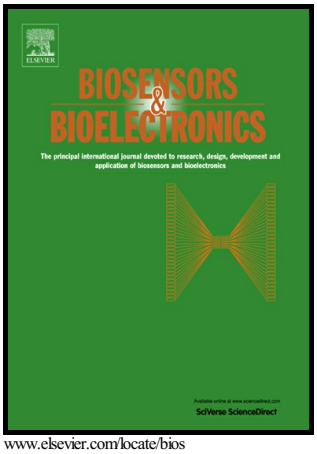
Creatinine and urea biosensors based on a novel ammonium ion-selective copper-polyaniline nano-composite

M. Zhybak, V. Beni, M. Vagin, E. Dempsey, A.P.F. Turner, Y. Korpan



www.elsevier.com/locate/bios

PII: S0956-5663(15)30474-7
DOI: http://dx.doi.org/10.1016/j.bios.2015.10.009
Reference: BIOS8045

To appear in: *Biosensors and Bioelectronic*

Received date: 22 July 2015
Revised date: 29 September 2015
Accepted date: 3 October 2015

Cite this article as: M. Zhybak, V. Beni, M. Vagin, E. Dempsey, A.P.F. Turner and Y. Korpan, Creatinine and urea biosensors based on a novel ammonium ion-selective copper-polyaniline nano-composite, *Biosensors and Bioelectronic* http://dx.doi.org/10.1016/j.bios.2015.10.009

Creatinine and urea biosensors based on a novel ammonium ion-selective copper-polyaniline nano-composite

M. Zhybak[1, 2], V. Beni[2*], M. Vagin[3], E. Dempsey[4], A. P. F. Turner[2], Y. Korpan[1**]

[1]*Laboratory of Biomolecular Electronics, Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, 03680, Kyiv, Ukraine;*

[2]*Biosensors & Bioelectronics Centre, IFM, Linköping University, 58183, Sweden*

[3]*Applied physics division, IFM, Linköping University, Linköping, 58183, Sweden*

[4]*Centre for Research in Electroanalytical Technologies, Department of Science, ITT Dublin, Tallaght, Dublin 24, Ireland;*

Corresponding authors

*Valerio Beni

Email: valbe@ifm.liu.se

Phone: +46(0)13 286629

**Yaroslav I. Korpan

Email: korpan@imbg.org.ua

Phone: +380445222824

Abstract

The use of a novel ammonium ion-specific copper-polyaniline nano-composite as transducer for hydrolase-based biosensors is proposed. In this work, a combination of creatinine deaminase and urease has been chosen as a model system to demonstrate the construction of urea and creatinine biosensors to illustrate the principle. Immobilisation of enzymes was shown to be a crucial step in the development of the biosensors; the use of glycerol and lactitol as stabilisers resulted in a significant improvement, especially in the case of the creatinine, of the operational stability of the biosensors (from few hours to at least 3 days). The developed biosensors exhibited high selectivity towards creatinine and urea. The sensitivity was found to be 85±3.4 mA M^{-1} cm^{-2} for the creatinine biosensor and 112±3.36 mA $M^{-1}cm^{-2}$ for the urea biosensor, with apparent Michaelis-Menten constants (K_M^{app}), obtained from the creatinine and urea calibration curves, of 0.163 mM for creatinine deaminase and 0.139 mM for urease, respectively. The biosensors responded linearly over the concentration range 1 to 125 µM, with a limit of detection of 0.5 µM and a response time of 15 s.

The performance of the biosensors in a real sample matrix, serum, was evaluated and a good correlation with standard spectrophotometric clinical laboratory techniques was found.

Keywords: creatinine, urea, amperometric ammonium detection, copper polyaniline composite

1. INTRODUCTION

Chronic kidney disease (CKD) is a common condition with a high risk of death. The National Health and Nutrition Examination Survey in America has estimated the prevalence of the CKD to be about 16% (Haynes and Winearls 2010). One of the main consequences of CKD is a significant increase in the level of metabolic waste products in the blood, including urea and creatinine, , that are normally excreted by the kidney (Vaidya *et al*. 2008). In CKD patients, urea and creatinine levels can reach up to 10-fold those recorded in healthy patients, rising from 1.7 – 8.1 mM (Eggenstein *et al*. 1999) to 50 – 70 mM for urea (Walcerz *et al*. 1998) and from 40-150 µM to 1-1.4 mM for creatinine (Ashwood *et al*. 2006). Consequently, frequent creatinine and urea monitoring, with rapid availability of results, can be expected to be of significant benefit for critically ill patients with remarkable improvement in quality of life for peritoneal dialysis patients (Udy *et al*. 2009).

Detection of creatinine dates back to the beginning of the 20th century when Jaffe method was developed (Jaffe 1886). However, this approach is quite complex, time consuming and has limited sensitivity/specificity. Ongoing demand for simple-to-use, cost effective and decentralised diagnostic tools for clinical practice has significantly boosted the development of alternative methods. In this context, over the past few decades, electrochemical sensors/biosensors have been making inroads. Among the electrochemical biosensors developed, potentiometric transduction based on the combination of ion-selective electrodes and ammonium ion-generating enzymes, is the most common approach used(Magalhaes and Machado 2002; Osaka *et al*. 1998; Radomska *et al*. 2004a; Radomska *et al*. 2004b). The first example of a potentiometric biosensor for the detection of creatinine was proposed by Meyerhoff and Rechnitz, who used creatinine deiminase (CDI) coupled with an ammonia gas-sensing electrode (Meyerhoff and Rechnitz 1976). Latter, CDI immobilised in a bovine serum albumin (BSA) (Soldatkin *et al*. 2002a) and PVA/SbQ membrane (Soldatkin *et al*. 2002b) was used in combination with ion-sensitive field-effect transistors (ISFETs) for creatinine detection and hemodialysis control (Zinchenko *et al*. 2012). However, these biosensors suffer from the inherent limitations of potentiometry, i.e. vulnerability to interference from other ions present in the sample solutions, relatively slow response times, and high detection limits in biological fluids (Lad *et al*. 2008).

