicate dangerous kidney disease. Regular level of urea in serum varies from 15 to 40 mg/dl (2.5 – 6.7 mM), while in patients suffering from renal failure urea concentrations in serum can reach 180 – 480 mg/dl (30 – 80 mM), and patients with such elevated concentrations have to undergo blood dialysis treatment (Dhawan et al., 2009). It is a dangerous condition – 5 year survival of men older than 64 years who are starting dialysis is worse than that of men with colon cancer and prostate cancer (Parfrey and Foley, 1999).

For such reasons the methods for assessment of urea concentration in blood, serum and spent dialysate solutions are being developed. Urea measurements are important in medical diagnostics for clinical evaluation of renal function and monitoring the effectiveness of dialysis treatment. One of the first methods for urea determination was based on spectrophotometric measurements after sample treatment with specific compounds leading to coloured solution with distinctive spectra (Patton and Crouch, 1977; With et al., 1961). Such methods are fairly accurate and are being used in medical practice, but they are not suitable for real-time sample analysis.

Alternative technologies for urea measurements being developed are based on biosensor electrodes. They have different design approaches such as potentiometric (Kuralay et al., 2005, Liu et al., 1993; Ahuja et al., 2011; Boubriak et al., 1995), conductometric (Soldatkin et al., 2014; Chen et al., 1994; Sangodkar et al., 1996) and amperometric (Sangodkar et al., 1996; Tiwari et al., 2009), they usually employ urease (EC 3.5.1.5) as a catalyst for urea breakdown, which is immobilized by a number of methods on electrode surface (Dhawan et al., 2009). The urease catalyses the hydrolysis of urea to

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yield ammonia and carbamic acid (equation 1) which spontaneously decomposes into carbonic acid and a second ammonia molecule (equation 2) (Carter et al., 2009), as shown below:

$$H_2N\text{-}C(O)\text{-}NH_2+H_2O+H^+ \xrightarrow{\text{Urease}} NH_4^+ + H_2N\text{-}COOH \quad (1)$$

$$H_2N\text{-}COOH+H_2O \rightarrow NH_4^+ + HCO_3^- \quad (2)$$

The principle of enzyme based action of amperometric and potentiometric detection systems are presented in figure 1.

Monitoring of electrolyte composition in blood during dialysis treatment is also very important. Serum electrolytes (sodium, potassium, calcium, phosphate) are usually elevated in chronic dialysis patients, they have a high survival risk ratio (Iseki et al., 1996). While sodium and potassium can be auto-regulated during long dwell continuous ambulatory peritoneal dialysis (CAPD) (Nolph et al., 1980), patients undergoing hemodialysis exhibit high incidence of cardiac arrhythmias during dialysis days particularly due to sudden imbalance of blood electrolytes (Ramirez et al., 1984). While the strategies of retaining sodium and potassium levels in blood and extracellular fluid are being constantly developed (Much and Wilcox, 1982; Maduell et al., 2013; Mc Causland et al., 2012), real-time monitoring of sodium and potassium concentrations during hemodialysis could be crucial to determine rapid change of such electrolytes in order to take according revival actions.

The purpose of the study is to find best sensor candidates for point-of-care urea, sodium and potassium analyser, useful for monitoring of patients undergoing hemodialysis treatment. The electrodes and bioelectrodes (biosensors) used in such equipment should be accurate, not expensive, stable, simple to use in field measurement, etc. In case of possible urea biosensor, our scientific group until recently has been developing several types of urea biosensors (Boubriak et al., 1995; Soldatkin et al., 2014; Mc Causland et al., 2012; Laurinavicius et al., 2013). In this case a specific attention is paid to previously developed and published potentiometric (Kulys et al., 1986) and amperometric (Mc Causland et al., 2012) urea biosensors that were further developed, as described in this work. In case of sodium and potassium measurements, we will adapt to our needs one of many commercially available electrolyte measurement system, consisting of potentiometric flow-through electrode cell. It is used in several electrolyte analysers, which electronics and signal analysis algorithms are hardcoded into microcontrollers, subject to copyright material, so in this study we will devise our own signal amplification and processing system.

