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The Study of Urea Nitrogen Biosensor Based on the Disposable Screen-Printed Carbon Electrode

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A novel urea nitrogen(UN) biosensor based on disposable screen-printed carbon electrode mixed with potassium ferrocyanide was developed. The biosensor was covered with enzyme ink contained urease, glutamate dehydrogenase and reduced nicotinamide adenine dinucleotide(NADH). Then the electrochemical behavior was studied according to the electrochemical methods and the results revealed that the electrochemical method for detection of UN is feasible. Under optimized conditions, there was a good linear relationship of the UN concentration from 0.05 to 40 mM and the detection limit was 12 μM. The UN biosensor revealed good anti-interference ability and storage stability for more than 6 months when kept at room temperature. Moreover, the UN biosensor was employed to measure the real samples compared with automatic biochemical analyzer, the results suggested the UN biosensor could be applied to UN measurement in renal function.

Keywords: Urea nitrogen; screen printed carbon electrode; reduced nicotinamide adenine dinucleotide

1. INTRODUCTION

Urea is one of the end-product of protein metabolism in human body, and the determination of urea is one of the most frequent analyses in clinical diagnosis. The concentration of urea nitrogen(UN) in normal people blood is 1.8~7.1 mM related to the concentration of urea is 8~20 mg/dl[1]. It is one of diagnostic indicators of uremia when the concentration of UN reached to 21.4 mM (60 mg/dl)[2]. Thus determination of the UN concentration has great significance in nephropathy clinical diagnosis, treatment and prognosis[3,4].

Numerous analytical techniques have been developed for the determination of UN[3,5], and the main method is employed the reaction with diacetylmonoxime in clinical laboratories[6]. However, such a method requires heating and some of reagents are quite noxious. Thus, some clinical

laboratories employ urease to hydrolyze UN and the products, especial of ammonium, is determined at present[7,8].

The ammonium ion can be detected using electrochemical technology of potentiometric[9-12], amperometric urea biosensor [13,14] and conductometric urea biosensor [15,16]. The above method is basically used the traditional electrode, such as PH electrode[17] or the ammonia sensitive electrode[18], which have problems of bad selectivity, slow response and enzyme instability. The development of analytical methods that respond to the growing need to perform rapid in situ analyses shows the disposable screen-printed electrodes (SPEs) as an alternative to the traditional electrodes[19,20]. Compared to the common electrode, SPEs, low cost, easy to prepare and high reproducibility, have attracted extensive interest in various field.

The aim of this work is to introduce a simple, rapid and sensitive UN detection method based on the disposable screen-printed carbon electrode technology. In this method, urease is employed to hydrolyze UN into ammonia and carbon dioxide, then the ammonia and α-ketoglutaric acid react to glutamic acid under the catalytic action of glutamate dehydrogenase, and reduced nicotinamide adenine dinucleotide(NADH). In this process, NADH is oxidized into NAD^+, and the rest of the NADH can react with potassium ferrocyanide at the electrode surface. The consumption of NADH is proportional to the UN concentration in sample, thus the UN concentration in sample can be measured according to measure the oxidation current changes of potassium ferrocyanide. In this paper, such a method was applied to UN determination of real samples in whole blood and it can be applied in the portable system to renal function.

2. EXPERIMENTAL

2.1 Materials

Carbon ink and insulation ink were acquired from JUJO (Tokyo, Japan). The hydrophilic film and double sided adhesive tape were from 3M China Co., Ltd(Shenzhen, China). Urease, glutamate dehydrogenase were purchased from TOYOBO(Tokyo, Japan). NaCl, $CaCl_2$, potassium hydrogen phosphate, potassium dihydrogen phosphate, Potassium perchlorate, polyethylene glycol(PEG-400), Cabosil M5 amorphous untreated fumed silica powder and hydroxyethyl cellulose(HEC), uric acid, ascorbic acid(AA), potassium ferrocyanide, glutathione(GSH), Acetaminophen(ACP), 4-Aminoantipyrine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aqueous solutions were prepared using Millipore water (Simplicity Model, Billerica, MA, USA).

2.2 Electrode Preparation

Carbon ink was printed onto polyethylene terephthalate(PET) constructed a working electrode and a reference/counted electrode, dried 15 min at 110 ℃. The react area was formed according to printed the insulation ink at conductive parts of electrode, dried 15 min at 75 ℃. Then the react area was printed by the enzyme layer under the humidity adjusted to 65-80%RH and dried 15 min at 55 ℃.

