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Simultaneous detection of purine metabolites by membrane modified electrochemical sensors

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Abstract: Purine metabolites are important for metabolic and cellular processes. Deregulation of purinergic signaling leads to pathological accumulation of purine degradation products in extracellular fluids and indicates various diseases. In clinical diagnosis at early stages of related diseases, accurate detection of Uric acid and Xanthine is of high importance. Electrochemical methods are fast, simple, sensitive, more convenient, and cost-effective compared to other analytical methods used in purine metabolites signaling. Electrochemical sensors are able to detect more compounds simultaneously. Modification of a glassy carbon electrode sensor with external protective membranes was used in this study to avoid unwanted signal interferences from analyte matrices. Polyvinyl alcohol, Chitosan, and Nafion membranes were selected for sensor modification to compare the electro-neutral, positive and negative charged setting of the Xanthine and Uric acid detection. All three membrane modified sensors showed adequate stability in the phosphate buffer solution after 5 min of incubation and are thus suitable for simultaneous detection of purine metabolites. The best results in anodic peak current response values were observed using the Nafion membrane modified glassy carbon electrode sensor. The approach reported here can be useful for the detection of purine metabolites from various matrices at early stages of clinical diagnosis.

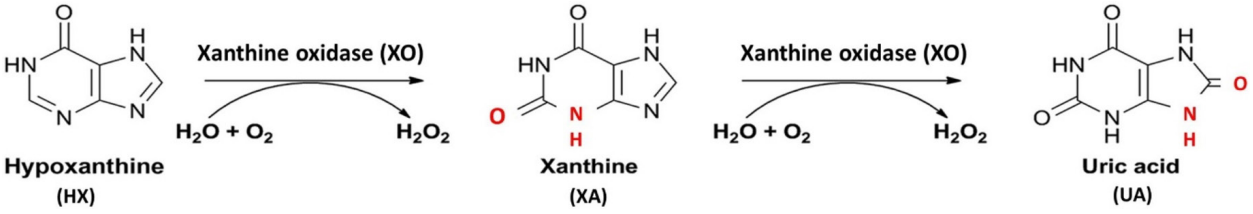
Keywords: electrochemistry, GCE, Chitosan, Nafion, purine metabolites, PVA

Introduction

Purine compounds play a key role in metabolic processes, often acting as a co-factors related to enzymes and receptors involved in cell signaling (Raj and John, 2013), and regulation of cellular proliferation, differentiation, and death (Huang, 2021). Products of purine degradation metabolism in humans and higher primates are uric acid (UA), xanthine (XA), and hypoxanthine (HX). UA is produced from HX and XA intermediates by enzyme xanthine oxidase (XO) (Siegel, 1999) (Scheme 1).

Pathological deregulation of purinergic signaling contributes to various diseases including neurodegeneration, rheumatic immune diseases, inflammation, and cancer (Huang, 2021). Products of purine degradation metabolism can accumulate in extracellular fluids like urine and human serum. Their concentration levels serve as an indicator of severe clinical diseases, including perinatal asphyxia, cerebral ischemia, hyperuricemia (Jin, 2015), xanthinuria (Mraz, 2015), gout (Martinon, 2006), preeclampsia (Chun Lam, 2005), and Parkinson's disease (Von Seggern, 2020). For this reason, accurate detection, and subsequent determination of UA, XA and HX in body fluids is of high importance in the clinical diagnosis at early stages of these diseases.

Many analytical methods have been reported for simultaneous detection of purine derivates in real samples including high performance liquid chromatography (HPLC) (Safranow, 2000; Wijemanne, 2018), capillary electrophoresis (CE) (Adam, 2002; Chen, 2002), chemiluminescence (Kather, 1987), and electrochemical methods (Jesny, 2017; Mukdasai, 2018; Zhang, 2019). Electrochemical methods are simple, sensitive, selective, more convenient, and less costly than classical analytical methods requiring complicated sample pre-treatment, extended analysis time, and expensive materials and in-



Scheme 1. Schematic representation of purine degradation mechanism catalyzed by xanthine oxidase.

strumentation. Electrochemical sensors can determine more compounds simultaneously. Electrodes used for purine metabolites determination are usually carbon-based materials especially glassy carbon electrode (GCE), carbon paste electrode, graphite electrode, screen printed carbon electrode, and others (Sheng, 2012; Tavana, 2012; Lian, 2014; Bukkitgar, 2016; Jankovska-Sliwinska, 2017). Carbon based electrodes display low background current, large potential range, and simple surface regeneration procedure (Bukkitgar, 2016).