2 EXPERIMENTAL

2.1 Chemicals and Reagents

In this work the enzyme used for assembly of urea amperometric biosensors was urease from *Canavalia ensiformis* (E.C. 3.5.1.5.), activity of 343.0 U/mg from Calbiochem (Germany). The enzyme substrate was used as the phosphate buffer solution, pH 7.2, containing 1 M of urea. Thermally reduced graphene oxide (TRGO) was used as electrode materials. TRGO have been synthesized by us as proposed in the protocol (Razumiene et al., 2015).

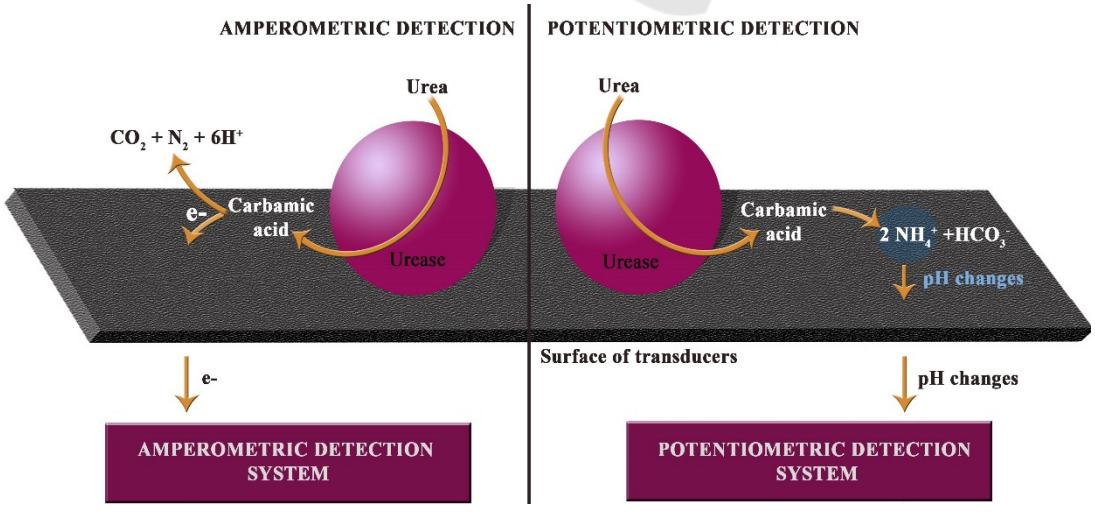

Figure 1: The principle of enzyme based action of amperometric and potentiometric urea detection systems.

For potentiometric biosensor creation recombinant urease (R. urease, E.C. 3.5.1.5) from USBiological (USA) expressed in *E. coli*, was used, its activity was 150 U/mg. Bovine serum albumin (BSA, fraction V) and urea were obtained from Sigma-Aldrich Chemie; poly(vinyl alcohol) containing styrylpyridinium (PVA-SbQ) from Toyo Gosei Kogyo Co. Ltd (Japan). The working phosphate buffer (KH_2PO_4-NaOH), pH 7.4, was prepared by using reagents from Helicon (Moscow, Russia). Sensor chips with differential pair of pH-sensitive field effect transistors produced at the JSC "Kwazar" facilities (Kiev).

The samples of blood dialysate and serum for the potentiometric measurements of urea content were obtained from Kiev municipal scientific and practical centre of nephrology and hemodialysis (Ukraine). All others chemical reagents were obtained from Sigma-Aldrich and were of analytical grade unless otherwise mentioned.

