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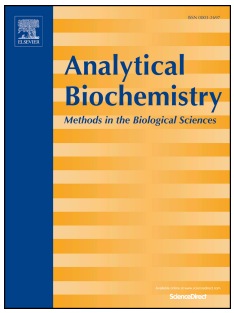

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Electrochemical methods

Electrochemical detection of uric acid via uricase-immobilized graphene oxide

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ABSTRACT

Measurement of the uric acid level in the body can be improved by biosensing with respect to the accuracy, sensitivity and time consumption. This study has reported the immobilization of uricase onto graphene oxide (GO) and its function for electrochemical detection of uric acid. Through chemical modification of GO using 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (NHS) as cross-linking reagents, the enzyme activity of the immobilized uricase was much comparable to the free enzyme with 88% of the activity retained. The modified GO-uricase (GOU) was then subjected to electrocatalytic detection of uric acid (UA) via cyclic voltammetry (CV). For that reason, a glassy carbon electrode (GCE) was modified by adhering the GO along with the immobilized uricase to facilitate the redox reaction between the enzyme and the substrate. The modified GOU/GCE outperformed a bare electrode through the electrocatalytic activity with an amplified electrical signal for the detection of UA. The electrocatalytic response showed a linear dependence on the UA concentration ranging from 0.02-0.49 mM with a detection limit of 3.447 μM at 3σ/m. The resulting biosensor also exhibited a high selectivity towards UA in the presence of other interference as well as good reproducibility.

Keywords*:* uricase; immobilization; GO; electrocatalytic detection; uric acid; redox reaction

Introduction

Determining the uric acid (UA) concentration in human body fluids (e.g., serum and urine) is required for the diagnosis of several diseases and determination of UA is a clinically

valuable diagnostic indicator [1]. UA is the primary end product of purine metabolism in the human body. Physiological level of UA in serum is between 0.13 mM and 0.46 mM (2.18-77 mg dl^{-1}) [2][3]. High level of UA in the body will lead to several diseases including gout, hyperucemia and Lesh-Nyhan syndrome [4–6]. A few studies have reported that the production of excess UA in serum is also a risk factor for cardiovascular and neurological diseases [7][8]. UA can be determined through the oxidation of its purine ring into allantoin, carbon dioxide and hydrogen peroxide with the presence of enzyme called uricase [9]. This enzyme is found in most organisms, however it is absent in human due to gene evolution and it plays different metabolic roles depending on its host organism [10]. Uricase assay is one of the most useful enzymatic determination of UA by coupling it with 4-aminoantipyrine-peroxidase system [11].

Most UA detection are electrochemical [12–14], UV and colorimetric [15–17], and chemiluminescence [18–20]. These methods, however, have exhibited certain drawbacks in terms of interferences, requirement of expensive instrumentation, selectivity and also sensitivity which may limit the intended applications. Current methodologies in electrochemical biosensing based on wide variety of nanostructures such as reduced graphene oxide (rGO), nanotubes and nonporous materials have attracted great interest due to their outstanding properties like non toxicity, excellent electrical conductivity, high sensitivity and low cost with ease of fabrication [21–24]. Graphene oxide (GO) introduced excellent multi-purpose functions and characteristics in many research fields. This material possesses incredibly large specific surface area, has an abundant oxygen functional groups such as carboxylic at the edges, hydroxyl and epoxide groups at the basal plane and is highly dispersible in water which provide numerous promising applications [25–29]. It is a flexible two-dimensional single layer carbon atom with the application is not just confined to electronic devices instead GO has been introduced to many reported works as seen in literature including as a biosensing medium. In biological systems, GO has received much attention due to its inertia, low toxicity under physiological conditions and good biocompatibility [30,31]. However, further research is needed to study the immobilization of enzymes on GO solely and its functions electrochemically.

Today, numerous methods of immobilization of uricase onto various supports have been reported such as adsorption onto cellulose acetate membrane [32], polyaniline film [18], physisorption onto polypyrrole and polyaniline [33], crosslinking onto chitosan membrane [1] and ZnO nanorods [34]. However, these methods required complex stages of preparation and provide less stability that may limit the fabrication and application of uric acid biosensors. Combination of immobilized enzyme with electrochemical biosensing provides unique advantages as they can maintain the enzymatic activity in a microenvironment and enhance the direct electron transfer between the active sites and the electrode [35].

In this study, we have reported the synthesis of GO through simplified Hummer's method and a simple immobilization approaches of uricase onto GO (GOU) as a voltammetric uric acid biosensor. Basic principle of uricase assay was conducted to test the immobilized enzyme activity. The electrocatalytic analysis of the GOU sensor on the detection of UA was investigated

through cyclic voltammetry. This study highlights promising features in the development of a low cost, simple design, highly sensitive and specific, fast response, less interference uric acid biosensor.

Materials and methods

Materials

Graphite powder was acquired from Ashbury Graphite Mills Inc. (New Jersey, USA). Sulphuric acid (H_2SO_4, 95-98%), phosphoric acid (H_3PO_4, 85%), potassium permanganate ($KMnO_4$, 99.9%), hydrochloric acid (HCl, 37%) and hydrogen peroxide (H_2O_2, 30%) were purchased from Systerm (Malaysia). Uricase (EC 232-655-5, 19.7 units/mg, from *Arthrobacter globiformis* and uric acid (99% purity) were purchased from Sigma (USA). Other reagents used in this work were of at least analytical grade and used as received without further purification. Phosphate buffer solution (PBS) was prepared by mixing the stock standard solutions of Na_2HPO_4 (99% purity) and NaH_2PO_4 (99% purity). All solutions were prepared using deionized water obtained from Millipore milli-Q system.