The enzyme ink was prepared as followed procedure. 1 wt% $CaCl_2$, 1 wt% PEG-400, 4 wt% Cabosil M5 amorphous untreated fumed silica powder and 2.5 wt% hydroxyethyl cellulose (HEC) were added into phosphate buffer solution (0.1M, pH=7.4) and stired for 2 h at 2000 r/min, then hydrated for 6 h at room temperature. The prepared enzyme ink in addition of 0.2 g potassium ferrocyanide, 1.5 k units urease, 1 k units glutamate dehydrogenase, 0.2 g NADH and 0.1 g α-ketoglutaric acid to each gram of reagent ink, mixed for 15 minutes. The viscosity of enzyme ink was 5400 mPas by this method.

2.3 Detection of UN at UN biosensor

All electrochemical measurements were carried out with a model CHI832C Electrochemical Workstation(CH Instruments, Austin, TX, USA). The UN biosensor contained two carbon electrode, one printed enzyme ink as the working electrode, and the other as the reference electrode, then we used the electrochemical workstation to measure. The electrochemical behaviors of disposable screen-printed carbon electrode of UN was acquired from cyclic voltammetry(CV) and chronoamperomerty, and the electrolyte was 0.1 M NaCl in PBS solution(pH=8.0). For detection of UN at UN biosensor, the applied potential was set at 0.20 V and the value of the current was obtained at 10 s at I-t curve after waiting 40 s when the sample was added. All experiments were carried out at room temperature unless otherwise stated.

2.4 Real sample preparation and analysis

Blood samples were acquired from venous blood collected in heparin anticoagulant tube. 100 μL serum samples were tested by automatic biochemical analyzer(Beckman Instruments, Inc., California, USA), another 2 μL whole blood samples were measured by UN biosensor without any treatment.

In order to determine the recovery of electrodes, UN was dissolved in PBS solution to prepare 1 mM and 5 mM UN stock solution, and the stock solution were immediately mixed to the same volume of blood samples without any treatment. For the stability of UN biosensor, the same batch of electrode was conserved in a sealed packaging at room temperature, then electrodes were investigated using 5 mM UN every month.

3. RESULTS AND DISCUSSION

3.1 Electrochemical analysis of UN biosensor

Electrochemical behavior of UN biosensor was studied by CV. As was shown in the curve a of figure 1, a pair of fine redox peaks were observed when potential was from -0.1 V to 0.45 V, with the cathodic peak potentials (Epc) and anodic peak potentials (Epa) at 0.181 and 0.273 V, respectively. The redox peak was mainly attributed to redox reaction of potassium ferrocyanide on the surface of the

electrode. From curve b in figure 1, the oxidized and reduced current of potassium ferrocyanide obviously reduced when 5 mM UN added to phosphate buffer solution.

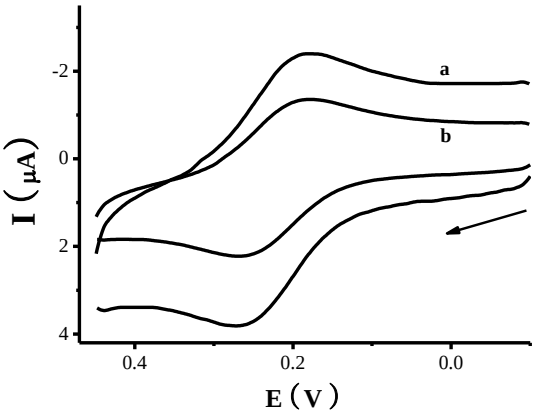

Figure 1. Cyclic voltammetry of different UN concentrations at UN biosensor, the arrow indicates the start scanned direction. The scan rate was 100 mV/s.

In order to verify electrochemical properties of potassium ferrocyanide and NADH at the electrode surface, CV behavior of basic electrode mixed with potassium ferrocyanide was researched in different NADH concentrations. When the test solution contained different concentrations of NADH (0, 0.1, 0.2, 0.3 and 0.5 mM), the oxidized current of potassium ferrocyanide increased with the increasing of NADH as shown in figure 2. The results showed that the redox reaction of NADH was effectively catalyzed by the basic electrode doped potassium ferrocyanide, also proved that the electrochemical method to detect UN was feasible.