In order to overcome the limitations of these potentiometric sensors, several authors have explored the use of multiple enzymes systems in combination with amperometric detection. One of the first examples of this approach was proposed in a paper by Tsuchida and Yoda (Tsuchida and Yoda 1983) in which the combination of three enzymes; creatininase, creatinase and sarcosine oxidase, was used for the amperometric detection of creatinine. Electrochemical transduction was achieved via the oxidation of the hydrogen peroxide, generated as part of the enzymatic reaction, at a platinum electrode. A similar enzymatic reaction sequence was explored by Nguyen *et al.* (Nguyen *et al*. 1991), who coupled the three-enzyme system with a Clark oxygen electrode. Schneider *et al.* (Schneider *et al*. 1996) and Erlenkotter *et al*.(Erlenkotter *et al*. 2002) reported a poly(carbamoyl)sulfonate (PCS) hydrogel biosensor that presented significant improvement in storage stability (ca. 3 months at 8 C) and dynamic range. The use of nano-composite electrodes based, for example, on zinc oxide or iron oxide nanoparticles or carbon nano-tubes, has been also explored as a way of improving the performance of these biosensors (Pundir *et al*. 2013). However, the electrochemical detection of oxygen and/or hydrogen peroxide continued to present several limitations in terms of sensitivity and specificity.

In order to overcome these issues, the use of mediators together with the three-enzyme cascade system was explored. Ramanavicius (Ramanavicius 2007) was one of the first to report on the use of a mediator, ferricyanide, for a creatine biosensor. This was despite the fact that mediated electron transfer from sarcosine oxidase had been reported by a several authors earlier on (Taniguchi *et al*. 1988; Turner *et al*. 1987). However, the dominance of point-of-care analysers (where sensors are required to be re-used) as opposed to home-use devices for this particular analyte has meant that this approach has not found favour to date. This led authors to explore the use of a four enzyme system in which peroxidase (horseradish peroxidase) was added as biocatalyst for hydrogen peroxide to promote more effective electron transfer (Nieh *et al*. 2013). Unfortunately, when multiple reactions are run in the same membrane, optimal conditions for each reaction can often not be satisfied and this means that in practice, none of the reactions runs at its optimum in terms of reaction rate and stability (Ricca *et al*. 2011).

Unlike creatinine biosensors, urea biosensors are commonly constructed using only one enzyme, urease (E.C. 3.5.1.5), as the biorecognition component, which catalyses the hydrolysis of urea to ammonium and bicarbonate ions (Singh *et al*. 2008). Among the electrochemical approaches used for urea determination, potentiometry is one of the most popular; several types of transducers, like CO_2 electrode (Guilbault and Shu 1972), ammonia gas-selective electrodes (Guilbault and Tarp 1974), or ISFETs (Munoz *et al*. 1997; Senillou *et al*. 1999), have been used to develop potentiometric urea biosensors. Guilbault and Montalo were the first to fabricate a potentiometric urea enzyme biosensor (Guilbault and Shu 1972; Guilbault and Tarp 1974) based on a cation-selective glass electrode for detection of ammonium ion activity. The original glass electrode was

soon replaced with a more selective, neutral carrier-type ion-selective ammonium electrode (Eggenstein *et al*. 1999; Walcerz *et al*. 1998). Given that the enzymatic hydrolysis of urea causes an increase in pH of the system, pH-sensitive ISFETs have been widely used for this detection (Dhawan *et al*. 2009). As already mentioned above, potentiometric approaches pose several drawbacks and to avoid these a number of amperometric urea biosensors have been developed over the last two decades.

Conducting polymers, like polyaniline (PANi) and polypyrole, have become the materials of choice for recent technological advances in amperometric sensors for urea detection. Adeloju *et al*. (Adeloju *et al*. 1996, 1997) reported on the use of polypyrrole-entrapped urease as an amperometric urea biosensor. The linear range of this biosensor was between 50 and 250 μM, but it suffered from significant limitations when applied to biological samples, requiring pretreatment with anion exchange separators for the removal of interferants (Rajesh *et al*. 2005).

Polyaniline-Nafion composites have been used to develop amperometric urea biosensors (Cho and Huang 1998), both on Au (Luo and Do 2004) and Pt support electrodes (Stasyuk *et al*. 2012). Experimental work over the past decade has shown that PANi-film, deposited on a metallic electrode (Au, Pt), and in combination with a cation exchange matrix (Nafion), is highly sensitive to ammonium ions (Strehlitz *et al*. 2000) .

In the current paper, we report the use of a novel ammonium ion-selective composite material, based on polyaniline and copper (Zhybak *et al*. 2015) for use as a transducer in electrochemical hydrolase-based urea and creatinine biosensors. The experimental designs and analytical performance of the biosensors are detailed together with their application for the determination of creatinine and urea in serum samples from patients with CKD.

2. MATERIAL AND METHODS

2.1 Reagents

Creatinine deiminase (EC 3.5.4.21, microbial, ≥25 U/mg) was purchased from Sorachim (Spain). Urease (EC 3.5.1.5, from *Canavalia ensiformis* (Jack Beans), ≥600,000 U/g), urea, creatinine (anhydrous), hydrochloric acid, copper nitrate, nitric acid, sodium chloride, KH_2PO_4, Na_2HPO_4, 5% Nafion® perfluorinated resin solution, glutarealdehyde (GA) (50% w/v aqueous solution), ethanol, D-Lactitol monohydrate, glycerol, bovine serum albumin (fraction V, 96%) were purchased from Sigma-Aldrich (Germany). Aniline (99%) was purchased from Sigma-Aldrich (Germany) and redistilled before use (**Important:** only freshly prepared aniline should be used for electropolymerisation). Ammonium chloride was purchased from Fluka (Germany). All the chemicals and solvents used were of analytical-reagent grade and used without further purification.

20 mM phosphate buffer solution (PBS) (prepared by mixing and diluting 20 mM disodium hydrogen phosphate, 20 mM potassium dihydrogen phosphate and 20 mM sodium chloride) pH 7.4,

was used as the supporting electrolyte during all the electrochemical measurements. The composition of the buffer solution was chosen in order to mimic, in terms of electrolyte composition, those of physiological samples. All solutions were prepared using 18.2 MΩ purified water produced using a Simplicity water purification system (Millipore, France). All glassware and polyethylene materials were rinsed with ultrapure water and dried before use.