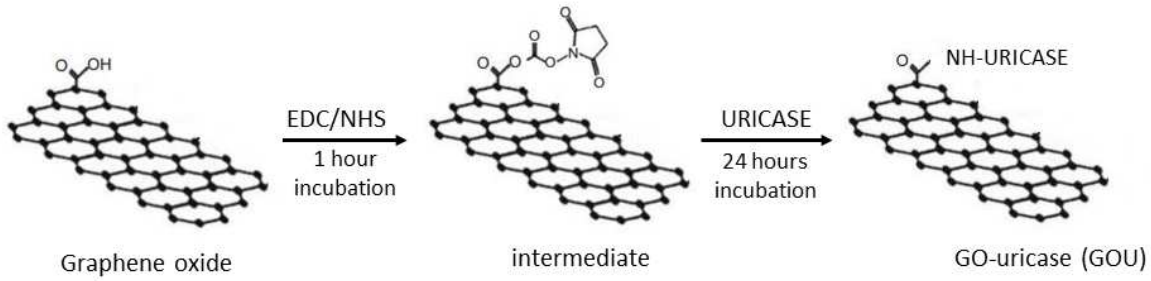
Synthesis of GO

Graphene oxide synthesis was based on simplified Hummer's method [36]. Strong oxidizing agents were used to oxidize the graphite flakes. In this study, H_2SO_4:H_3PO_4 (320:80 ml) was added into graphite flakes along with 18 g of $KMnO_4$ and stirred with magnetic bar. The mixture was continuously stirred for three days to allow complete oxidation of graphite. During oxidation the colour of the mixture changed from purplish green to dark brown. Then H_2O_2 solution was added into the mixture to stop the oxidation process. The colour changed again to bright yellow, indicating high oxidation degree of graphite. The mixture formed known as graphite oxide was then washed three times with 1 M of HCl aqueous solution and followed by deionized water repeatedly until pH 4-5 was obtained. The washing steps were carried out using simple decantation of supernatant using a centrifugation technique. During the washing process, the graphite oxide underwent exfoliation resulting in thickening the GO solution and lastly forming a GO gel. To proceed for the enzyme immobilization part, the GO gel was freeze-dried to obtain the GO solid [37].

Immobilization of uricase onto GO using EDC-NHS esters as crosslinking reagents

Scheme 1 is the schematic diagram for the preparation of the graphene oxide-uricase (GOU) complex. Firstly, chemical modification of GO was carried out by introducing the compound to two cross-linking reagents, N-hydroxysulfosuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). 1 mg/ml of GO was incubated with 1 ml of 50 mM of NHS and 1 ml of 20 mM EDC for 1 h. Next, the modified GO was centrifuged for 10,000 rpm for 10 min and washed using PBS to remove any crosslinkers residue. 1 ml of uricase (1.315 µg/ml) was then added into the modified GO and incubated at 4^oC for 15 h with mild shaking on

a rotator. The mixture was then centrifuged again at 10,000 rpm for 10 min and the precipitate which was the immobilized uricase on GO (GOU) was collected for construction of the biosensor platform.



Scheme 1. Schematic diagram of uricase immobilization onto graphene oxide using EDC-NHS as cross-linkers.

Enzyme activity determination of immobilized uricase

The enzyme activity of the immobilized uricase was determined through uricase assay [38]. The principle of enzyme activity measurement was that uricase could catalyze the oxidation of uric acid into allantoin and H_2O_2 which was then measured by using a chromogen reaction system containing 4-aminoantipyrine, phenol, and peroxidase. In this study, 0.1 ml of GOU was incubated with a mixture of 0.6 ml PBS (pH 6.5, 10 mM) containing 4 mM uric acid, 0.15 ml of 30 mM 4-aminoantipyrine, 0.15 ml 0f 1.5% phenol and 0.05 ml of 15 U/ml peroxidase at 37^{o}C for 20 min. The reaction was then stopped by addition of 1.0 ml of absolute ethanol and finally the absorbance at 540 nm was read through a spectrophotometer. One unit of enzyme was defined as the rate of product produced for 1 mM of H_2O_2 under standard assay conditions.

Fabrication and electrochemical analysis of GO-uricase modified GC electrode

The GOU (5 uL) was drop-casted onto the surface of GCE and allowed to evaporate for 15 min under the air stream at room temperature. Once the GOU was completely dried, the electrode was then labelled as GOU/GCE and served as working electrode (WE) for the electrochemical analysis. Cyclic voltammetry (CV) were performed using Autolab (PGSTAT101) electrochemical workstation with a three electrode system. A silver/silver chloride (Ag/AgCl) electrode was used as reference electrode while a platinum electrode was used as an auxiliary electrode. All experiments were done in a three-electrode electrochemical cell and carried out using 0.1 mol/L PBS (pH = 6.5). Several experiment parameters were optimized to obtain a suitable working potential range from -1.0 to 1.0 V and optimal CV response. A reproducible CV was obtained under steady-state conditions after about 5 cycles. All CV measurements unless otherwise stated were recorded at a constant scan rate of 50 mV per

second and potential window ranging from -1.0 to 1.0. Electrolyte used for these electrochemical measurements was uric acid. 100 µl of 4 mM UA solution was spiked into 20 ml of 10 mM PBS a few times until a final concentration of UA up to 0.154 mM. The CV was recorded for each UA solution spiked in. For the selectivity study, ascorbic acid (4 mM) was used in similar method with UA measurement.