2.2 Construction and Electrochemical Measurements of Biosensors

2.2.1 Amperometric Urea Biosensors

Amperometric measurements were performed using an electrochemical system PARSTAT 2273 (Princeton Applied Research) with a conventional three-electrode system composed of an auxiliary platinum plate electrode, a reference Ag/AgCl electrode and working TRGO (Ø 3 mm) electrodes as transducer for amperometric biosensor (Razumiene et al., 2015). Aiming to design amperometric biosensor TRGO were extruded by forming a tablet. The tablet was sealed in a Teflon tube then tablet surface was covering by the semipermeable terylene membrane containing immobilized urease from *Canavalia ensiformi*. The response of the prepared amperometric biosensors to the addition of substrate was investigated under potentiostatic conditions at +200 mV (vs. Ag/AgCl) in a stirred phosphate buffer solution, pH 7.2, 20 ^{o}C.

2.2.2 Potentiometric Urea Biosensor

Potentiometric biosensor was based on pH-sensitive field effect transistors as current transducers (Sheliakina et al., 2014; Pavluchenko et al., 2011). Each transducer contained a differential pair of pH-sensitive field effect transistors placed on a single crystal with the total area of 8 mm × 8 mm. Signals were recorded from both transistors and then signal from reference transistor (covered with BSA membrane) was subtracted from the signal of transistor covered with enzyme membrane. Transistors demonstrated pH-sensitivity of approximately 40 mV/pH and transconductance of 400–500 mkA/V. More information about transistor structure and their photo can be found in (Sheliakina, 2014) and description of a portative measuring device – in (Pavluchenko et al., 2011).

A bioselective membrane on the transducer surface was formed by immobilisation of R. urease in PVA/SbQ photopolymeric membrane. 66 % of PVA/SbQ and 10 % of R. urease were mixed at 1:1 ratio and the mixture (0,1 µl) was deposited on the surface of the ISFET. Then sensor chip was exposed under the UV lamp KF-4M (Ukrainian production) of 3.4V/m^2 for 20 min.

Measurements were carried out in the 5 mM potassium phosphate buffer solution (KH_2PO_4-NaOH), pH 7.4, with intensive stirring at room temperature. The biosensor and Ag/AgCl reference electrode were placed into an open 1.5 ml measuring cell. The urea concentrations in the working cell were obtained by the addition of aliquots of concentrated stock solution or real samples.

2.2.3 Na^+ and K^+ Selective Electrodes – Cell Construction and Signal Processing

Measurements of electrolytes – sodium and potassium ions – concentration in blood or standard solutions were performed by using commercially available potentiometric Sensa K and Sensa Na electrodes in dedicated five electrode cell containing integrated Sensa reference electrode, bubble detector and three blank electrodes, forming a single flow-through channel for measurement of solutions of interest. The whole set was purchased from Sensa Core Medical Instrumentation Pvt Ltd, India. The idea of the study was to not use any dedicated commercial electronic amplification and signal analysis equipment, thus such system was designed from ground up.

The voltamperometric measurements of electrodes revealed that the resistance of sodium, potassium and reference electrodes was in range of 4 – 10 MΩ, thus two stage, closed loop operational amplifier (op-amp) based circuit was used to amplify the signals – one op-amp was used to follow the changes of electrode potential difference without voltage gain, and the output signal was further amplified at 11-fold gain by second op-amp. The gain level was selected to allow optimal voltage measurements with 18-bit analogue-to-digital converter (ADC) (for example, MCP3424 from

Microchip Technology Inc.), capable of measuring potential differences up to 3.3 V in our electronic setup. To avoid electromagnetic interference, op-amp circuit was encased in dedicated small metal shield box and placed on top of electrode contacts. The constructed urea analyser employs ADCs with dedicated computer hardware and software for signal analysis and implementation of measurement algorithms; however, in this study we used bench type UT804 multimeter with USB data output (Uni-Trend Group Limited, Hong Kong) for analysis of amplified signals.

The measurements were performed by following procedure. First, the electrode cell was assembled and connected; inner fluid path and tubing were rinsed by de-ionised water. Then the solution of interest was aspired into fluid channel and amplified output signal (voltage) was recorded by computer software controlled multimeter. The fluid path was rinsed by de-ionised water between measurements, after work the electrodes were disassembled, their channels dried and stored in room temperature. In case of sodium ions concentration measurements, standard referenc solutions with NaCl concentrations 100 – 200 mM were used to measure potential difference dependence on electrolyte concentration. In case of potassium ions concentration measurements, KCl solutions from 2 to 7 mM were used, they also contained 150 mM NaCl in order to maintain ionic strength. In blood measurements, equal amounts of solutions with different NaCl or KCl concentrations or pure water were added to blood samples. In result several batches of blood samples were obtained with varying concentration of sodium and potassium by 10 or 1 mM, respectively.

