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Construction of ferrocene modified conducting polymer based amperometric urea biosensor

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ABSTRACT

Herein, an electrochemical urea sensing bio-electrode is reported that has been constructed by firstly electropolymerizing 4-(2,5-Di(thiophen-2-yl)-1H-pyrrol-1-yl)aniline monomer (SNS-Aniline) on Pencil Graphite Electrode (PGE), then modifying the polymer coated electrode surface with di-amino-Ferrocene (DAFc) as the mediator, and lastly Urease enzyme through glutaraldehyde crosslinking. The effect of pH, temperature, polymer thickness, and applied potential on the electrode current response was investigated besides performing storage and operational stability experiments with the interference studies. The resulting urea biosensor's amperometric response was linear in the range of 0.1–8.5 mM with the sensitivity of 0.54 μA/mM, detection limit of 12 μM, and short response time of 2 s. The designed bio-electrode was tested with real human blood and urine samples where it showed excellent analytical performance with insignificant interference.

1. Introduction

Urea is the final non-toxic product of nitrogen metabolism where the amino acid degradation born ammonia is converted into urea in liver after which being sent to the kidneys through blood to be eliminated from the body. Unbalanced fluctuations from normal blood/urine urea level that are between 3.3–6.7 mM [1] and ranges between 12 and 20 g/24 h respectively, may signify physiological abnormalities including renal, hepatic, and blood circulatory system together with the need of dialysis. Additionally, excessive amount of urea may cause acute or chronic renal failure, dehydration, burns, urinary tract obstruction, shock, and gastrointestinal bleeding [2,3] where although decreased urea levels are not common, it might provide evidence for acute liver disease or unsatisfactory protein uptake [4].

Methods have been used for the determination of urea from various samples include colorimetric [5,6], spectrophotometric [7], chemiluminometric [8,9], fluorimetric [10], chromatographic [11,12], and electrochemical methods [13–16] amongst which most of the techniques necessitate long and tedious sample preparation procedures along the requirement of expensive and complex operational equipment. In order to overcome the aforementioned obstacles, biosensors are favored promising satisfactory performance factors such as high sensitivity and reusability, short response time, and long shelf life together with low cost. Biosensors are analytical devices composed of biological analyte recognition element, transduction element enabling the conversion of biochemical signal into electrical one, and signal display unit. Conducting polymers have been widely used in the construction of electrochemical biosensors due to the intrinsic electrical conductivity property resulting from electrochemical redox activity and charge transfer rate next to the chemical properties allowing to act as an outstanding immobilization platform for biological elements [17,18]. Poly(aniline) (PANI), poly(pyrrole) (PPy), poly(thiophene) (PTh), poly (3,4-ethylenedioxythiophene) (PEDOT), and poly(acetylene) meet the biocompatibility, fast and efficient electron transfer capability, electrode protection from interfering compounds, and ability of electrochemical deposition on desired electrode type acquirements of conducting polymers to be used for the biosensing applications [19]. Additionally, the electron transfer efficiency at the electrode surface could be further enhanced by the incorporation of mediator compound that acts as the electron carrier by participating in the redox reactions. Ferrocene is one of the most widely used mediators in the electrochemical biosensors [20–23] providing lower operational potential and