Results and discussion

Immobilization of uricase on GO using crosslinking reagents

Uricase was immobilized on GO sheets through diimide-activated amidation reaction of the amine groups on uricase with the carboxyl groups of GO under ambient condition using EDC-NHS as crosslinking reagents. Through a standard protein assay using Bradford's reagent kit, 100% uricase was successfully immobilized onto GO. EDC alone reacts with carboxylic acid groups on the GO to form GO-*O*-acylurea intermediate that is easily relocated by nucleophilic attack from primary amino groups in the reaction mixture of the uricase. The primary amine of uricase forms an amide bond with the original carboxyl group and EDC by-product is released as a soluble urea derivative. The *O*-acylurea intermediate is unstable in aqueous solutions. Thus, failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls and the release of an N-unsubstituted urea. Therefore, NHS is regularly included in EDC coupling protocols to improve efficiency and create dry stable intermediates. EDC couples NHS to carboxyl groups of GO forming a NHS ester that is considerably more stable than the *O*-acylurea intermediate while allowing for efficient conjugation to primary amines of uricase at physiological pH [39–41].

Enzymatic activity of immobilized uricase on GO

Without using any cross-linkers, no protein was found to be immobilized on the GO and no activity of the uricase can be detected. However, through chemical modification of GO using EDC-NHS, the immobilized uricase was able to perform a much comparable activity with the free enzyme, where 88% of the activity was retained. This proved that the uricase was covalently bonded on the GO and still able to retain its enzymatic activity.

A comparison of the colour intensity of the reaction of the chromogen and the enzyme in the presence of its substrate, uric acid was shown in Figure 1. The figure showed various colour intensities after the addition of ethanol as to stop the reaction which can be seen in the following mediums; blank, free uricase, GO without immobilized uricase and GOU. Basically, the reaction of uricase and uric acid will give out H_2O_2. With the presence of 4-aminoantipyrine, phenol and peroxidase, concentration of H_2O_2 can be determined through colour changes of the chromogen and followed by measurements done spectrophotometrically. Higher colour intensity indicated higher enzymatic activity of uricase. Through immobilization, the uricase showed a similar colour intensity when compared to the free uricase. This once again proved that the immobilized uricase was able to retain its enzymatic activity

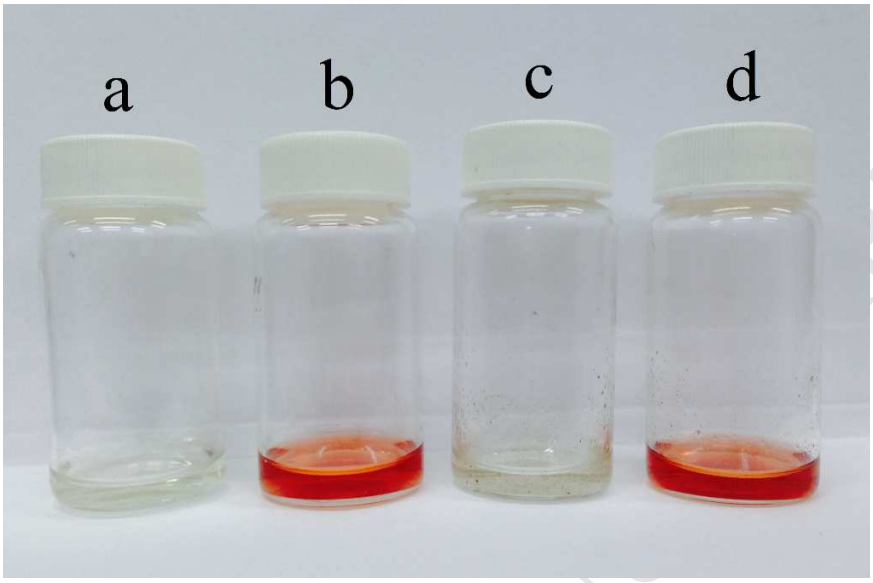


Fig.1. Reaction mixture of chromogen and uricase in the presence of uric acid after adding ethanol; (a) blank, (b) free enzyme, (c) control-GO without immobilization of uricase (d) GO with immobilized uricase using sulfo-NHS and EDC as crosslinking reagents.

Electrocatalytic activity of GOU/GCE

The electrocatalytic activity of GOU/GCE was carried out using UA as the electrolyte. Cyclic voltammetry (CV) for the detection of UA is shown in Figure 2A. A clear and significant potential peak of the sensor was observed on the voltammogram (0.47 V). The current and also the anodic peak increase with increasing concentration of UA spiked into PBS, pH 6.5 (0.02 mM to 0.154 mM). Highest current obtained on the anodic peak is on 18 µA. The electrochemical detection of uric acid through this biosensor is based on enzymatic reaction catalyzed by uricase according to the following;

$$C_5H_4N_4O_3(\text{uric acid}) + 2H_2O_2 + O_2 \xrightarrow{\text{Uricase}} C_4H_6N_4O_3\ (\text{allantoin}) + H_2O_2 + CO_2$$

When uric acid is oxidized in the presence of uricase, allantoin along with hydrogen peroxide and carbon dioxide will be produced. GO has a large specific surface area, this resulting in uricase immobilized on the GO has more spatial freedom in its orientation, which aid in assisting direct electron transfer between the active site of the enzyme and the electrode surface [34].

Chronoamperometric response also was recorded to extend the detection range of UA in lower and higher concentrations. Figure 2B shows the calibration curve plotted using the chronoamperometric values in the presence of excess uric acid concentration in the reaction

medium. Concentration of uric acid spiked in the medium is in within the range of 0.020 mM to 0.491 mM. The detection limit calculated from the analysis is 3.45 μM at 3σ/m.