2.2 Electrochemical measurements

All biosensors were built using three-electrode screen-printed platforms (DS C110; "SPE") with a carbon working electrode (4 mm of diameter) a carbon counter and an Ag pseudo-reference electrode.

Amperometric measurements were performed using a portable potentiostat (μStat 400, "DropSens", Spain), controlled by the DropView 2.0 software supplied by DropSens and used in accordance with the producer's manual guide. Amperometric detection was performed at -0.35 V vs the internal Ag pseudo-reference electrode and under vigorous stirring.

Differential pulse voltammetric measurements (DPV) were carried out using an Autolab model PGSTAT 30 potentiostat/galvanostat controlled with the General Purpose Electrochemical System software program (Eco Chemie, The Netherlands). DPV measurements were performed according to the following protocol: equilibration pretreatment 300 seconds at -0.3 V; measurement potential range: 0.6 to -0.5 V (modulation time 0.15 s; interval time 0.6 s; step potential 0.004 V; modulation amplitude 0.01 V). The use of a pretreatment step (-0.3 V for 300 s) prior to DPV measurement was adopted to improve the reproducibility (SD ca. 4% for 3 repetitions) of the experimental responses. All DPV measurements were performed without stirring.

Prior to use, the biosensors were soaked in PBS for 30 minutes at room temperature to equilibrate the sensing composite. The sensor response to a particular substrate concentration was taken as the average of three separate measurements.

2.3 Electrode modification with PANi-Nafion-Cu composite

Preparation of the PANi-Nafion-Cu SPE was carried out according to the protocol proposed by the authors and described in detail in a prior manuscript (Zhybak *et al*. 2015). Briefly: (i) SPEs were modified with Cu by cyclic voltammetry (10 cycles between -0.9 to 0.7 V at 0.05 V s^{-1}) in a 0.05 M copper (II) nitrate and 0.1 M HNO_3. (ii) Following rinsing thoroughly with distilled water and drying in air for 15 min. The electrodes were drop-cast with a 2 µl aliquot of 2 wt % neutralised Nafion solution (prepared from 5% sample by 90% ethanol/water dilution) and left to dry in air for 15 min. (iii) Finally the PANi layer was electrodeposited from a 0.2 M aniline solution in 0.5 M HCl by cyclic voltammetry (10 cycles between -0.4 and 1.0 V at 0.05 V s^{-1}). The PANi-Nafion-Cu-

modified SPE was then rinsed thoroughly with distilled water and dried in air for 15 min. All treatments and measurements were carried out at room temperature.

2.4 Enzyme immobilisation procedure

Fresh solutions of enzyme were prepared in PBS. The stock solutions used in this work contained 0.1 mg μl^{-1} (10%) for urease and 0.2 mg μl^{-1} (20%) for creatinine deiminase (CDI). Aliquots of enzyme solution were mixed with PBS containing BSA, lactitol and glycerol to give final concentrations of 2% for urease and 5% for CDI, and 5% BSA, 2% lactitol and 5% glycerol. Enzyme immobilisation was performed by drop-casting onto the PANi–Nafion-Cu-modified SPE 2 µl of a solution of the enzyme. Following drop-casting, the prepared sensor was cross linked in the presence of saturated GA (25% w/v) vapour in an exhaust fume hood at room temperature for 25 minutes. Prior to use, the biosensors were dried for 1 hour at 4 ºC. The functionalised electrodes were then rinsed 3 times with 20 mM PBS and stored in the same buffer at 4 ºC until use.

2.5 Preparation of serum samples and standard addition assay

Serum samples from patients with chronic kidney disease were obtained from the Kyiv Research and Practice Center of Nephrology and Hemodialysis (Kyiv, Ukraine) following the guidelines of the European Group on Ethics in Science and New Technologies of the European Commission (especially No 17, 4th February 2003) and those of the Ethics Committee of the American Psychological Association. The Ukrainian Government personal data protection rules as well as the Ethics Committee of the Institute of Molecular Biology and Genetics NAS of Ukraine rules (approved on 25th April 2012 to conduct the relevant research in the frame of SMARTCANCERSENS Project, PIRSES-GA-2012-318053) were followed during collection, delivery and investigation of Human biological samples.

Venous blood (2 ml) was collected in a heparinised cold tube. The tube was centrifuged at 1500 g for 10 min and cell-free supernatant removed. All serum samples were kept cold in an ice box during the time between collection and measurement. The assay of creatinine and urea in the serum samples was performed by means of amperometry at -0.35 V with the elaborated biosensors using the standard addition method. An aliquot of the serum sample was directly injected into the measuring vessel in order to obtain a 100-500 and a 1000-2000 fold dilution of the serum sample, respectively, for creatinine and urea determination.

Reference data on creatinine and urea concentration in patient serum samples were obtained from a commercial laboratory specialising in medical analyses; concentration of creatinine and urea were obtained by the standard colorimetric assay based on creatininase, creatine amidinohydrolase, sarcosine oxidase multiple enzymatic reactions and urease enzymatic reaction, respectively.

3. RESULTS AND DISSCUSSION

3.1 Optimisation of the ammonium ion-selective composite for use in biosensors

The authors recently reported on the development of an ammonium ion-sensitive PANi-Nafion-Cu composite that was shown to be suitable for the voltammetric/amperometric detection of ammonium in serum samples (Zhybak *et al*. 2015). This composite was also shown not to respond to model primary amine containing biomolecules (BSA) such as creatinine or urea, thus demonstrating its high specificity towards ammonium ions as it can be seen in Fig 1SA. These results were highly significant, in demonstrating that the combination of PANi and copper provides improvement in the selectivity of the sensor compared to other copper-based electrodes, which have been shown to be sensitive to a variety of primary amines (Lin *et al*. 2011), including creatinine (Chen and Lin 2012). Furthermore the PANi-Nafion-Cu-nanocomposite showed significant increase in sensitivity when compared to the only Cu or only PANi electrodes (Fig. 1SB) (Zhybak et al. 2015). These results indicate the high potentiality of the PANi-Nafion-Cu-nanocomposite to serve as transducer in the development of highly specific creatinine/urea biosensors, based on the appropriate hydrolase enzymes. Hence, to demonstrate this possibility, the development of a creatinine and of a urea biosensor, via the coupling of the PANi-Nafion-Cu-modified SPE with creatinine deiminase or urease, was undertaken and reported in the current manuscript.