3 RESULTS

3.1 Study of Amperometric Urea Biosensor

Amperometric biosensor after addition of urea into electrochemical cell shows substrate-dependent anodic response. The biosensor response was fast: 90% of steady state current achieved in 20 s. The response was measured as a difference between the steady state and the background current. The urea calibration curve with marked linear range is shown in figure 2 and main characteristics of the biosensor are shown in Table 1.

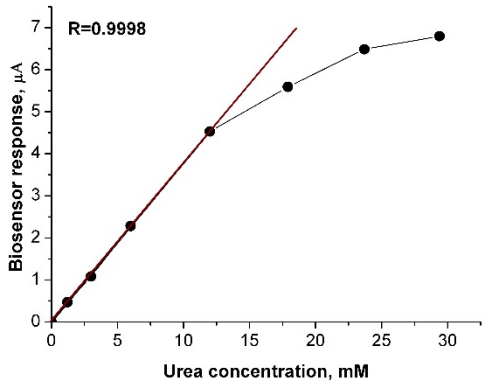

Figure 2: The urea calibration curve and the linear range (solid line) obtained using the amperometric biosensor. Applied electrode potential of 0.2 V, phosphate buffer solution, pH 7.2.

Operational stability of the biosensors was tested by consecutive measurements of the current response to urea solution of 3 mM. At least twenty measurements of the biosensor activity per day were done during three weeks. Between the experiments the biosensor was stored at room temperature. The averaged data are shown in figure 3.

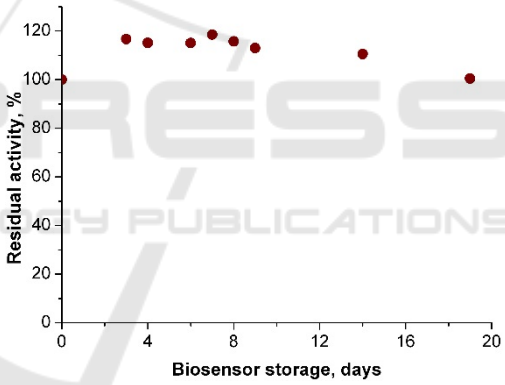

Figure 3: Operational stability of the amperometric biosensor.

As can be seen in figure 3 after a period of three weeks the decreasing of sensitivity of the amperometric biosensor was negligible.

The amperometric biosensor has been tested for urea measurements in dialysate as well. Aiming to validate responses of the biosensor measurements the samples of dialysate in parallel were examined at the hospital laboratory. The testing has been carried out by investigating dialysate of four patients after one hour of hemodialysis. Urea concentration data obtained by both methods are presented in figure 4.

The results shown in figure 4 exhibited good correlation between two methods.

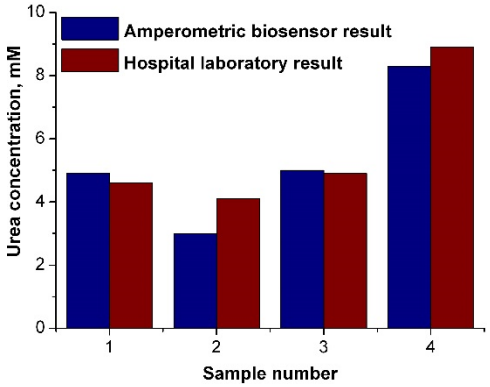

Figure 4: Comparison of results of urea determination in dialysate after one hour of hemodialysis. The dialysates of four patients were examined by the amperometric biosensor and in the hospital laboratory.