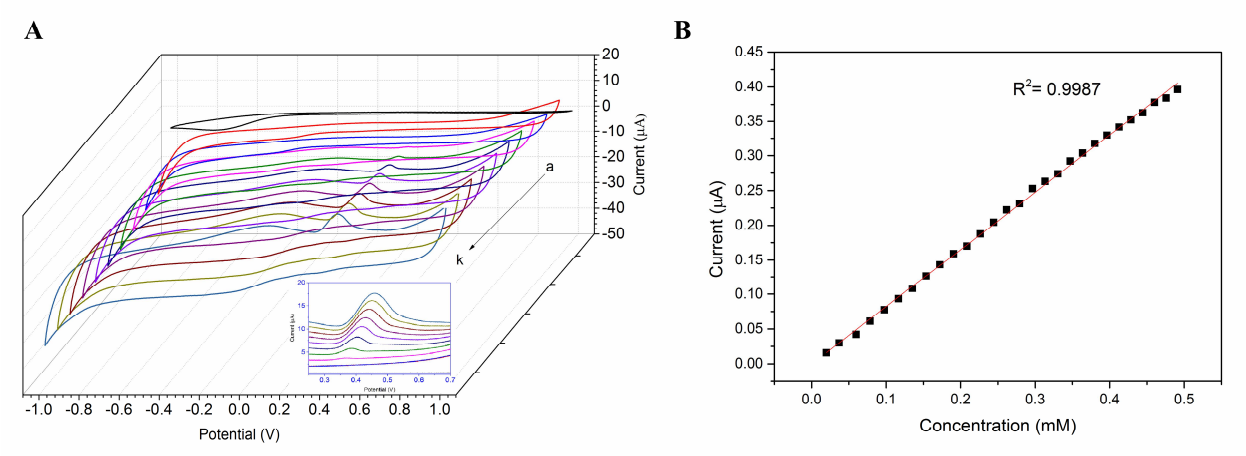


Fig.2A (left). Cyclic voltammograms of GOU on detection of UA in PBS, pH 6.5, scan rate 50 mV s^{-1} (a → k). (a) bare gc, (b) GO, (c) GOU, (d) GOU in 0.020 mM of UA, (e) GOU in 0.037 mM of UA, (f) GOU in 0.060 mM of UA, (g) GOU in 0.079 mM of UA, (h) GOU in 0.098 mM of UA, (i) GOU in 0.117 mM of UA, (j) GOU in 0.135 mM of UA and (k) GOU in 0.154 mM of UA. Inset is the oxidation peak of UA based on 2D cyclic voltammetry. **Fig.2B (right).** Calibration plot of uric acid with increasing concentrations in the range of 0.020 mM to 0.491 mM.

Selectivity, stability and reproducibility analyses

Selectivity study for the UA biosensor was conducted against ascorbic acid (AA). Basically the selectivity of a uric acid sensor depends on two factors; the enzyme-analyte reaction and the selective mechanism. The enzyme analyte reaction is very specific with respect to the nature of the uricase functionality that it only reacts with UA. The possible interferences present in blood that normally disrupt the amperometric uric acid detection is AA [35]. A cyclic voltammogram (Figure 3) was recorded using a fixed concentration of UA (0.154 mM) but with varying concentration of AA spiked in the range of 0.020 mM to 0.098 mM of AA. A peak of 0.47 V was obtained for UA whilst a 0.15 V peak was observed for AA.

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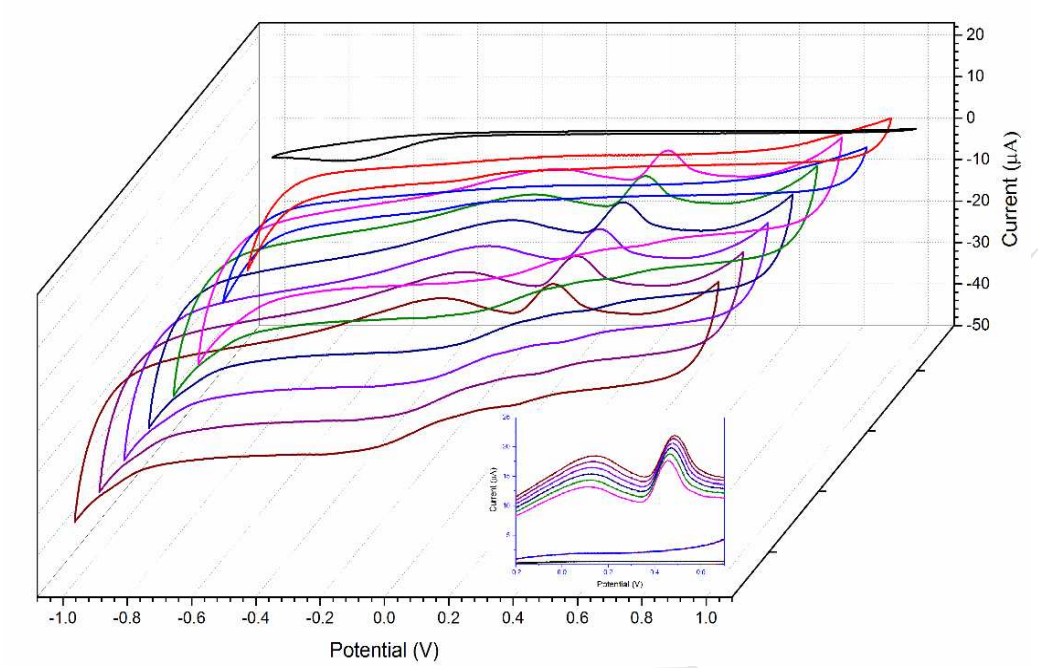

Fig.3. Cyclic voltammogram for interference study between UA and AA. Inset is the oxidation peak of UA and AA in cyclic voltammetry.