Biological matrices usually contain low and high molecular weight substances that interfere with the detection of analytes and diminish the sensor response by unwanted signal interferences (Svitkova and Labuda, 2017). These effects can be eliminated by a modification of the sensor surface with protective membranes (Ambrozy, 2013). Polyvinyl alcohol (PVA), Chitosan (CHIT), and Nafion (NAF) belong to protective membranes with film-forming ability and good mechanical properties possessing chemical inertness, long-term stability, and biocompatibility.

Polyvinyl alcohol is an electroneutral polymer with excellent chemical resistance (Tripathi, 2010) and hydrophilic properties (Park MJ, 2018). Results of the search and optimization of polymer protective films at a glassy carbon electrode show that a polyvinyl alcohol membrane is an effective barrier stabilizing the sensor response in urine (Svitkova, 2018; Rahmanian, 2018).

Nafion is a negatively charged copolymer with a polytetrafluoroethylene backbone and perfluorinated ether side chains terminated with sulfonic acid groups (Kondratenko, 2017). Nafion is widely used as a barrier for diffusion of small neutral or negatively charged interfering species. (Ambrozy, 2013). It has both hydrophilic and hydrophobic properties, and it exhibits fast response and excellent long-term stability (Liu, 2011). Nafion can be used as a durable membrane in biological environment (Kondratenko, 2017). It was utilized as a protective membrane for the biosensor on the glassy carbon electrode surface improving thus the biosensor stability (Yang, 2013). Zhao et al. described protective effect of the nafion membrane on a glassy carbon surface when detecting analytes even from undiluted serum (Zhao, 2013).

Chitosan is a polysaccharide with —OH and —NH_2 functional groups. Because of the amino groups in the backbone, chitosan is a positively charged biopolymer and can dissolve in aqueous solution at pH below 6 (Seidi, 2021), it is a highly hydrophilic (Kushwaha, 2018) eco-friendly material, which is biocompatible, biodegradable, and nontoxic (Chang, 2021). Chitosan was used for the construction of a urea biosensor with the enzyme urease resulting in CO_2 formation (Mulyasuryani, 2010). Hui-Ting Lian et al. reported an improved imprinted film-based electrochemical sensor with CdS quantum dots (QDs) doped chitosan for urea recognition in human blood serum samples. (Lian HT, 2012). The possibility of chitosan electrodeposition as a thin coating on electrode surface in a potential controlled way provides an immense immobilization matrix for an extensive range of miniaturized biosensors (Annu and Raja, 2020).

In this paper, a novel electrochemical approach to sensitive determination of purine metabolites is demonstrated as a simple, fast, and low-cost alternative to other analytical methods. GCE was chosen as the most appropriate sensor due to its carbon-based composition often used for purine metabolites determination. Usefulness of the proposed chemical methodology is shown by determining the XA and UA in solutions using protective membranes with various properties allowing the elimination of complicated sample pre-treatment.

Material and Methods

Chemicals

XA and UA were purchased from Sigma-Aldrich (Germany). Acetic acid, ethanol, $[Fe(CN)_6]^{3-/4-}$, Na_2HPO_4 and NaH_2PO_4 were obtained from Lachema (Czech Republic). PVA and Nafion were purchased from Sigma-Aldrich (Germany), and Chitosan from Fluka (Germany). All chemicals were of analytical reagent grade. Deionized water with resistivity above 18 MΩ cm (Milipore Milli-Q system) was used for the preparation of all solutions.

Apparatus

All voltammetric experiments were conducted on an Autolab PGSTAT12 Potentiostat/Galvanostat Electrochemical System (Metrohm, Netherlands) driven by the software NOVA version 1.10.23 (Metrohm, Netherlands). The three-electrode system consisted of a glassy carbon working electrode (GCE, Metrohm, Netherlands) with a disc diameter of 3 mm, Ag/AgCl/3 M KCl reference electrode, and a platinum wire counter electrode (L-CHEM, Czech Republic). All measurements were performed in 20 mL glass cells at ambient temperature.