3.2 Immobilisation of enzymes

The final target of the developed biosensors was the detection of creatinine and urea in serum sample; for this reason physiological pH (7.4) was chosen to perform all experimental work.

Despite this, prior to continue the biosensors development the influence of pH on the response of the PANi-Nafion-Cu nano-composite was performed. As it can be seen from Fig. 2S no significant variations in the response in the pH range 5 – 8 (Zhybak *et al*. 2015) was recorded.

These results, together with the aim of developing the biosensors to work in serum sample forced us to optimise both biosensors for operating at a pH of 7.4. Conveniently, this also fits well with the optimum working/stability pH for urease, but unfortunately, this is not the case for the CDI (pH optimum 8.5-9.5). In order to improve the operational stability of the enzymes, different strategies for their immobilisation were investigated as part of this work. At first, immobilisation of the enzymes from a 5% BSA solution in PBS followed by cross linking in saturated GA (25% w/v) vapour was attempted. Stability and performance of the biosensors were evaluated by monitoring their responses, upon measurement of creatinine or urea standard solutions (5, 25 and 50 μM), over a period of 72 h. The urease-based biosensor presented quite good stability and conversion ability, especially at low concentrations, but the CDI-based biosensor suffered from high noise, low response and poor stability, with loss of ca. 90% of its efficiency after 3 hours from preparation

(Data not shown). To improve stability (operation and storage) of the enzyme biosensors, the use of D-lactitol monohydrate and glycerol as stabilisers was explored (Gibson *et al.* 1992). The use of glycerol prevents loss of the enzyme activity during the immobilisation process and results in a better homogeneity and adhesion of the membrane (Zhylyak *et al.* 1995). D-lactitol and glycerol (2 and 5 % respectively) were added in both CDI and urease solutions; their use improved considerably the performance of the creatinine biosensor delivering good operation stability over 72 h (Fig. 3S). As can be seen from Fig. 3S, no significant loss in the electrochemical response for both the biosensors was recorded over this period.

3.3 Biosensor performance in model media (PBS pH 7.4).

The addition of urea or creatinine in the PBS solution resulted in a significant change, in a voltammetric experiment, of both the cathodic and the anodic currents (Fig. 1A, Fig. 4S). Initially, the use of DPV measurements was investigated as the transduction approach. As it can be seen from Fig. 1B and Fig. 5S the addition of increasing concentration of the analytes (creatinine, urea or ammonium) resulted in a significant increase in the cathodic current between -0.15 and -0.45 V (vs. Ag pseudo reference electrode).

In order to improve the quality of the analytical response and to gain better understanding of the signal generation process, the use of background compensation was explored (O'Mahony *et al.* 2005). As it can be seen from Fig. 6S (A and B), when the background response (no analyte in the solution) was subtracted from the different curves, a clear cathodic peak appeared between -0.15 and -0.25 V. In Fig. 6SC the calibration results obtained for the creatinine and urea biosensors are reported and compared with those obtained for ammonium.

The similarity of the calibration curves, regardless of the analyte, seems to indicate that the enzyme conversion rate is not the limiting factor in these biosensors. The developed biosensors had linear ranges between 8 and 90 µM for creatinine and 5 and 50 for urea, respectively. Sensitivity was estimated to be 95±6.7 mA M^{-1} cm^{-2} for the creatinine biosensor and 91±7.8 mA M^{-1} cm^{-2} for the urea biosensor.

Despite the fact that the background-subtraction DPV was proved to be rather sensitive, this transduction approach was quite time consuming and operationally complicated; subsequently the use of amperometric measurement was explored.

3.4 Amperometric assay

Accordingly to the DPV results -0.35 V was identified a suitable generic potential for the detection of the different analytes studied in this work. In Fig. 2AB the amperometric responses to increasing concentrations of creatinine and urea, respectively, are reported. As can be seen, a

consistent increase in the cathodic current was observed as a result of increased analytes concentration.

Fig. 2C shows that for both enzyme electrodes, the typical Michaelis-Menten kinetic mechanism, with signal saturation upon the addition of high concentrations of the substrate, was recorded. The apparent Michaelis-Menten constant (K_M^{app}) was calculated from Lineweaver-Burk plot taking into account the current induced by the enzymatic activity and found to be 0.163 mM for CDI and 0.139 mM for urease.

To estimate the conversion level of the target analytes in the biocatalytic layer, the response to ammonium was also evaluated (Fig. 2C and Fig. 7S). The yield of the enzymatic reactions was calculated from the I_{max} values obtained from this calibration; the yield of the conversion of creatinine and urea to ammonium was estimated as 83% and 94% for CDI and urease, respectively. The relatively low conversion rate can be explained by non-optimal pH (especially for CDI) and temperature (optimal temperature for both enzymes in aqueous solution is ca. 60 °C) conditions. GA vapours could also be expected to partly inhibit enzymes during the cross-linking step.

The analytical performances of the developed creatinine/urea biosensors were also evaluated. As can be seen from Fig. 2 A,B, the system presented very low background noise (5-8 nA); this facilitated (Fig. 2C) a low limit of quantification (ca. 1 µM) and accurate detection of analytes in the concentration range between 1 µM and 125 µM for both creatinine and urea. The sensitivities for the biosensors were calculated as 85±3.4 mA M^{-1} cm^{-2} for the creatinine biosensor and 112±3.36 mA $M^{-1}cm^{-2}$ for the urea biosensor, respectively. The limit of detection (3×SD/sensitivity) was estimated to be 0.5 µM for both biosensors. The developed biosensors also had fast response times (15s). The standard deviation of the amperometric responses was 8-9 % in the range 1 – 15 µM; 6-8% in range 20 – 50 µM and 5-6% in the range 75 – 200 µM of creatinine or urea (n=11), respectively.

3.5 Comparison of biosensor performance

A comparison of the key analytical characteristics of the developed amperometric creatinine (Table 1) and urea (Table 2) biosensors with those previously decsribed in the literature illustrates that the our biosensors possess higher sensitivity.