Cyclic voltammogram of AA also was recorded to confirm the oxidation peak of the compound (Figure 4). Immobilized uricase was not used in the detection, to ensure that the enzyme itself does not react with the AA to give out oxidation peak. However, the oxidation of AA was recorded by the electrode. This may be due to the surface of GO being not fully saturated with uricase and that there was still available site of reaction on the electrode. A peak was produced at 0.15 V for the oxidation of AA alone.

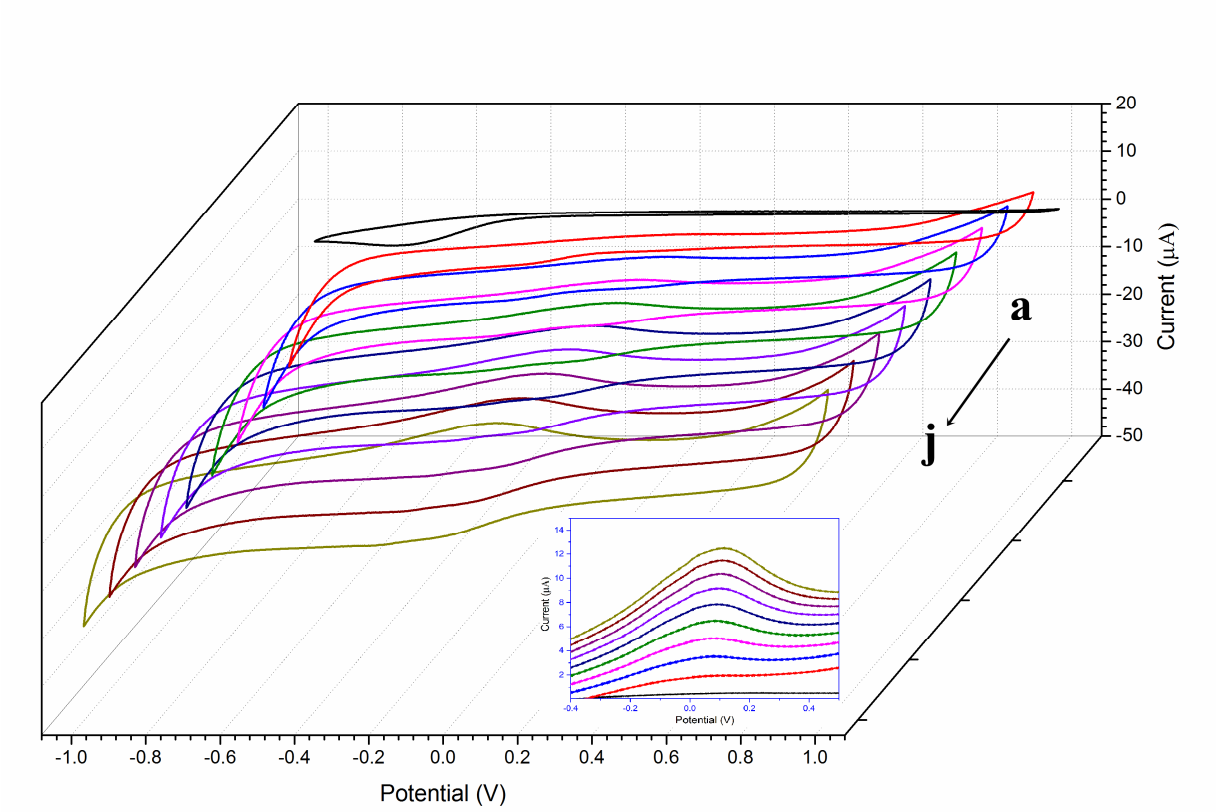

Fig.4. Cyclic voltammogram of GO on detection of AA in PBS, pH 6.5, scan rate 50 mV s^{-1} (a) bare gc, (b) GO, (c) GO in 0.020 mM of AA, (d) GO in 0.037 mM of AA, (e) GO in 0.060 mM of AA, (f) GO in 0.079 mM of AA, (g) GO in 0.098 mM of AA, (h) GO in 0.117 mM of AA, (i) GO in 0.135 mM of AA and (j) GO in 0.154 mM of AA. Inset is the oxidation peak of AA at 0.15 V in cyclic voltammetry.

Lastly, to prove the specificity of the reaction is between immobilized GOU with UA only, a cyclic voltammogram was recorded using a fixed concentration of AA but increasing concentration of UA was spiked in (Figure 5). Based on the figure, when UA was spiked into the 0.098 mM of AA solution, a significant peak was recorded on the voltammogram at 0.47 V. This shows that it was indeed the GOU that react and record the oxidation of UA in the medium.

The GOU/GCE also underwent stability and reproducibility test. A few scan rates ranging from 10, 30, 50, 70, 100, 130 and 150 mV/s were used to determine the stability. The test was recorded through CV in the presence of 0.098 mM uric acid solution in 10 mM PBS (pH 6.5). From the voltammogram the GOU/GCE was able to retain its CV pattern with an oxidation peak of UA at 0.47 V. It showed that the GOU/GCE can operate under a few different scan rates either high or low but still able to retain its oxidation peak and pattern.

For reproducibility study, the GOU was kept at 4^{o}C and the CV was recorded for 0.098 mM UA for a total of 30 days. At day 10, a decrease of 15 % of the oxidation peak of UA was found when compared to an oxidation peak of UA using freshly prepared GOU. These observations proved that the GOU/GCE was stable and reproducible for the detection of UA. A comparison of various properties of GOU based sensor with those uricase immobilized onto different supports is summarized in Table 1.