Preparation of membrane modified GCE sensor

Surface of the GCE was mechanically cleaned by alumina polishing suspension (0.3 μm, Metrohm, Netherlands) on the surface of polishing cloth suitable for GCE (BUEHLER, UK). GCE was pretreated by applying a potential of 1.6 V for 120 s

to a 20 ml electrochemical cell filled with PB solution to oxidize possible contaminants present on the electrode surface. Polyvinyl alcohol (PVA) was dissolved in nanopure water (18 MΩ cm) at 98 °C to the concentration of 0.1 mg mL^{-1}, 0.5 % w/v solution of chitosan was prepared in 1 % v/v acetic acid, and 1 % w/v solution of nafion was dissolved in 50 % v/v ethanol. Chemical modification of the electrode was carried out by covering the pretreated GCE surface with a layer of 2 µL of NAF, CHIT or PVA solution. The deposited membrane films were let to evaporate to dryness.

Sample preparation

The 1 mol L^{-1} stock solution of purine metabolites was prepared by dissolving XA and UA in nanopure water. The final concentration of 10^{-5} mol L^{-1} XA and 10^{-5} mol L^{-1} UA were prepared by diluting the stock solution with PB pH 7.4.

Experimental methods

Cyclic voltammetric (CV) scans of 1 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ redox indicator in PB with pH 7.4 were recorded for bare GCE within a potential range from –0.4 V to 0.8 V at the scan rate of 100 $mV \cdot s^{-1}$ with the potential step of 2 mV. The membrane modified electrodes were immersed into 0.1 M PB with pH 7.4 for 5 min under stirring. Subsequently, GCE, NAF/GCE, CHIT/GCE and PVA/GCE CV records in PB, XA, and UA solutions were obtained within the potential range from –0.4 to 0.8 V at the scan rate of 100 $mV \cdot s^{-1}$ and potential step of 2 mV. The membrane modified sensors were incubated in the mixture of purine metabolites (see sample preparation) at ambient temperature for a given time (2 and 5 min) under stirring. Then, CV measurements were performed again under the same conditions. Each potential reported in this paper is given against the Ag/AgCl/3 M KCl reference electrode at the laboratory temperature of 21 °C.

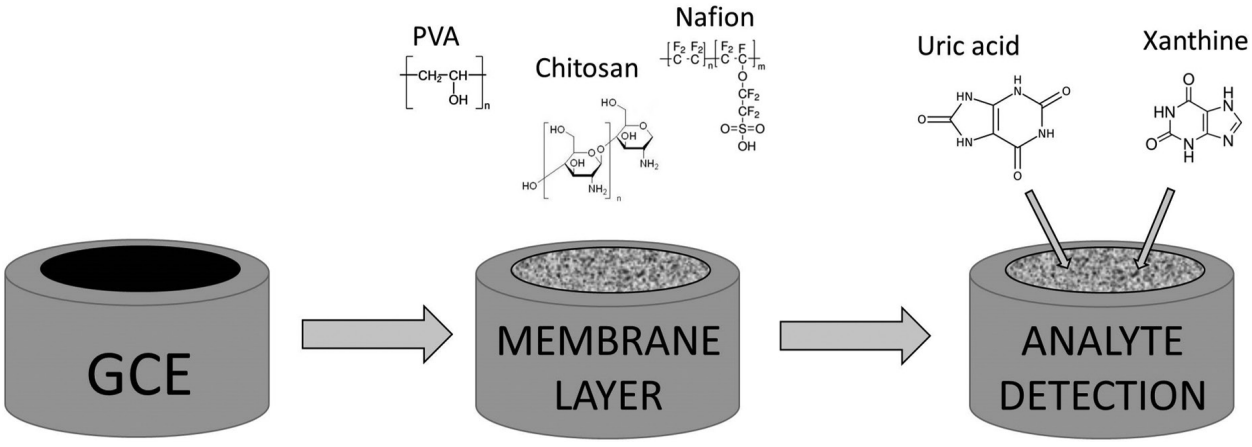
Results and discussion

The GCE sensor was modified with protective membranes to avoid diminished response due to unwanted signal interferences often caused by low or high molecular weight substances involved in biological matrices. PVA, CHIT, and NAF were selected for the GCE modification to compare the electro-neutral, positive, and negative charged settings of the experiment. Subsequently, simultaneous detection of XA and UA purine metabolites was performed. The experimental workflow is shown in Scheme 2.

Evaluation of membrane modified GCE sensor stability

Protective membranes formed of polyvinyl alcohol (PVA), Nafion (NAF), and Chitosan (CHIT) polymers were studied during the measurement. Four types of sensors, namely GCE, PVA/GCE, NAF/GCE, and CHIT/GCE, were tested with respect to their response after incubation in 1 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$ solution. The modified GCE sensors were rinsed with water and incubated in 0.1 mol L^{-1} PB for 2 and 5 min, the sensors were transferred to $[Fe(CN)_6]^{3-/4-}$ redox indicator and CV measurements were performed.