3.2 Study of Potentiometric Urea Biosensor

Firstly an investigation of analytical characteristics of the potentiometric biosensor based on R. urease at determination of urea concentration in model solutions was done. The calibration curve for urea determination by the potentiometric biosensor is presented in figure 5 and main characteristics of the biosensor are shown in Table 1.

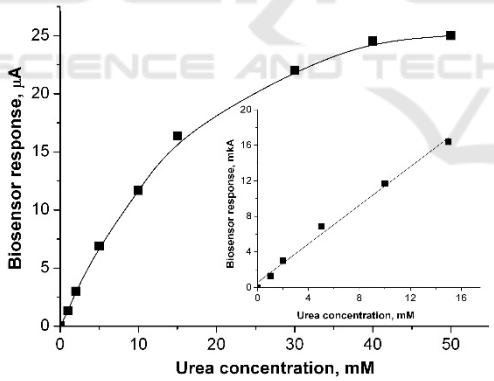

Figure 5: The urea calibration curve – dependence of current versus final urea concentration. Inset shows measurements in linear response region. Measurements were performed in 5 mM potassium-phosphate buffer, pH 7.4, at room temperature.

The signal reproducibility of the biosensor was studied, in order to verify electrode suitability for measurement of urea concentration both in model solution and real samples. For this task the biosensor responses to urea concentration of 5 mM in model solution and 150 μl of the serum or blood dialysate in working buffer were recorded during one workday at 30 minutes intervals. As shown in Figure 6, potentiometric biosensor demonstrated high degree of signal reproducibility in all cases (the relative standard deviation of responses did not exceed 5 %).

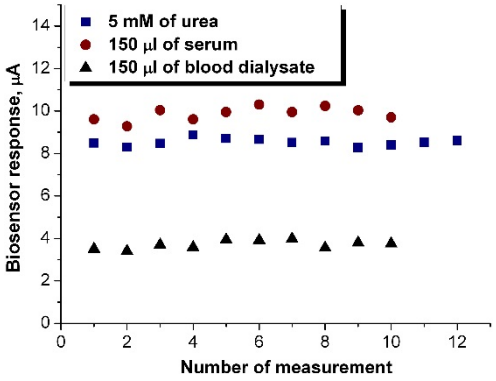

Figure 6: Reproducibility of responses of potentiometric biosensor to model and real samples. Measurements were performed in 5 mM phosphate buffer, pH 7.4, at room temperature.

The developed biosensor based on R. urease was tested by the analysis of samples of blood dialysate taken from patients with renal failure. For this task the concentration of urea was analyzed in 10 samples of blood dialysate and in 10 samples of serum. The samples were added to the working cells (10-fold dilution), the responses of biosensor were measured and compared with the previously plotted calibration curve.

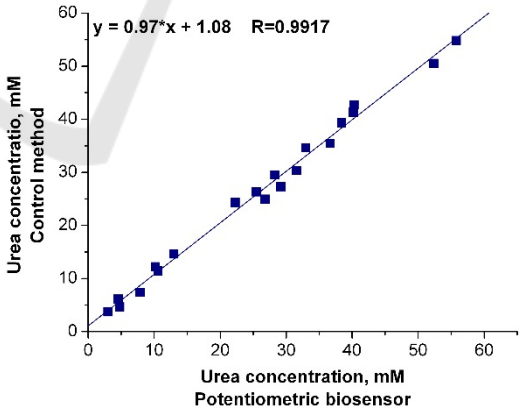

Figure 7: Correlation between the data on urea concentration in real samples obtained by biosensor and control method.

To check the accuracy of urea determination by the biosensor, the samples were analyzed in a diagnostic laboratory by the control method - the commercial Vitros 250 (Johnson & Johnson) analyzer, based on "dry chemistry" technology – urea

content being measured by using urease reaction with colorimetric detection of produced ammonia. The results of biosensor analysis and those obtained by control method are shown in figure 7. The study revealed high correlation between methods used (R = 0.9917).

3.3 Characterization of Amperometric and Potentiometric Urea Biosensors

The most important characteristics in terms to apply the amperometric or potentiometric biosensors in analytical device are summarized in Table 1.