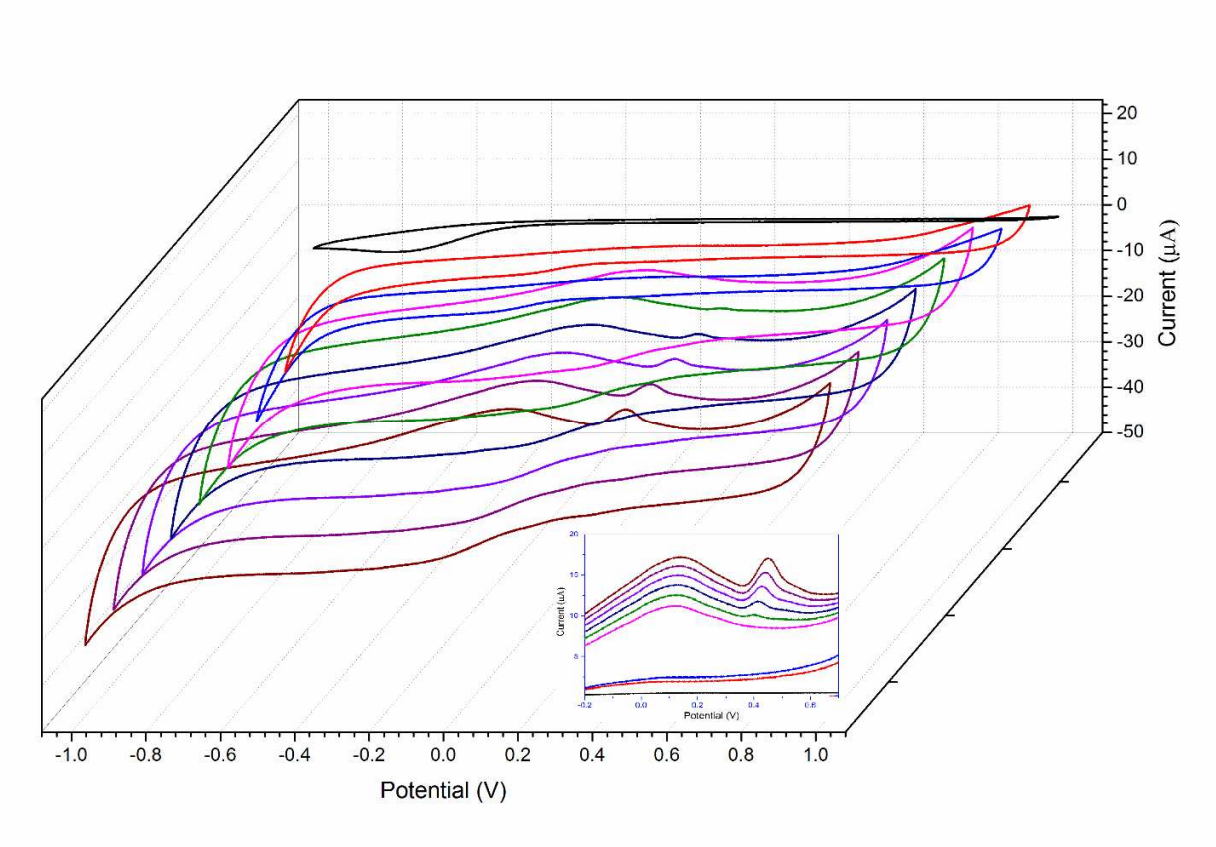

Fig.5. Cyclic voltammogram of AA in fixed concentration (0.098 mM) but spiked in with increasing concentration of UA. Inset is the the oxidation peak for both AA (0.15 V) and UA (0.43) in 2D cyclic voltammetry.

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265 Table 1: A comparison of present uric acid biosensor with earlier reported sensors.

Source of uricase	Immobilization support	Method of immobilization	Working range	Detection limit	Mode of measurement	References
Arthrobacter globiformis	Egg shell	Covalent bonding	4.0-6.40 μM	2 μM	Dissolved O_2 estimation	Zhang et al., 2007
Black-eyed cowpea leaf	Araldite membrane	Hydrogen bonding	0.005-1 mM	25.22 μM	Dissolved O_2 estimation	Arora et al., 2009
Bacillus fastidious	PANI/MWCNT	Covalent bonding	0.005-0.06 mM	5 μM	Amperometric	Bhambi et al., 2010
Uric acid kit	AuNPs/MWCNT	Covalent bonding	0.01-0.8 mM	0.01 mM	Amperometric	Chauhan and Pundir, 2011
Did not use uricase	Reduced GO	-	0.8–2500 μM	0.20 μM	Amperometric	Ping et al., 2012
Lactobacillus plantarum	Natural zeolite	Cells immobilization	0.001-0.05 mM	-	Amperometric	Iswantini et al., 2014
Did not use uricase	Partially electro-reduced GO	-	100 nM-10 μM	0.05 μM	Amperometric	Zhang and Yin, 2014
Arthrobacter globiformis	GO	Covalent bonding	0.02–0.49 mM	3.45 μM	Amperometric	This work

Conclusion

In conclusion, the immobilization of uricase onto GO was successfully carried out and the immobilized enzyme was able to retain the activity to 88% as compared to the free enzyme. The constructed biosensor also has achieved a direct electron transport from the reaction of uricase and UA based on cyclic voltammetry study. The linear range of the obtained electrochemical sensor for UA detection was found to be 0.02–0.49 mM, with a detection limit of 3.45 μM at 3σ/m. Besides, the resulting biosensor also showed a good selectivity toward UA even in the presence of another interfering compound i.e. ascorbic acid. In terms of stability and reproducibility the biosensor exhibited a remarkable result. The attractive features of GO also play an important role in providing a favourable microenvironment for enzyme immobilization which may lead to the production of a simple, fast-response and accurate uric acid biosensor.