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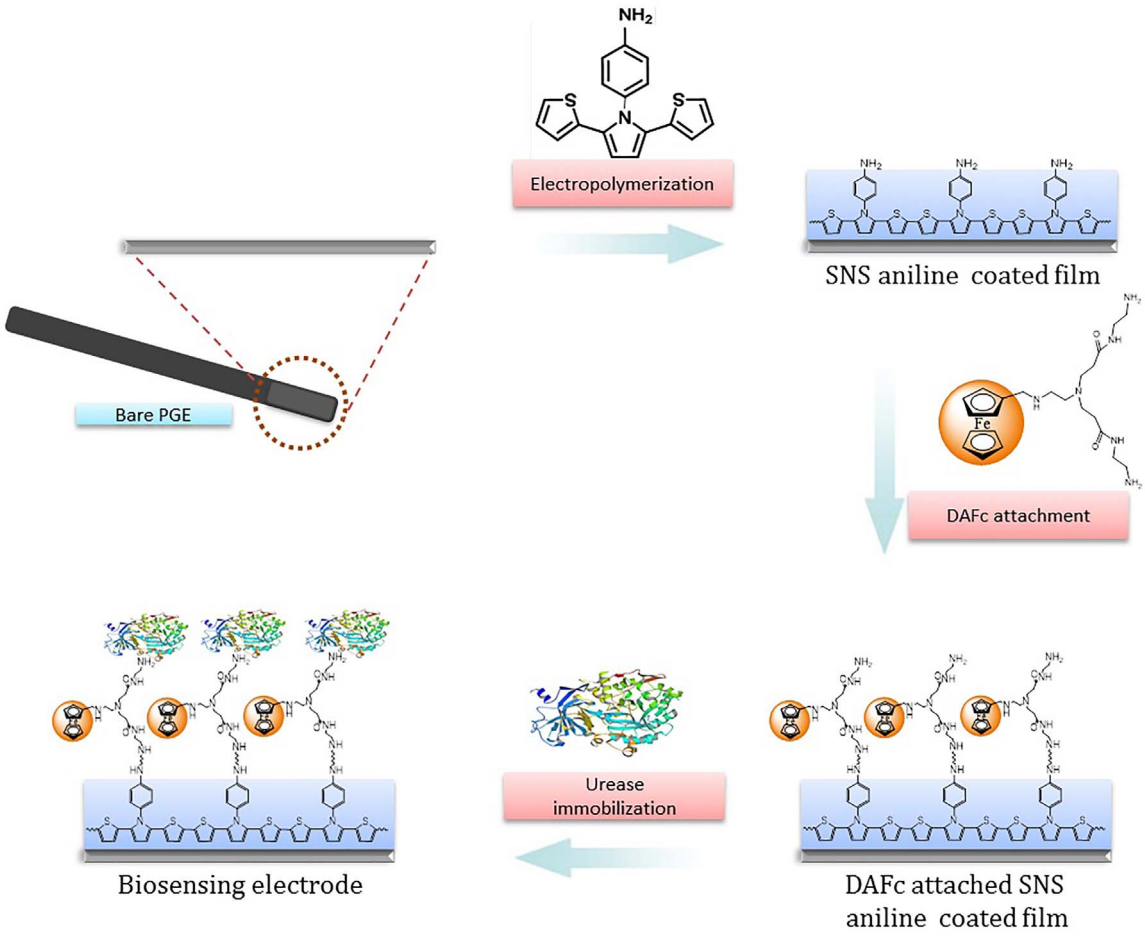

Fig. 1. Schematic illustration of preparation of Urease immobilized biosensing electrode.

so lower interference signal next to the faster electron transfer property [24].

This work reports an amperometric bio-sensing electrode constructed by using electropolymerized 4-(2,5-Di(thiophen-2-yl)-1H-pyrrol-1-yl)aniline monomer (SNS-NH_2), 3,3′-(2-(ferrocenylamino)ethylazanediyl)bis(N-(2-aminoethyl)propanamide) (DAFc), and urease enzyme in which glutaraldehyde was used as the crosslinking agent. The bio-electrode showed outstanding analytical properties of high sensitivity, selectivity, and reliability being applied to real human blood and urine samples for urea determination.

2. Experimental part

2.1. Materials

Urease (EC 3.5.1.5. from jack beans), Urea, Glucose, Cholesterol, Ascorbic acid, Uric acid, and Lactic acid were purchased from Sigma Aldrich. Glutaraldehyde 25%, tetrabutylammonium hexafluorophosphate ($TBAPF_6$) and acetonitrile (ACN) was purchased from Sigma Aldrich. Ferrocene cored PAMAM dendrimer generation one (G1) was synthesized as described in previous study [25]. The synthesis of monomer SNS-Aniline (4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine) was reported in our previous study [26]. All the chemicals employed in this study were of analytical grade and used as received without purification.