Table 1: Analytical characteristics of amperometric and potentiometric urea biosensors.

Biosensor characteristic	Amperometric	Potentiometric
Linear range, mM	0.1-12	0.5-15
Operational range, mM	0.1-30	0.1-40
Detection limit, mM	0.1	0.1
Response reproducibility (relative standard deviation of responses), %	2-3	3-5
Response time, s	20	60-120
Sensitivity after 20 days, %	100	110
Dilution of the sample	10-20	10
References	Present work	Marchenko et al., 2015 and present work

Comparison of characteristics of the amperometric and the potentiometric biosensors shows that both electrodes have adequate linearity and operational range, detection limit and reproducibility, which means they both could be considered as good candidates for commercialization.

3.4 Studies of Na^+ and K^+ Selective Electrodes

Commercial sodium and potassium electrodes were used to analyse samples of standard solutions and blood. This study was essential to mimic the measurement procedure hardcoded into electrolyte analysers which algorithms are sensitive copyright material. However, the commercial electrodes we purchased are being used in various applications, each manufacturer having their own algorithms, and, as consulted by representative of manufacturer, it is legal to use them as long as one follows recommended sample measurement procedure, which is similar to ours as described before.

Consequently, the purpose of our study is to analyse dependences of electrode potentials on electrolyte concentrations in various solutions, also to determine if our selected signal processing and amplification method is adequate to obtain readable data. Since the manufacturer provides calibration information about each electrode and is responsible for the quality of the product, we assumed that it is of minor importance to perform extended time dependent electrode response repeatability measurements.

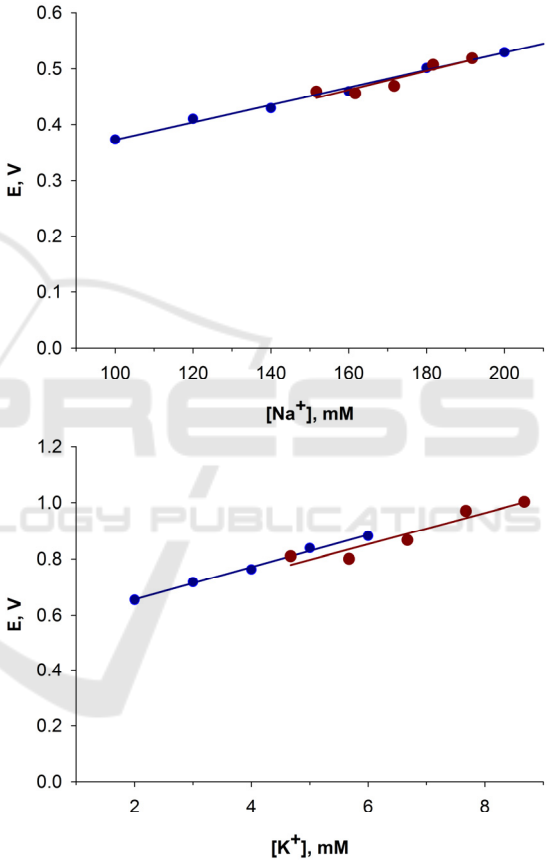

Figure 8: Sodium (top) and potassium (bottom) ion selective electrode 11-fold amplified response dependence on concentration. Standard solutions with known concentrations are marked blue, blood with incremental additions of sodium and potassium by 10 or 1 mM, respectively, are marked red. Concentration of sodium or potassium in unaltered blood samples are calculated from measured potential and linear function from measurements of standard solutions.

The measurements revealed that voltage signals were stable (noise floor less than 2 mV of amplified signal), immune to electromagnetic interference. In

fact, when one stage closed loop op-amp system with direct 11-fold gain was used instead of two stage system, signals were stable as well. After aspiration of solution into electrode path, the signal in either electrode setup asymptotically approaches stability in 2 – 5 minutes in exponential decay manner, at about 40 seconds the signal reaches 95 % of its final value. For our study the responses were mathematically fitted by exponential decay function, final offset values were calculated and used in analysis.