3.6 Creatinine and urea determination in real serum samples

To demonstrate the suitability of the developed urea and creatinine biosensors for the assessment of analyte concentrations in biological fluids, serum samples from patients with CKD were collected and analysed.

Creatinine and urea detection in serum samples was performed using the amperometric detection technique, via the use of a portable potentiostat (µStat 400), following the standard

addition approach. Due to the fact that serum samples were collected from patients with CKD, their dilution with PBS prior to quantification was needed in order to bring the analyte concentrations into the linear range of the biosensors. Dilution factors needed to achieve this goal were estimated using prior clinical/literature data; in the case of creatinine, dilutions of 100 – 500 fold were decided, while in the case of urea, due to the high concentration of this analyte expected in the patient sample, dilutions of 1000 – 2000-fold were envisaged. A lower dilution factor was adopted initially; if saturation upon addition of the stock solution of sensor was recorded, the measurement was repeated adopting a higher dilution factor.

The concentrations of creatinine and urea in 5 serum samples from patients undergoing renal dialysis were determined. A typical amperometric response (Fig. 8S A and Fig. 9S A) and the linear approximation curve (Fig. 8S B, 9S B) for the detection, via standard addition analysis of creatinine and urea, respectively, are reported.

The results obtained with the biosensors showed a very good correlation (slope ≈ 1 and R=0.99 for both biosensors) with the standard spectrophotometric method for creatinine and urea measurement (Fig. 3 A and B). Furthermore the RSD (RSD = 4-8 % for n=3) of the developed biosensors were comparable with those obtained for the standard spectrophotometric methods (RSD = 2.5 %). The results obtained demonstrated the possibility of exploiting the proposed biosensors for the amperometric detection of creatinine and urea in serum, which could be used for further development of analytical devices for distributed diagnostics.

4. CONCLUSIONS

Novel amperometric creatinine and urea biosensors based on a new PANi-Nafion-Cu composite and immobilised hydrolase enzymes (CDI and urease) have been developed and optimised. The biosensors were characterised by a fast response to the analytes (15 s), high selectivity and sensitivity (85±3.4 mA M^{-1} cm^{-2} for creatinine and 112±3.36 mA $M^{-1}cm^{-2}$ for urea) with a limit of detection of 0.5 µM and with RSD < 8%. The responses of the biosensors were linear over the concentration range 1 to 125 µM. The use of glycerol and lactitol in the enzymatic membrane significantly improved the operational stability of the biosensors; this was particularly important in the case of the creatinine biosensor. Measurements obtained with the biosensors correlated well with the standard spectrophotometric laboratory assays for the urea and creatinine in CDK patient serum samples.

5. ACKNOWLEDGEMENTS

This work was partially funded by the “SMARTCANCERSENS” project from the European Communities Seventh Framework Program under the Grant Agreement PIRSES-GA-2012-318053 and by the NATO Science for Peace (SFP) Project CBP.NUKR.SFPP 984173.

6. REFERENCES

Adeloju, S.B., Shaw, S.J., Wallace, G.G., 1996. Polypyrrole-based amperometric flow injection biosensor for urea. Analytica Chimica Acta 323(1-3), 107-113.

Adeloju, S.B., Shaw, S.J., Wallace, G.G., 1997. Pulsed-amperometric detection of urea in blood samples on a conducting polypyrrole-urease biosensor. Analytica Chimica Acta 341(2-3), 155-160.

Ashwood, E.R., Bruns, D.E., Burtis, C.A., Tietz, N.W., 2006. Textbook of clinical chemistry and molecular diagnostics. Elsevier/Saunders, St. Louis, Mo.

Bertocchi, P., Compagnone, D., Palleschi, G., 1996. Amperometric ammonium ion and urea determination with enzyme-based probes. Biosensors & Bioelectronics 11(1-2), 1-10.

Chen, C.-H., Lin, M.S., 2012. A novel structural specific creatinine sensing scheme for the determination of the urine creatinine. Biosensors & Bioelectronics 31(1), 90-94.

Cho, W.J., Huang, H.J., 1998. An amperometric urea biosensor based on a polyaniline-perfluorosulfonated ionomer composite electrode. Analytical Chemistry 70(18), 3946-3951.

Choi, S.H., Lee, S.D., Shin, J.H., Ha, J., Nam, H., Cha, G.S., 2002. Amperometric biosensors employing an insoluble oxidant as an interference-removing agent. Analytica Chimica Acta 461(2), 251-260.

Dhawan, G., Sumana, G., Malhotra, B.D., 2009. Recent developments in urea biosensors. Biochemical Engineering Journal 44(1), 42-52.

Eggenstein, C., Borchardt, M., Diekmann, C., Grundig, B., Dumschat, C., Cammann, K., Knoll, M., Spener, F., 1999. A disposable biosensor for urea determination in blood based on an ammonium-sensitive transducer. Biosensors & Bioelectronics 14(1), 33-41.

Erlenkotter, A., Fobker, M., Chemnitius, G.C., 2002. Biosensors and flow-through system for the determination of creatinine in hemodialysate. Analytical and Bioanalytical Chemistry 372(2), 284-292.

Gibson, T.D., Higgins, I.J., Woodward, J.R., 1992. Stabilization of analytical enzymes uzing a novel polymer carbohydrate system and the production of a stabilized, single reagent for alcohol analysis. Analyst 117(8), 1293-1297.

Guilbault, G.G., Shu, F.R., 1972. Enzyme electrodes based on the use of a carbon dioxide sensor. Urea and L-tyrosine electrodes. Analytical chemistry 44(13), 2161-2166.

Guilbault, G.G., Tarp, M., 1974. A specific enzyme electrode for urea. Analytica chimica acta 73(2), 355-365.

Haynes, R.J., Winearls, C.G., 2010. Chronic kidney disease. Surgery (Oxford) 28(11), 525-529.

Hsiue, G.H., Lu, P.L., Chen, J.C., 2004. Multienzyme-immobilized modified polypropylene membrane for an amperometric creatinine biosensor. Journal of Applied Polymer Science 92(5), 3126-3134.

Jaffe, M., 1886. Über den Niederschlag, welchen Pikrinsäre in normalem Harn erzeugt und über eine neue Reaction des Kreatinins. Zeitschrift für physiologische Chemie 10(5), 391-400.