2.2. Preparation of bio-sensing electrode

Pencil graphite electrode (PGE) prior to usage was sonicated in 95% ethanol in order to remove all adsorbent materials from the electrode surface. In contrast to electrochemical pretreatment of PGE that is widely used to generate oxygenated functional groups and increase the sensitivity together with electrocatalytic activity of the electrode surface [39], in this study the aim is only to get rid of adsorbed materials from the surface without changing the electrocatalytic properties of the electrode itself as the analyte triggered current change is focused to be only due to the enzyme and substrate interaction. Then the electrode was immersed in electrochemical cell and electro-polymerization was carried out in 0.1 M solution of tetrabutylammonium hexafluorophosphate ($TBAPF_6$)/acetonitrile (ACN) containing 10 mg/ml SNS-Aniline monomer, at a scan rate of 100 mVs^{-1}, in the range of −0.5 to 1.5 V. Afterwards, electrode was immersed in 2.5% glutaraldehyde for 60 min than in 10 mM DAFc solution for 90 min each performed on orbital shaker set at 160 rpm at RT. At the end, electrode was rinsed with dH20 and once more treated with glutaraldehyde for 1 h than immersed in 20 mg/ml urease solution for 3 h (Fig. 1.).

2.3. Urea measurements in human blood and urine

Real sample application was performed using healthy 34-year-old male human blood sample and healthy 26-year-old male human urine sample. After obtaining samples, blood serum sample has been isolated while urine sample was used directly. In order to efficiently test proposed electrode and investigate its reliability, samples were spiked with a known concentration of standard urea solution after which recovery of electrode has been investigated.