Based on the voltammograms depicted in Fig. 1, neutral PVA film caused diminished response of the $[Fe(CN)_6]^{3-/4-}$ redox indicator due to the PVA/GCE surface modification compared to bare GCE. The shift of anodic peak potential (*Epa*) to more positive values can be seen only for the PVA modification of the GCE surface. Anodic peak current of the redox indicator (*Ipa*) decreased from 12.01 µA to 8.94 µA, 8.68 µA and 8.84 µA for bare GCE and PVA/GCE measured immediately and after 2 or 5 min of incubation in PB with pH 7.4, respectively. The obtained CV response of the redox indicator within the PVA/GCE sensor showed improvement for the



Scheme 2. Experimental workflow.

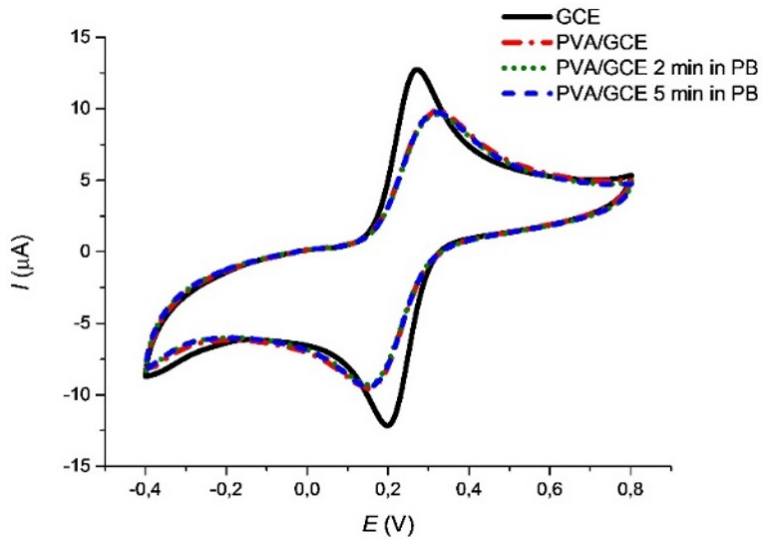


Fig. 1. CV response of $[Fe(CN)_6]^{3-/4-}$ redox indicator for bare GCE and PVA/GCE immediately and after 2 and 5 min of incubation in PB with pH 7.4.

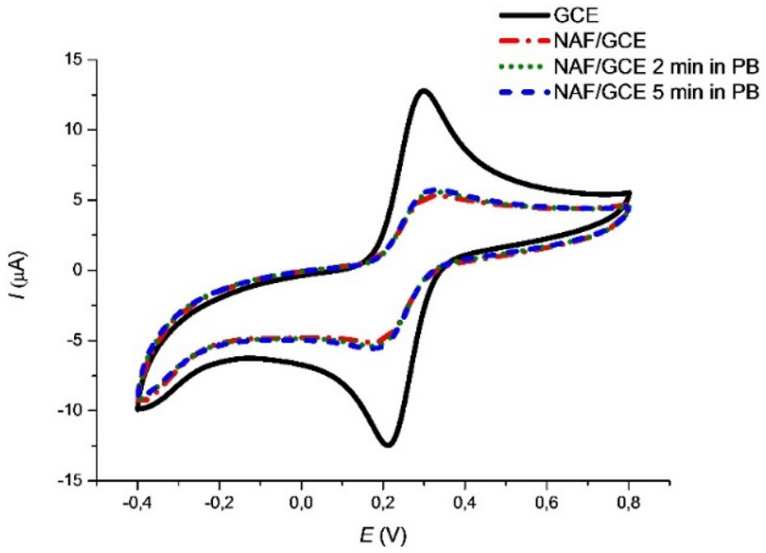


Fig. 2. CV response of $[Fe(CN)_6]^{3-/4-}$ redox indicator for bare GCE and NAF/GCE immediately and after 2 and 5 min of incubation in PB with pH 7.4.

anodic peak current signal stability in comparison to NAF/GCE.