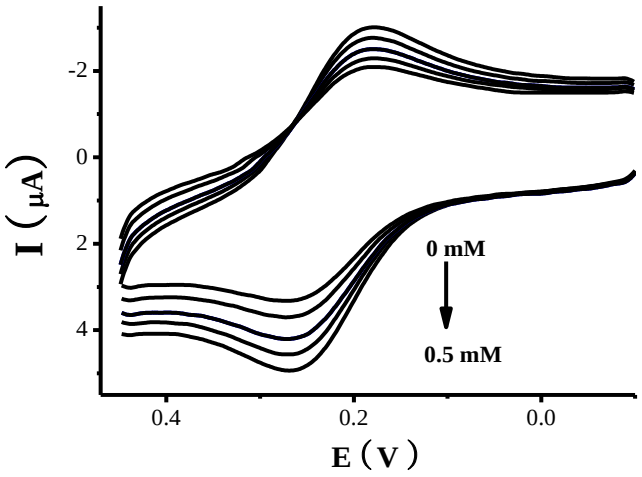

Figure 2. Cyclic voltammetry of various concentrations of NADH at basic electrode doped potassium ferrocyanide. The scan rate was 100 mV/s.

3.2 Detection of UN at UN biosensor

Chronoamperomerty was employed to measure oxidized current of UN at UN biosensor mixed

with potassium ferrocyanide. The applied potential catalyzed NADH was set at 0.2 V, the value of the oxidized current was obtained at 10s after adding different concentrations of UN(0-100 mM) and waiting 40 s. The result of oxidized current related with concentration of UN was shown in figure 3.

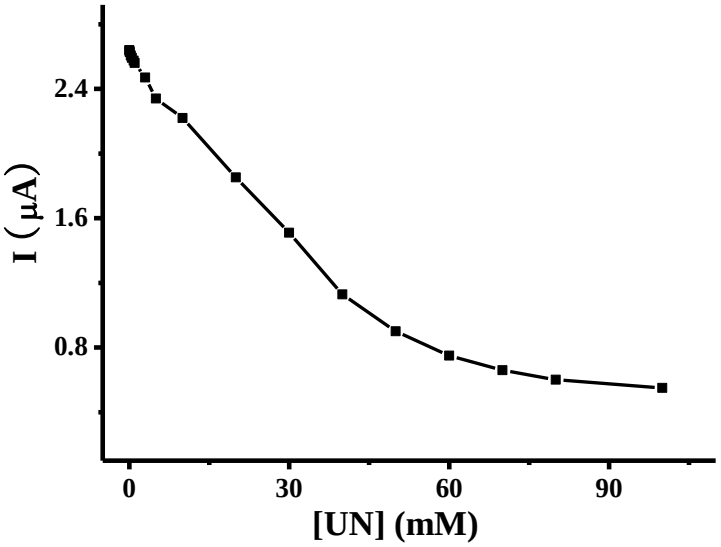

Figure 3. The relationship between different UN concentration and current with the potential of 0.2 V, and the test started after waiting 40 s when sample was added.

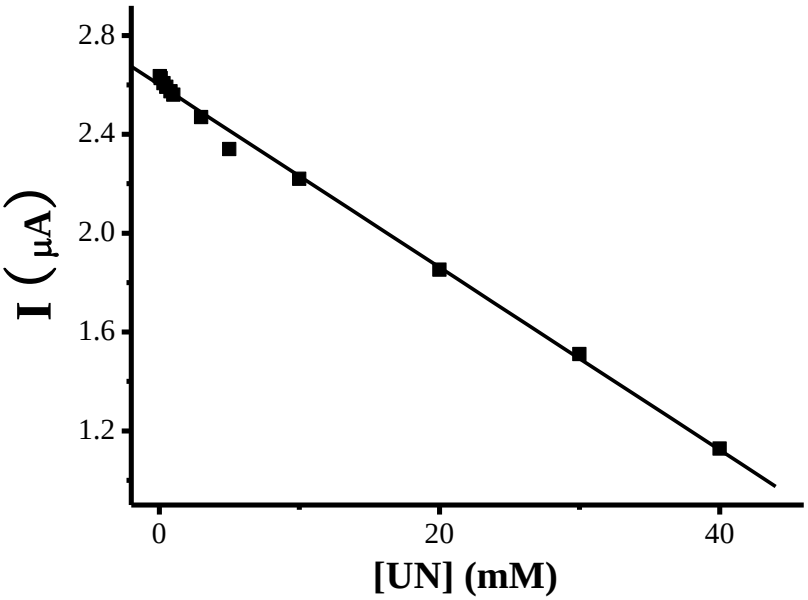

Figure 4. The calibration curve of UN concentration of 0.05-40 mM with current, the test started after waiting 40 s when sample was added, and the values were acquired at 10 s.