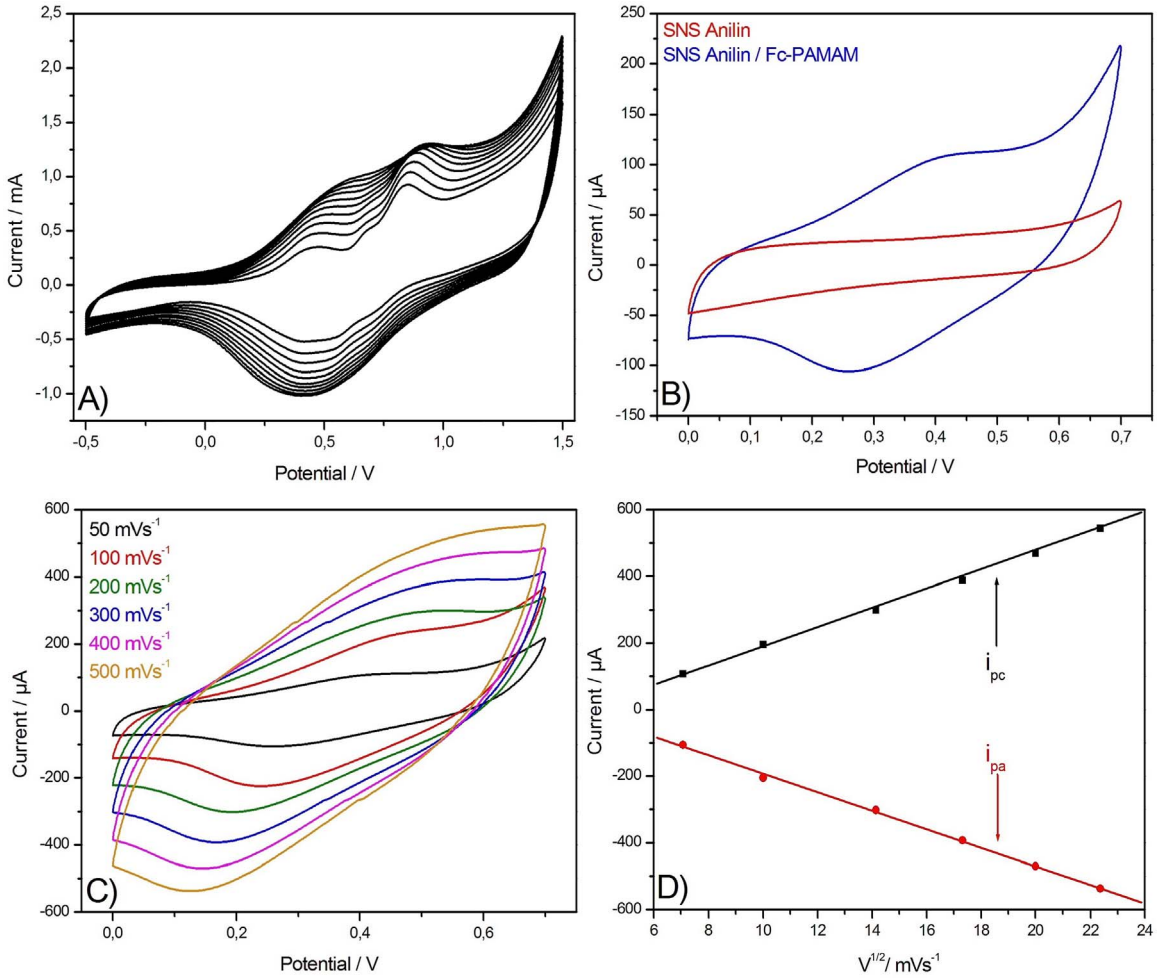

Fig. 2. A) Electro-polymerization of SNS-Aniline on PGE electrode by cyclic voltammogram in the rage of −0.5 to 1.5 V at 100 mVs^{-1}, B) Cyclic voltammogram comparison of SNS-Aniline and SNS-Aniline/FcPAMAM in the range of 0.0–0.7 V at 50 mVs^{-1}, C) Cyclic volammorgram of PGE/SNS-Aniline/DAFc electrode under different scan rates in 10 mM PBS, pH 7.4, D) Plots of anodic and cathodic peak currents versus square root of the scan rate.

2.4. Instrumentation

Electrochemical measurements were conducted by using CopactStat (*Ivium Technologies*) portable electrochemical interface and impedance analyzer having conventional three electrodes electrochemical cell where Ag/AgCl was used as reference, Platinum as counter, and Pencil Graphite Electrode as working electrode. After adding urea solution in phosphate buffer saline (PBS) filled, ceaselessly stirred, and steady-state reached electrochemical cell, the urea concentration was chrono-amperometrically recorded.

3. Results and discussion

3.1. Characterizations

Electro-polymerization of SNS-Aniline monomer was carried out by cyclic voltammetry in 0.1 M solution of $TBAPF_6$/ACN as the electrolyte/solvent system, at a scan rate of 100 mVs^{-1}, in the range of −0.5 to 1.5 V (Fig. 2A). CV of SNS-Aniline indicated two consecutive oxidation peaks at 0.45 V and 0.88 V and reduction peak at 0.43 V which corresponds to monomer oxidation and polymer reduction, respectively. As the polymerization continuous, a drop in the oxidation peak is observed that might be attributed to the loss of electro-activity of the thickening polymer layer.

After PGE was electrochemically modified with SNS-Aniline, DAFc (G1) was immobilized. Fig. 2B illustrates cyclic voltammogram of SNS-Aniline alone and SNS-Aniline/DAFc(G1) electrode in the range of 0.0–0.7 V at 50 mVs^{-1} scan rate (10 mM PBS). It is observed that SNS-Aniline electrode has no oxidation or reduction peaks in the set potential range in contrast to Aniline/DAFc(G1) electrode in which it is observed that Fe(II) is oxidized into Fe(III) at 0.4 V and then Fe(III) is reduced into Fe(II) at 0.25 V potential [27].

Electrochemical properties of fabricated electrode were analyzed by cyclic voltammetry (CV) by recording number of oxidation states and electron transfer behavior under the effect of different scan rates. Fig. 2C illustrates CV measurements which were performed in scan rate range of 50 mVs^{-1}–500 mVs^{-1} in 10 mM PBS pH 7.4 and in the potential range of 0.0 V–0.7 V. As seen from Fig. 2C, increase in scan rate results in both anodic and cathodic peaks potential increment indicating electrochemical quasi reversible process [27] where peak potentials were shifting to opposite directions, positive for anodic and negative for cathodic. Anodic peak potential (Epa) was obtained at 0.35 V which is caused by the presence of ferrocene and cathodic peak (Epc) is obtained at 0.25 V at scan rate of 50 mVs^{-1}.