The negatively charged NAF film diminished the CV *Ipa* response of the $[Fe(CN)_6]^{3-/4-}$ redox indicator more significantly, up to 4.28 μA in comparison with bare GCE *Ipa* of 12,01 μA, due to the electric repulsion of the $[Fe(CN)_6]^{3-/4-}$ anion (Fig. 2). *Epa* value of NAF/GCE remained stable. The *Ipa* response value of the redox indicator increased after 2 and 5 min of incubation to 4.6 μA and 4.85 μA, respectively, compared to 4.28 μA of NAF/GCE response measured immediately, which indicates the slightly lower stability of NAF/CGE and the need of longer time interval for the membrane layer regeneration. The 2—5 min time interval was sufficient for membrane rehydration and CV signal stabilization. (Fig. 2)

The CHIT/GCE *Ipa* redox indicator response increased in comparison with bare GCE from 12.01 μA to 14.25 μA, what can be described by the electrostatic pre-concentration of the $[Fe(CN)_6]^{3-/4-}$ anion within the positively charged CHIT polymer. The CV signal of CHIT/GCE was stable within the selected time intervals of incubation (Fig. 3). No *Epa* value shift was detected.

Based on these results, all three membrane modified sensors, namely PVA/GCE, NAF/GCE, and CHIT/GCE, showed sufficient stability after 2—5 min incubation in PB with pH 7.4 containing $[Fe(CN)_6]^{3-/4-}$ redox indicator for further experi-

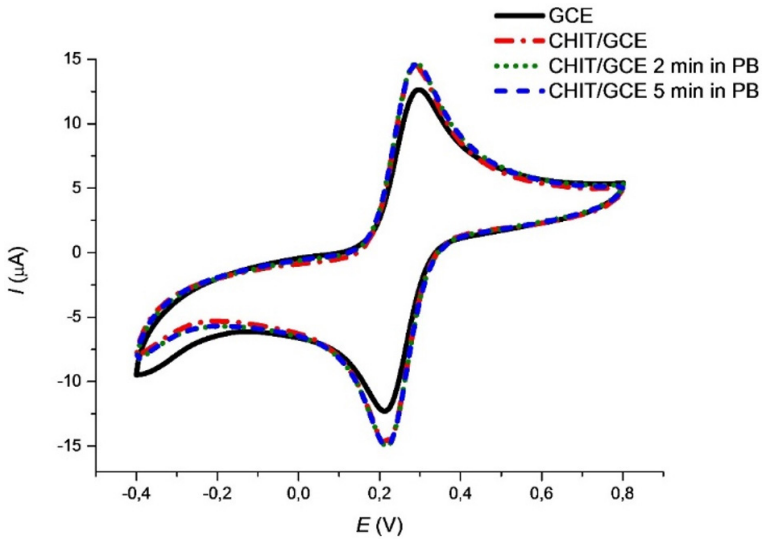


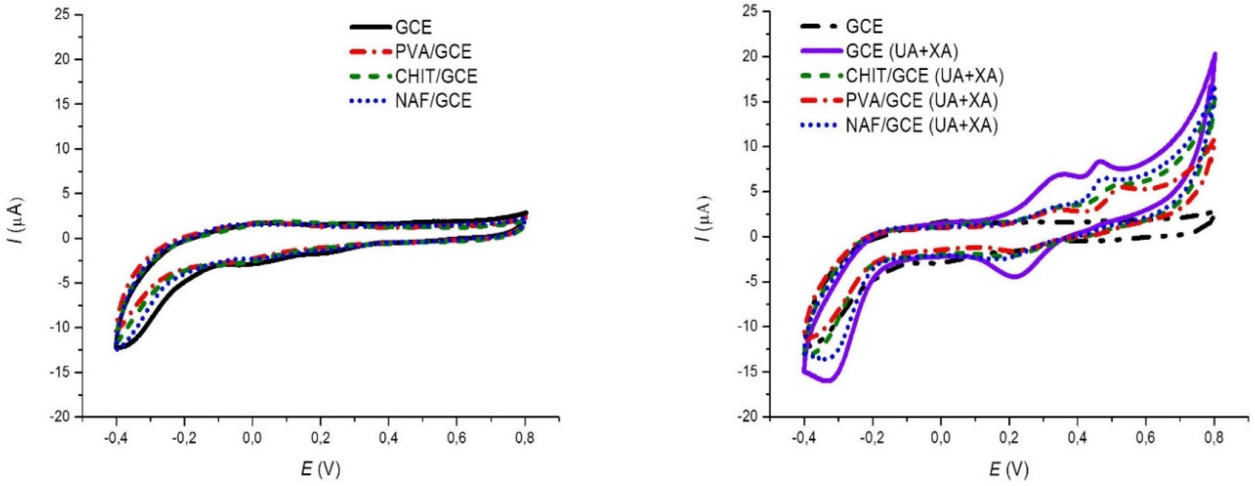
Fig. 3. CV response of $[Fe(CN)_6]^{3-/4-}$ redox indicator for bare GCE and CHIT/GCE immediately and after 2 and 5 min of incubation in PB with pH 7.4.

ments. The hydrophilic property of CHIT, NAF and PVA makes them suitable candidates for purine metabolites detection in body fluids.