Khan, G.F., Wernet, W., 1997. A highly sensitive amperometric creatinine sensor. Analytica Chimica Acta 351(1-3), 151-158.

Kuralay, F., Ozyoruk, H., Yildiz, A., 2006. Amperometric enzyme electrode for urea determination using immobilized urease in poly (vinylferrocenium) film. Sensors and Actuators B-Chemical 114(1), 500-506.

Lad, U., Khokhar, S., Kale, G.M., 2008. Electrochemical Creatinine Biosensors. Analytical Chemistry 80(21), 7910-7917.

Lin, M.-S., Chen, C.-H., Chen, Z., 2011. Development of structure-specific electrochemical sensor and its application for polyamines determination. Electrochimica Acta 56(3), 1069-1075.

Luo, Y.C., Do, J.S., 2004. Urea biosensor based on PANi(urease)-Nation (R)/Au composite electrode. Biosensors & Bioelectronics 20(1), 15-23.

Magalhaes, J., Machado, A., 2002. Array of potentiometric sensors for the analysis of creatinine in urine samples. Analyst 127(8), 1069-1075.

Meyerhoff, M., Rechnitz, G.A., 1976. Activated enzyme electrode for creatinine. Analytica Chimica Acta 85(2), 277-285.

Munoz, J., Jimenez, C., Bratov, A., Bartroli, J., Alegret, S., Dominguez, C., 1997. Photosensitive polyurethanes applied to the development of CHEMFET and ENFET devices for biomedical sensing. Biosensors & Bioelectronics 12(7), 577-585.

Nguyen, V.K., Wolff, C.M., Seris, J.L., Schwing, J.P., 1991. Immobilized enzyme electrode for creatinine determination in serum. Analytical Chemistry 63(6), 611-614.

Nieh, C.-H., Tsujimura, S., Shirai, O., Kano, K., 2013. Amperometric biosensor based on reductive H2O2 detection using pentacyanoferrate-bound polymer for creatinine determination. Analytica Chimica Acta 767, 128-133.

O'Mahony, A.M., Scanlon, M.D., Berduque, A., Beni, V., Arrigan, D.W.M., Faggi, E., Bencini, A., 2005. Voltammetry of chromium(VI) at the liquid vertical bar liquid interface. Electrochemistry Communications 7(10), 976-982.

Osaka, T., Komaba, S., Amano, A., 1998. Highly sensitive microbiosensor for creatinine based on the combination of inactive polypyrrole with polyion complexes. Journal of the Electrochemical Society 145(2), 406-408.

Osborne, M.D., Girault, H.H., 1995. The micro water 1,2-dichloroethane interface as a transducer for creatinine assay. Mikrochimica Acta 117(3-4), 175-185.

Pizzariello, A., Stredansky, M., Stredanska, S., Miertus, S., 2001. Urea biosensor based on amperometric pH-sensing with hematein as a pH-sensitive redox mediator. Talanta 54(4), 763-772.

Pundir, C.S., Yadav, S., Kumar, A., 2013. Creatinine sensors. Trac-Trends in Analytical Chemistry 50, 42-52.

Radomska, A., Bodenszac, E., Glab, S., Koncki, R., 2004a. Creatinine biosensor based on ammonium ion selective electrode and its application in flow-injection analysis. Talanta 64(3), 603-608.

Radomska, A., Koncki, R., Pyrzynska, K., Glab, S., 2004b. Bioanalytical system for control of hemodialysis treatment based on potentiometric biosensors for urea and creatinine. Analytica Chimica Acta 523(2), 193-200.

Rajesh, Bisht, V., Takashima, W., Kaneto, K., 2005. An amperometric urea biosensor based on covalent immobilization of urease onto an electrochemically prepared copolymer poly (N-3-aminopropyl pyrrole-co-pyrrole) film. Biomaterials 26(17), 3683-3690.

Ramanavicius, A., 2007. Amperometric biosensor for the determination of creatine. Analytical and Bioanalytical Chemistry 387(5), 1899-1906.

Ricca, E., Brucher, B., Schrittwieser, J.H., 2011. Multi-Enzymatic Cascade Reactions: Overview and Perspectives. Advanced Synthesis & Catalysis 353(13), 2239-2262.

Schneider, J., Grundig, B., Renneberg, R., Cammann, K., Madaras, M.B., Buch, R.P., Vorlop, K.D., 1996. Hydrogel matrix for three enzyme entrapment in creatine/creatinine amperometric biosensing. Analytica Chimica Acta 325(3), 161-167.

Senillou, A., Jaffrezic-Renault, N., Martelet, C., Cosnier, S., 1999. A miniaturized urea sensor based on the integration of both ammonium based urea enzyme field effect transistor and a reference field effect transistor in a single chip. Talanta 50(1), 219-226.

Shih, Y.T., Huang, H.J., 1999. A creatinine deiminase modified polyaniline electrode for creatinine analysis. Analytica Chimica Acta 392(2-3), 143-150.

Singh, M., Verma, N., Garg, A.K., Redhu, N., 2008. Urea biosensors. Sensors and Actuators B-Chemical 134(1), 345-351.

Soldatkin, A.P., Montoriol, J., Sant, W., Martelet, C., Jaffrezic-Renault, N., 2002a. Creatinine sensitive biosensor based on ISFETs and creatinine deiminase immobilised in BSA membrane. Talanta 58(2), 351-357.

Soldatkin, A.P., Montoriol, J., Sant, W., Martelet, C., Jaffrezic-Renault, N., 2002b. Development of potentiometric creatinine-sensitive biosensor based on ISFET and creatinine deiminase immobilised in PVA/SbQ photopolymeric membrane. Materials Science & Engineering C-Biomimetic and Supramolecular Systems 21(1-2), 75-79.

Stasyuk, N., Smutok, O., Gayda, G., Vus, B., Koval'chuk, Y., Gonchar, M., 2012. Bi-enzyme L-arginine-selective amperometric biosensor based on ammonium-sensing polyaniline-modified electrode. Biosensors & Bioelectronics 37(1), 46-52.