Fig. 2D demonstrates diffusion controlled redox process during forward scan of Fe(II) oxidized to Fe(III) and reverse scan of reduced Fe(III) which can be seen at both anodic and cathodic linear relation of peak currents with the increase of square root of scan rate ($V^{1/2}$). The regression equations for bio-sensing electrode were expressed as $Ipa = -27.691\,\nu^{1/2} + 84.019$ (µA, mVs^{-1}, R = 0.9983) and $Ipc = 28.143\,\nu^{1/2} - 92.449$ (µA, mVs^{-1}, R = 0.9986), respectively. As such, it is confirmed that the kinetics of overall process is mainly

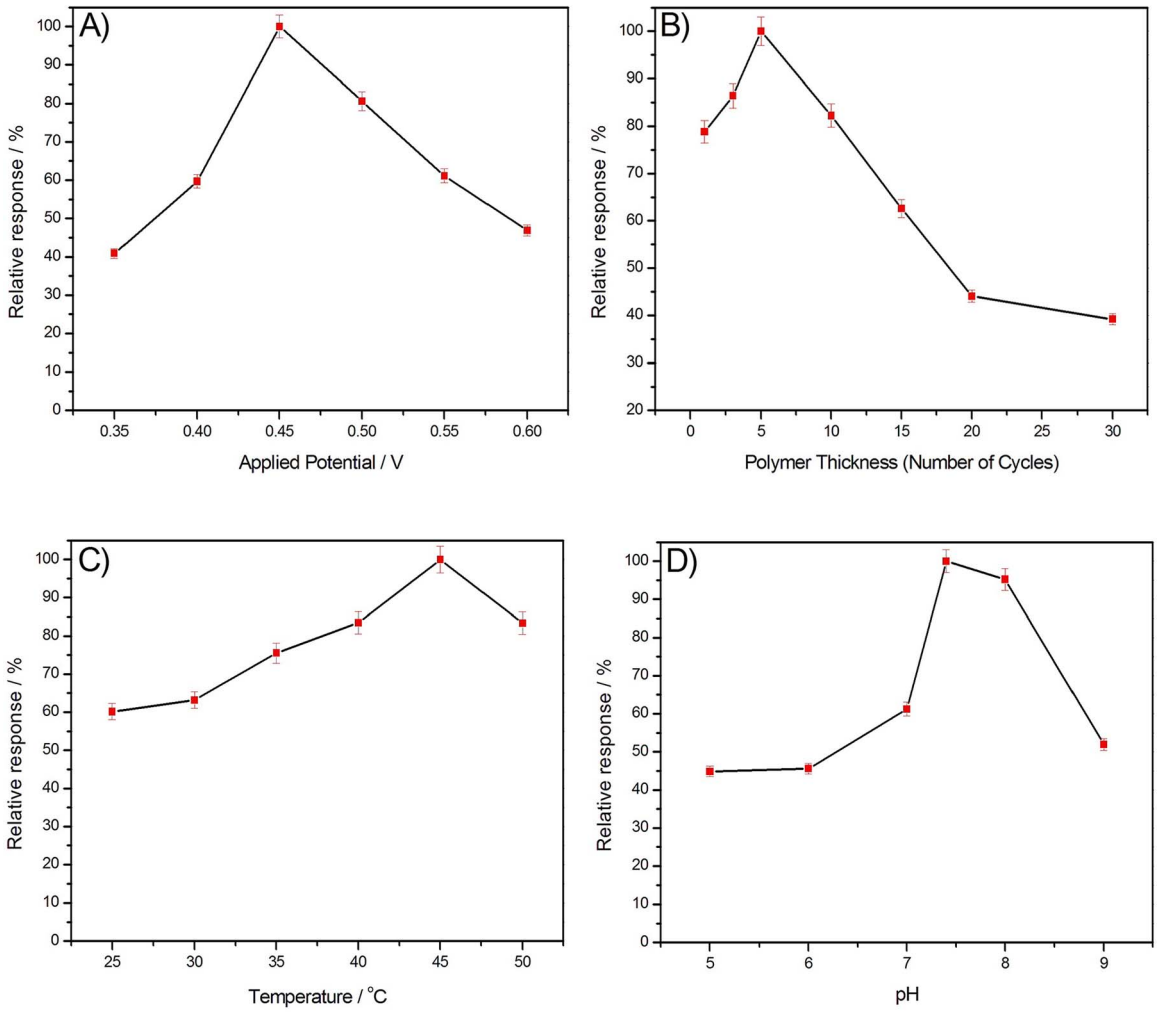

Fig. 3. A) Effect of applied potential of enzymatic biosensor on urea substrate addition in 10 mM PBS pH 7.4 (n = 3), B) Amperometric current response of bio-sensing electrode modified with different thickness of electropolymerized polymer (n = 3), C) Effect of temperature on the current response of enzyme electrode on urea substrate addition under different temperatures at 10 mM PBS, pH 7.4 and applied potential of 0.45 V (n = 3), D) Evaluation of optimum pH on amperometric response of bio-sensing electrode (10 mM PBS, applied potential 0.45 V) (n = 3).

controlled by the diffusion process.

3.2. Determination of experimental variables

Applied potential of biosensor was selected from the results illustrated in Fig. 3A, showing the effect of different applied potentials on current response of proposed bio-sensing electrode. Selected applied potentials were in the range of 0.3 V–0.6 V. The results show that the electrode response increased as the potential was increasing from 0.3 V to 0.4 V reaching the maximum current response at 0.45 V after which further increase in potential caused to decrease in amperometric response losing 50% of response at 0.6 V when compared to 0.45 V. Therefore, optimum applied potential for proposed bio-sensing electrode was selected to be 0.45 V and it was used throughout all amperometric experiments.