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References

[1] D. Martinez-Pérez, M.L. Ferrer, C.R. Mateo, A reagent less fluorescent sol–gel biosensor for uric acid detection in biological fluids, Anal. Biochem. 322 (2003) 238–242. doi:10.1016/j.ab.2003.08.018.

[2] S.H. Huang, Y.C. Shih, C.Y. Wu, C.J. Yuan, Y.S. Yang, Y.K. Li, T. K. Wu, Detection of serum uric acid using the optical polymeric enzyme biochip system., Biosens. Bioelectron. 19 (2004) 1627–1633. doi:10.1016/j.bios.2003.12.026.

[3] C.R. Raj, T. Ohsaka, Electroanalysis of ascorbate and dopamine at a gold electrode modified with a positively charged self-assembled monolayer, J. Electroanal. Chem. 496 (2001) 44–49. doi:10.1016/S0022-0728(00)00335-1.

[4] I.H. Fox, Metabolic basis for disorders of purine nucelotide degradation, Metabolism. 30 (1981) 616–634. doi:10.1016/0026-0495(81)90142-6.

[5] B. Ullman, M. a Wormsted, M.B. Cohen, D.W. Martin, Purine oversecretion in cultured murine lymphoma cells deficient in adenylosuccinate synthetase: genetic model for inherited hyperuricemia and gout., Proc. Natl. Acad. Sci. U. S. A. 79 (1982) 5127–5131.

[6] P.A. Simkin, Management of Gout, Ann. Intern. Med. 90 (1979) 812–816. doi:10.7326/0003-4819-90-5-812.

[7] H.J. Moallem, G. Taningo, C.K. Jiang, R. Hirschhorn, S. Fikrig, Purine Nucleoside Phosphorylase Deficiency: A New Case Report and Identification of Two Novel Mutations (Gly156A1a and Val217Ile), Only One of Which (Gly156A1a) is Deleterious, Clin. Immunol. 105 (2002) 75–80. doi:10.1006/clim.2002.5264.

[8] J.F. Baker, E. Krishnan, L. Chen, H.R. Schumacher, Serum uric acid and cardiovascular disease: recent developments, and where do they leave us?, Am. J. Med. 118 (2005) 816–826. doi:10.1016/j.amjmed.2005.03.043.

[9] K. Motojima, S. Kanaya, S. Goto, Cloning and sequence analysis of cDNA for rat liver uricase, J. Biol. Chem. 263 (1988) 16677–16681.

[10] X.W. Wu, C.C. Lee, D.M. Muzny, C.T. Caskey, Urate oxidase: primary structure and evolutionary implications., Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 9412–9416.

[11] N. Gochman, J.M. Schmitz, Automated Determination of Uric Acid, with Use of a Uricase-Peroxidase System, Clin. Chem. 17 (1971) 1154–1159.

[12] T. Hoshi, H. Saiki, J.-I. Anzai, Amperometric uric acid sensors based on polyelectrolyte multilayer films., Talanta. 61 (2003) 363–368. doi:10.1016/S0039-9140(03)00303-5.

[13] J.-C. Chen, H.-H. Chung, C.-T. Hsu, D.-M. Tsai, a. S. Kumar, J.-M. Zen, A disposable single-use electrochemical sensor for the detection of uric acid in human whole blood, Sensors Actuators B Chem. 110 (2005) 364–369. doi:10.1016/j.snb.2005.02.026.

[14] H. El Bouhouti, I. Naranjo-Rodríguez, J.L.H.-H. de Cisneros, M. ElKaoutit, K.R. Temsamani, D. Bouchta, L.M.C. Aguilera, Electrochemical behaviour of epinephrine and uric acid at a Sonogel-Carbon L-Cysteine modified electrode., Talanta. 79 (2009) 22–6. doi:10.1016/j.talanta.2009.02.057.

[15] N. Kagemaya, A direct colorimetric determination of uric acid in serum and urine with uricase-catalse system, Clin. Chim. Acta. 31 (1970) 421–426.

[16] P. Schrenkhammer, New Optical Biosensors for Uric Acid and Glucose, University of Regensburg, 2008.

[17] P. Schrenkhammer, O.S. Wolfbeis, Fully reversible optical biosensors for uric acid using oxygen transduction., Biosens. Bioelectron. 24 (2008) 1000–5. doi:10.1016/j.bios.2008.08.007.

[18] D. Yao, A.G. Vlessidis, N.P. Evmiridis, Microdialysis sampling and monitoring of uric acid in vivo by a chemiluminescence reaction and an enzyme on immobilized chitosan support membrane, Anal. Chim. Acta. 478 (2003) 23–30. doi:10.1016/S0003-2670(02)01484-8.

[19] C. Yang, Z. Zhang, A novel flow-injection chemiluminescence determination of uric acid based on diperiodatoargentate(III) oxidation., Talanta. 81 (2010) 477–81. doi:10.1016/j.talanta.2009.12.028.

[20] J. Yu, S. Wang, L. Ge, S. Ge, A novel chemiluminescence paper microfluidic biosensor based on enzymatic reaction for uric acid determination., Biosens. Bioelectron. 26 (2011) 3284–9. doi:10.1016/j.bios.2010.12.044.

[21] U. Harborn, B. Xie, R. Venkatesh, B. Danielsson, Evaluation of a miniaturized thermal biosensor for the determination of glucose in whole blood., Clin. Chim. Acta. 267 (1997)

225–37. http://www.ncbi.nlm.nih.gov/pubmed/9469255.

[22] M.K. Ram, M. Adami, S. Paddeu, C. Nicolini, Nano-assembly of glucose oxidase on the in situ self-assembled films of polypyrrole and its optical , surface and electrochemical characterizations, 112 (n.d.).