Typical dependencies of amplified voltage difference signals on electrolyte concentration from measurements of blood and standard solutions are presented in figure 8.

The study revealed that Na^+ and K^+ selective electrodes responses exhibit linear concentration dependence, as long as concentration range is within the ones present in physiological media. The slope values both in cases of standard and blood solutions are similar. To estimate the best value, the device algorithm should preferably contain exponential decay minimization procedure; however, measurement after waiting for about 40 seconds from sample aspiration should be adequate.

3.5 Insights into Construction of Multi-Parameter Urea Analyzer

Currently, urea measurement in blood and dialysate samples are performed in laboratory using commercial cumbersome equipment, the test itself is termed and standardized as blood urea nitrogen (BUN) measurement. Implementation of urea biosensors (Dhawan et al., 2009) gives promising leads to development of standalone point-of-care compact urea measurement devices. Consequently, our group together with colleagues has been developing commercially viable biosensor designs for about three decades (Boubriak et al., 1995; Soldatkin et al., 2014; Maduell et al., 2013; Mc Causland et al., 2012; Laurinavicius et al., 2013; Kulys et al., 1986; Marchenko et al., 2015). This study compares our best candidates to incorporate into such device. Comparison of characteristics of the amperometric and the potentiometric biosensors shows that both electrodes have adequate linearity and operational range, detection limit and reproducibility, which means they both could be considered as good candidates for commercialization. However, several features of the amperometric sensor such as short response time and higher accuracy due to better linearity in relevant concentration range, as well as technical benefits (lower sensitivity to background noise, ease of production, almost complete insensitivity to pH, etc.), not mentioned in this study, make the amperometric urea sensor a better option at this stage of designing of the multi-parameter analyzer.

The study revealed that Na^+ and K^+ selective electrode system is suitable for integration to device as well. The signal amplification and analysis techniques have been developed by technical cues implied in sodium and potassium electrode cell design. However, the amperometric biosensor and ions selective electrode systems are of different design nature and should be developed as different parts of the multi-parameter analyzer. Specific care must be taken when designing specimen and liquid handling system, keeping in mind that electrical amplification circuits would be separate – liquids from both cells should not touch in order to avoid signal interference from occurring common electric plane.

4 CONCLUSIONS

The amperometric and potentiometric urea biosensors based on urease and recombinant urease were developed and possibilities of the real samples analysis using the developed biosensors were shown. The working parameters of the urea biosensors were studied and optimized. Linear dynamic range of the potentiometric urea determination was 0.5 – 15 mM, detection limit - 0.1 mM. Urea concentrations were determined in 20 samples of blood dialysate and serum taken from patients with renal failure; the potentiometric biosensor demonstrated a high correlation of the results with the control method of urea determination. Linear range of the amperometric urea determination was 0.1 – 12 mM and detection limit of 0.1 mM. The amperometric biosensor has been tested for urea measurements in dialysate and results correlated with data obtained in the hospital laboratory. Both biosensors – the potentiometric and the amperometric demonstrated high degree of signal reproducibility in all cases (the relative standard deviation of responses did not exceed 5 %). Thus, both biosensors studied in this research can be effectively used to diagnose the patients with renal failure and to control the urea content in blood or dialysis fluid during hemodialysis.

In this study the commercial sodium and potassium ion selective electrodes were analysed aiming to integrate them in to the designing analyser. It was not used any dedicated commercial electronic amplification and signal analysis equipment, thus, such system was designed from ground up. The

dependences of electrode potentials on electrolyte concentrations in various solutions revealed that our selected signal processing and amplification method is adequate to obtain readable data.

Considering analytical and technical features of the biosensor designs, it seems that benefits of the amperometric sensor hold the edge over choosing the latter in designing the commercial analyser. Together with electrolyte electrodes, such multi-parameter point-of-care blood and dialysis fluid analyser would help in better outcomes and hemodialysis procedure corrections for patients diagnosed with various stage renal failures.

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