Figure 4 described the linear relationship of UN concentration with oxidized current at biosensor. The oxidized current at 10 s in the amperometric I-t curve declined with the increased of

UN concentration and exhibited a good linear relationship with the UN concentration in the range from 50 μM to 40 mM with the linear equation was I(μA)=2.62-0.04[UN](mM) and the limits of detection were calculated on the basis of three times of the background noise and the value was found to be 12 μM.

3.3 Selectivity and specificity of the UN biosensor

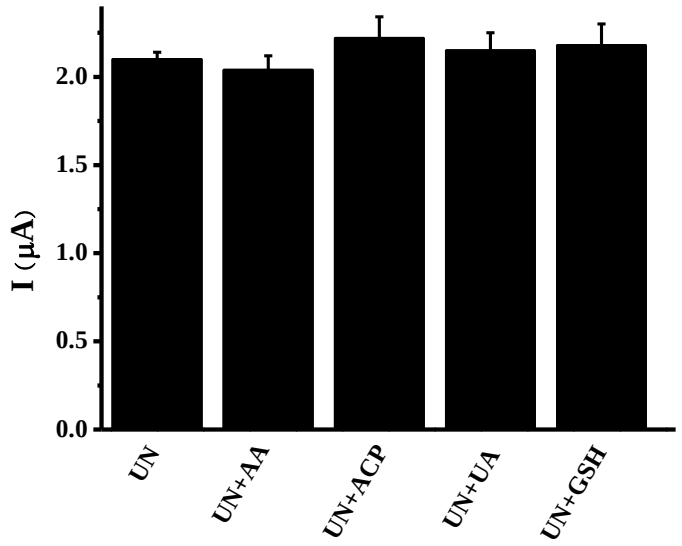

Figure 5. Selectivity of the UN sensor for UN detection in the absence or presence of redox-active species (AA, ACP, UA and GSH).

The electrochemical detection of UN were often affected by some interferences such as glutathione(GSH), glucose, uric acid(UA), acetaminophen(ACP) and ascorbic acid(AA). Therefore, we observed current changes at UN biosensor after adding different redox-active species of UA, AA, ACP and GSH. The results revealed that UN biosensor was basically not affected by the above species and had good selectivity and specificity(figure 5).

3.4 Reproducibility and stability of the UN biosensor

To examine the repeatability of the UN biosensor, 5 mM UN in PBS solution was detected. The results showed that the coefficient of variation of intra-assay and inter-assay were 1.44% and 5.67%, respectively, which suggested that UN biosensor had good reproducibility.

For assessment of the stability of UN biosensor, the electrodes were extracted to measure the current intensity with 5 mM UN in PBS solution every month, the electrodes were acquried from the same batch and stored at room temperature in a closed environment. As shown in figure 6, the catalytic current of electrodes still maintained 84.2% of the initial current after 6 months. The experimental results revealed that structure and activity of enzymes remained stable, which may due to the physical

adsorption, and adjunction of hydroxyethyl cellulose(HEC) and activators of enzymes such as $CaCl_2$. Meibodi *et al.* developed a Amperometric urea biosensor based on covalently immobilizedurease on an electrochemically polymerized film of polyanilinecontaining MWCNTs. And polyaniline-multiwalled carbon nanotubes (PANI/MWCNTs) composite were fabricated byelectropolymerization method as a matrix for entrapment of enzyme. The optimized urea biosensor shows a good sensitivity from 10^{-2} M to 10^{-5} M urea concentration range and a response time of about 50 s. The proposed biosensor retained 50% of its original response after 15 days[21]. Compared these results, our method is more simple and has a better storage stability.