Strehlitz, B., Grundig, B., Kopinke, H., 2000. Sensor for amperometric determination of ammonia and ammonia-forming enzyme reactions. Analytica Chimica Acta 403(1-2), 11-23.

Taniguchi, I., Miyamoto, S., Tomimura, S., Hawkridge, F.M., 1988. Mediated electron transfer of lactate oxidase and sarcosine oxidase with octacyanotungstate(IV) and octacyanomolibdate(IV). Journal of Electroanalytical Chemistry 240(1-2), 333-339.

Tombach, B., Schneider, J., Matzkies, F., Schaefer, R.M., Chemnitius, G.C., 2001. Amperometric creatinine biosensor for hemodialysis patients. Clinica Chimica Acta 312(1-2), 129-134.

Tsuchida, T., Yoda, K., 1983. Multi-enzyme membrane electodes for determination of creatinine and creatine in serum. Clinical Chemistry 29(1), 51-55.

Turner, A.P.F., Karube, I., Wilson, G.S., 1987. Biosensors: Fundamentals and Applications. Oxford University Press, Oxford.

Tyagi, M., Tomar, M., Gupta, V., 2013. NiO nanoparticle-based urea biosensor. Biosensors & Bioelectronics 41, 110-115.

Udy, A., O'Donoghue, S., D'Intini, V., Healy, H., Lipman, J., 2009. Point of care measurement of plasma creatinine in critically ill patients with acute kidney injury. Anaesthesia 64(4), 403-407.

Vaidya, V.S., Ferguson, M.A., Bonventre, J.V., 2008. Biomarkers of acute kidney injury. Annual Review of Pharmacology and Toxicology, pp. 463-493.

Vostiar, I., Tkac, J., Sturdik, E., Gemeiner, P., 2002. Amperometric urea biosensor based on urease and electropolymerized toluidine blue dye as a pH-sensitive redox probe. Bioelectrochemistry 56(1-2), 113-115.

Walcerz, I., Glab, S., Koncki, R., 1998. Potentiometric enzyme electrode in a flow injection system for the determination of urea in human serum samples. Analytica Chimica Acta 369(1-2), 129-137.

Zhybak, M., Beni, V., Vagin, M.Y., Dempsey, E., A. P. F. Turner, A.P.F., Korpan, Y., 2015. Electrochimica Acta Submitted.

Zhylyak, G.A., Dzyadevich, S.V., Korpan, Y.I., Soldatkin, A.P., Elskaya, A.V., 1995. Application of urease conductometric biosensor for heavy-metal ion determination. Sensors and Actuators B-Chemical 24(1-3), 145-148.

Zinchenko, O.A., Marchenko, S.V., Sergeyeva, T.A., Kukla, A.L., Pavlyuchenko, A.S., Krasyuk, E.K., Soldatkin, A.P., El'skaya, A.V., 2012. Application of creatinine-sensitive biosensor for hemodialysis control. Biosensors & Bioelectronics 35(1), 466-469.

FIGURE AND TABLE LEGENDS

Table 1: Analytical characteristics of different amperometric creatinine biosensors

Table 2: Analytical characteristics of different amperometric urea biosensors

Figure 1. **A)** Cyclic voltammograms recorded before and after addition of 0.5 mM urea (black solid and red dotted lines, respectively; scan rate 0.05 V s^{-1}). **B)** Typical DPV-response of the urea biosensor upon increasing additions of urea. PBS pH 7.4.

Figure 2. **A)** An example of the amperometric response of the creatinine biosensor to successive additions of creatinine (-0.35 V, PBS pH 7.4); **B).** An example of the amperometric response of the urea biosensor to successive additions of urea (-0.35 V, PBS pH 7.4); **C).** Calibration curve for creatinine, urea and ammonium ion detection obtained at creatinine and urea biosensors and PANi-Nafion-Cu-modified SPE.

Figure 3. Correlation of the creatinine **(A)** and urea **(B)** biosensor response in serum from CKD patients with standard reference methods. Nb. The red line represents a perfect correlation.

Tables

	Signal registration mode and sensing component	*LOD* μM	*Linear range* μM	*Sensitivity,* $mAM^{-1}cm^{-2}$
(Khan and Wernet 1997)	Pt SEC film with cross-linked CA-CI-SOx-enzyme cascade	1-2	10-5000	23
(Schneider *et al*. 1996)	CA-CI-SOx immobilised in PCS on Pt electrode	0.3	1-150	34
(Osborne and Girault 1995)	CDI immobilised in gas permeable membrane	-	20-1000	0.001
(Tombach *et al*. 2001)	CA-CI-SOx – cascade immobilised in PCS on Pt electrode	5	5-150	5
(Choi *et al*. 2002)	CA-CI-SOx-enzyme cascade in PVA on Pt electrode	10	10-1000	0.125
(Hsiue *et al*. 2004)	Graft polymerisation of CA-CI-SOx-enzyme cascade on Pt electrode	-	3.2-320	-
(Shih and Huang 1999)	CDI immobilised on PANi-Nafion-GCE	0.5	0.5-500	-
This work	CDI immobilised on PANi-Nafion-Cu-modified SPE	0.5	1-100	85±3.4

Table 1

	Signal registration mode and sensing component	*LOD* μM	*Linear range* μM	*Sensitivity,* $mAM^{-1}cm^{-2}$
(Bertocchi *et al*. 1996)	Urease encapsulated in nylon net	10	10-300	-
(Pizzariello *et al*. 2001)	Urease adsorbed on Graphite-Pt electrode	3	10-250	0.2
(Vostiar *et al*. 2002)	Urease adsorbed on TB/GCE	200	200-800	0.98
(Luo and Do 2004)	Urease-PANi-Nafion-Au-ceramic film	50	500-5000	31±2
(Kuralay *et al*. 2006)	Urease immobilised in poly(vinyl ferrocenium) matrix	1	1-250	-
(Stasyuk *et al*. 2012)	Urease-PANi-Nafion-Pt electrode	3	30-300	11.6±0.05
(Tyagi *et al*. 2013)	Urease-NiO-NPs-ITO-glass	-	830-16650	21.33
This work	Urease immobilised on PANi-Nafion-Cu-modified SPE	0.5	1-100	112±3.36