[23] B.M. Dixon, J.P. Lowry, R.D.O. Neill, Characterization in v itro and in v i v o of the oxygen dependence of an enzyme / polymer biosensor for monitoring brain glucose, 119 (2002) 135–142.

[24] C. Jianrong, M. Yuqing, H. Nongyue, W. Xiaohua, L. Sijiao, Nanotechnology and biosensors., Biotechnol. Adv. 22 (2004) 505–18. doi:10.1016/j.biotechadv.2004.03.004.

[25] D. Li, M.B. Müller, S. Gilje, R.B. Kaner, G.G. Wallace, Processable aqueous dispersions of graphene nanosheets., Nat. Nanotechnol. 3 (2008) 101–5. doi:10.1038/nnano.2007.451.

[26] S. Park, R.S. Ruoff, Chemical methods for the production of graphenes., Nat. Nanotechnol. 4 (2009) 217–24. doi:10.1038/nnano.2009.58.

[27] Y. Liu, D. Yu, C. Zeng, Z. Miao, L. Dai, Biocompatible graphene oxide-based glucose biosensors., Langmuir. 26 (2010) 6158–60. doi:10.1021/la100886x.

[28] M. Veerapandian, Y.-T. Seo, H. Shin, K. Yun, M.-H. Lee, Functionalized graphene oxide for clinical glucose biosensing in urine and serum samples., Int. J. Nanomedicine. 7 (2012) 6123–36. doi:10.2147/IJN.S38402.

[29] E.K. Goharshadi, G. Akhlamadi, S.J. Mahdizadeh, Investigation of graphene oxide nanosheets dispersion in water based on solubility parameters: a molecular dynamics simulation study, RSC Adv. 5 (2015) 106421–106430. doi:10.1039/C5RA19932H.

[30] Y. Wang, Z. Li, J. Wang, J. Li, Y. Lin, Graphene and graphene oxide: biofunctionalization and applications in biotechnology., Trends Biotechnol. 29 (2011) 205–12. doi:10.1016/j.tibtech.2011.01.008.

[31] X. Wu, Y. Zhang, C. Wu, H. Wu, Preparation and characterization of magnetic Fe3O4/CRGO nanocomposites for enzyme immobilization, Trans. Nonferrous Met. Soc. China. 22 (2012) s162–s168. doi:10.1016/S1003-6326(12)61703-8.

[32] J. Kan, X. Pan, C. Chen, Polyaniline-uricase biosensor prepared with template process., Biosens. Bioelectron. 19 (2004) 1635–40. doi:10.1016/j.bios.2003.12.032.

[33] F. Arslan, An Amperometric Biosensor for Uric Acid Determination Prepared From Uricase Immobilized in Polyaniline-Polypyrrole Film, Sensors. 8 (2008) 5492–5500. doi:10.3390/s8095492.

[34] F. Zhang, X. Wang, S. Ai, Z. Sun, Q. Wan, Z. Zhu, Y. Xian, L. Jin, K. Yamamoto, Immobilization of uricase on ZnO nanorods for a reagentless uric acid biosensor, Anal. Chim. Acta. 519 (2004) 155–160. doi:10.1016/j.aca.2004.05.070.

[35] S.M. Usman Ali, N.H. Alvi, Z. Ibupoto, O. Nur, M. Willander, B. Danielsson, Selective potentiometric determination of uric acid with uricase immobilized on ZnO nanowires, Sensors Actuators, B Chem. 152 (2011) 241–247. doi:10.1016/j.snb.2010.12.015.

[36] H.N. Lim, N.M. Huang, S.S. Lim, I. Harrison, C.H. Chia, Fabrication and characterization of graphene hydrogel via hydrothermal approach as a scaffold for preliminary study of cell growth., Int. J. Nanomedicine. 6 (2011) 1817–23. doi:10.2147/IJN.S23392.

[37] N.M. Huang, H.N. Lim, C.H. Chia, M. a Yarmo, M.R. Muhamad, Simple room-temperature preparation of high-yield large-area graphene oxide., Int. J. Nanomedicine. 6 (2011) 3443–8. doi:10.2147/IJN.S26812.

[38] A. Anderson, S. Vijayakumar, Purification and Optimization of Uricase Enzyme Produced by Pseudomonas aeruginosa, 2 (2011) 5–8.

[39] K. Nam, T. Kimura, A. Kishida, Controlling coupling reaction of EDC and NHS for preparation of collagen gels using ethanol/water co-solvents., Macromol. Biosci. 8 (2008) 32–7. doi:10.1002/mabi.200700206.

[40] S. Sam, L. Touahir, J. Salvador Andresa, P. Allongue, J.-N. Chazalviel, a C. Gouget-Laemmel, C. Henry de Villeneuve, a Moraillon, F. Ozanam, N. Gabouze, S. Djebbar, Semiquantitative study of the EDC/NHS activation of acid terminal groups at modified porous silicon surfaces., Langmuir. 26 (2010) 809–14. doi:10.1021/la902220a.

[41] S.K. Vashist, Comparison of 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide Based Strategies to Crosslink Antibodies on Amine-Functionalized Platforms for Immunodiagnostic Applications, Diagnostics. 2 (2012) 23–33. doi:10.3390/diagnostics2030023.

[42] G. Xie, J. Cheng, Y. Li, P. Xi, F. Chen, H. Liu, F. Hou, Y. Shi, L. Huang, Z. Xu, D. Bai, Z. Zeng, Fluorescent graphene oxide composites synthesis and its biocompatibility study, J. Mater. Chem. 22 (2012) 9308–9314. doi:10.1039/C2JM16678J.

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