Simultaneous detection of purine metabolites

In this work, CV was used to examine sensing performance of GCE, CHIT/GCE, NAF/GCE, and PVA/GCE sensor after 5 min of incubation in 0.1 M PB with pH 7.4 simulating physical conditions. Voltammograms in Fig. 4 show the effect of protective membrane layers. Solid line represents the voltammogram of bare GCE. CHIT, NAF, and PVA membrane layers (Fig. 4a, dashed line, dotted line and dash-dotted line, respectively) had no effect on the oxidation process at the scan rate of 100 $mV \cdot s^{-1}$ and step potential of 2 mV. All measurements were performed in 20 mL electrochemical cells at ambient temperature. All three membrane modified sensors showed sufficient stability in the PB pH 7.4 solution after 5 min of incubation.

Simultaneous electrochemical oxidation of XA and UA was thoroughly investigated using CV in 0.1 M PB with pH 7.4 as described in experimental methods. In the positive potential region, two well-defined anodic peaks corresponding to UA and XA oxidation were observed (Fig. 4b). The oxidation peak potentials of UA and XA on the surface of bare GCE were around 0.36 V and 0.47 V, respectively.



a) b)

Fig. 4. CV response of bare GCE (solid line), PVA/GCE (dash-dotted line), CHIT/GCE (dashed line), and NAF/GCE (dotted line) after 5 min of incubation in PB with pH 7.4 (a) or PB with pH 7.4 and 10^{-5} $molL^{-1}$ UA and 10^{-5} $molL^{-1}$ XA (b).

Tab. 1. *Ip* and *Ep* anodic peak response values for UA and XA for bare/modified GCE.

Sensor	UA		XA	
	***Ipa* (μA)**	***Epa* (V)**	***Ipa* (μA)**	***Epa* (V)**
GCE	4.43	0.36	5.37	0.47
PVA/GCE	1.39	0.36	3.79	0.53
CHIT/GCE	1.77	0.36	4.05	0.52
NAF/GCE	1.91	0.36	4.79	0.48

The *Ipa* and *Epa* values for UA and XA response for bare GCE and membrane modified GCE are shown in Table 1.

The best anodic peak current response values for UA and XA were observed with the NAF/GCE sensor. The NAF/GCE sensor showed no *Epa* shift to positive or negative values. *Epa* for UA showed no shift at all. An *Epa* shift towards more positive potential values was observed for XA detection within the CHIT and PVA membrane modified GCE sensor. In case of PVA, only the PVA modification of bare GCE in the absence of XA resulted in an *Epa* shift for redox indicator (Fig. 1). For the CHIT/GCE modified sensor, the *Epa* shift to the positive values in case of XA detection may be the result of electrostatic repulsion as XA can exist in the solution in the form of positively charged dimers (Paragi et al., 2011). Similar electrostatic repulsion and oxidation potential shift was obtained in dopamine detection in the presence of positively charged modified film (Chen and Chzo, 2006).

Conclusion

To avoid unwanted signal interferences caused by low or high molecular weight substances from the analyte matrices, modifications of the GCE sensor surface with electro-neutral PVA, positively charged CHIT, and negatively charged NAF was studied. Simultaneous detection of XA and UA purine metabolites by CV was performed. The time interval of 2 min was sufficient for the membrane regeneration and $[Fe(CN)_6]^{3-/4-}$ redox indicator signal stabilization in PB with pH 7.4. All three membrane modified sensors, namely PVA/GCE, NAF/GCE, and CHIT/GCE, showed adequate stability in PB with pH 7.4 after 5 min of incubation and were efficient in the fast, simple, and cost-effective simultaneous detection of XA and UA. The highest *Ipa* values were observed for the NAF/GCE sensor, showing the best results in the UA and XA detection. No *Epa* shift to positive or negative values was reported. The *Epa* shift to positive values in the detection of XA by PVA/GCE was due to the GCE modification by PVA alone. In case of CHIT/GCE, the *Epa* shift to positive values for XA was the result of electrostatic repulsion. The present study demonstrates the efficiency of external protective membranes in simultaneous detection of purine metabolites.

Acknowledgement

This research was financially supported by the Slovak Research and Development Agency APVV-20-0413.

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