Table 2

Figures

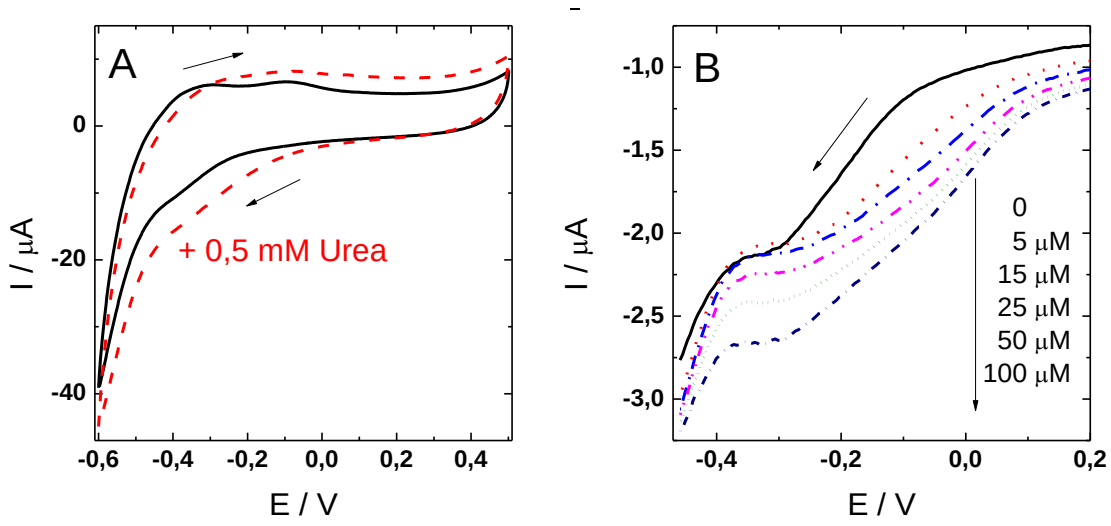


Figure 1

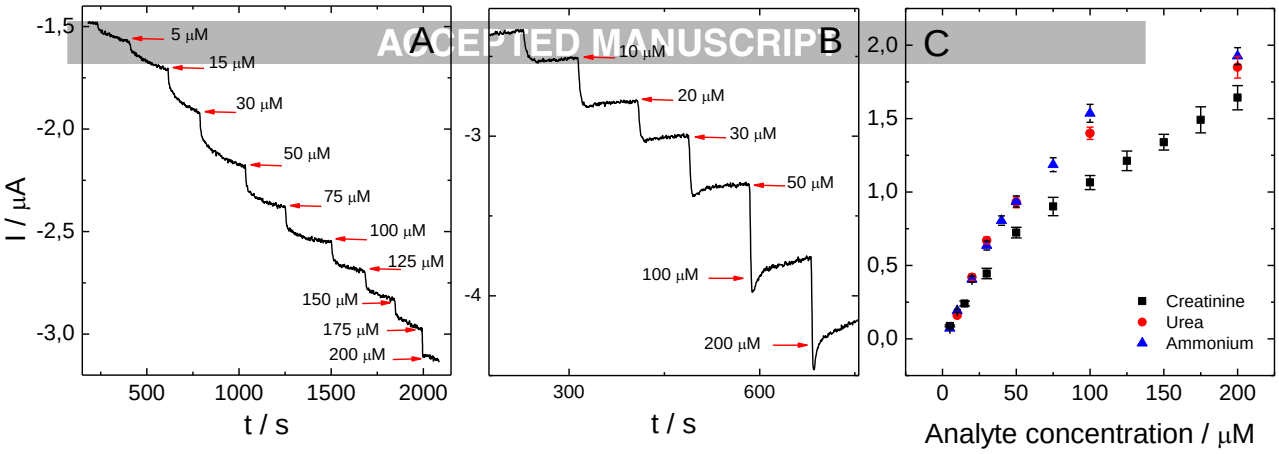


Figure 2

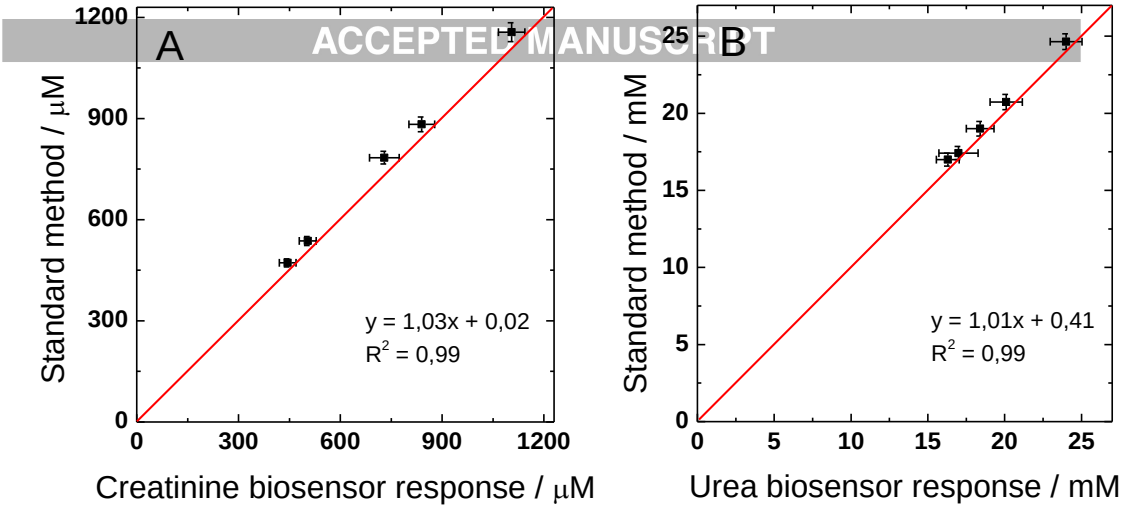


Figure 3

Highlights

Use of novel ammonium sensitive copper-polyaniline nano-composite in amperometric biosensing.

Fabrication and evaluation of ammonium based amperometric Creatinine and Urea biosensor.

Stable immobilisation of creatinine deaminase.

Sensitive and fast detection of creatinine and urea.

The developed biosensors have been validated with real patient samples.