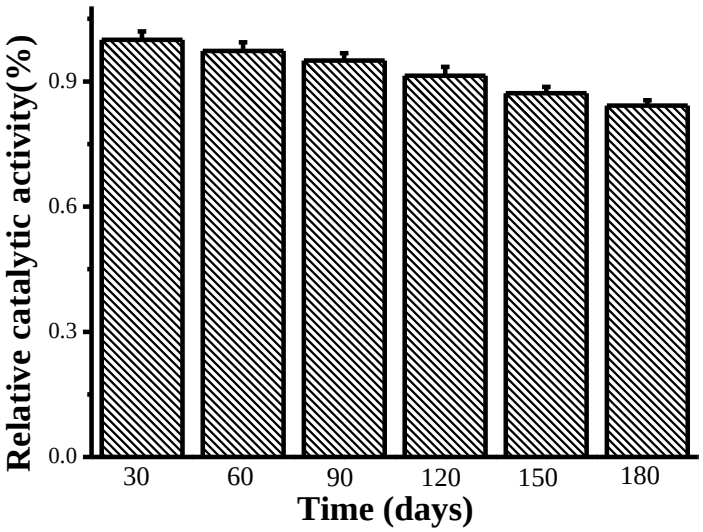

Figure 6. Stability of the UN biosensor.

3.5 Recovery of UN biosensor

The accuracy of UN biosensor can be reflected by the recovery. UN dissolved in PBS to prepare 1 mM and 5 mM as the stock solution, then added the same volume of whole blood specimens without any treatment, the recovery rate was in the range from 96.5% to 103.5% (cf. Table 1). The data suggested that UN biosensor had excellent accuracy, and the proposed screen-printed method was feasible for the detection of UN without the interference of other redox-active species in blood samples.

Table 1. Recovery of UN in blood sample

Samples	Add UN (mM)	Found (mM)	Recovery (%)	RSD (%)
1	0	2.42 ± 0.2	-	1.82
2	1.0	1.65 ± 0.1	96.5	1.25
3	5.0	3.84 ± 0.4	103.5	3.26

3.6 Real sample measurement

Automatic biochemical analyzer was usually employed by hospital to determine UN and the principle of measurement was showed as follows. UN was hydrolyzed by urease into ammonia and carbon dioxide, then the ammonia and α-ketoglutaric acid react to glutamic acid under the catalytic action of glutamate dehydrogenase, and NADH. In this process, NADH is oxidized into NAD^+ and has absorption peak in the wave of 340 nm, the decreasing rate of absorbance is proportional to the concentration of UN in the sample.

When we test the real samples, each sample was respectively measured three times and all the results were acquired by our electrochemical and hospital methods. In table 2, samples 1, 2 and 3 were collected from healthy donors, sample 4 was from patients with disease of renal function whose BUN concentration was higher than normal level (1.78-7.14 mM). The results revealed that the two methods had good consistency and there was no significant difference. Soldatkin *et al.* developed a enzyme/zeolite sensor for urea analysis in serum. The linear range of urea determination by using the biosensor was 0.003-0.75 mM. The method of standard addition was used for analysis of serum samples with 500-fold dilution. Total time of analysis was 10 min[22]. Compared these methods, our method has shorter response time and doesn't need any treatment, our method is more suitable for testing the real sample.

Table 2. Comparison of UN concentrations determined by hospital and our method

Samples	Hospital results (mM)	Our method (mM)
1	3.61 ± 0.30	3.42 ± 0.21
2	5.87 ± 0.42	6.01 ± 0.29
3	4.28 ± 0.18	4.33 ± 0.18
4	10.71 ± 0.23	11.24 ± 0.38

4. CONCLUSIONS

In this paper, we designed a new method of disposable UN biosensor mixed with potassium ferrocyanide successfully, and the electrochemical behavior of the UN biosensor based on the reaction of NADH, urease and glutamate dehydrogenase with UN, potassium ferrocyanide as an electron transfer agent was studied. The oxidated current at amperometric I-t curve exhibited a good linear relationship with UN concentration in the range of 50 μM ~ 40 mM, and the limit of detection was found to be 12 μM. Furthermore, the UN biosensor exhibited good reproducibility and coefficient of variation of intra-assay and inter-assay were 1.44% and 5.67%. In addition, this method was also successfully employed for UN detection in whole blood samples. Therefore, We concluded that the rapid and sensitive UN biosensor can be applied in the field of point-of-care testing(POCT) for renal function.

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