Fig. 3B illustrates the results of polymer thickness study. Interaction between enzyme and electrode surface plays important role in electrode electroanalytic performance. Experiments were conducted by measuring amperometric response of electrode which was modified with polymer having divergent thickness. Polymer was deposited on working electrode with 1, 3, 5, 10, 15, 20, 25 and 30 scans in the range of −0.5 to 1.5 V with scan rate of 100 mVs^{-1}. As seen in Fig. 2B, current response of bio-sensing electrode was increasing until the electropolymerization cycle number is 5, after which activity of bio-sensing electrode decreased as film thickness increased. Decrease in amperometric response after 5 cycle polymer coating can be mainly caused by possible diffusion problems arising from high polymer layer thickness [28]. As the optimum film thickness, 5 electro-polymerization cycles were used in all electrode modifications in this study.

Effect of temperature on amperometric bio-sensing electrode was investigated in the range of 25–50 °C as illustrated in Fig. 3C. Amperometric response of electrode was gradually increasing from 25 °C to 45 °C after which further increase in temperature decreased current response of electrode. There was 40% response difference between room temperature and 45 °C while at 50 °C current response loss was 20% according to the initial response. Loss of current response at higher temperatures can occur due to thermal deactivation of enzyme and decrement of molecular oxygen in solution. As a result, optimum temperature for urea bio-sensing electrode was found to be 45 °C.

Investigation of effect of pH on electrode response was performed using 10 mM PBS with different pH in the range of 5.0–9.0 with an applied potential of 0.45 V. Fig. 3D, demonstrates effect of pH on electrode response which is low in acidic pH and starts to increase as pH is increasing reaching maximum response plateau at pH 7.4. Then current response starts to decrease as pH is increasing. From results obtained it can be concluded that amperometric urea biosensor has optimum working pH of 7.4, which makes it suitable in measuring urea concentration at physiological pHs. As well' it can be seen that acidic

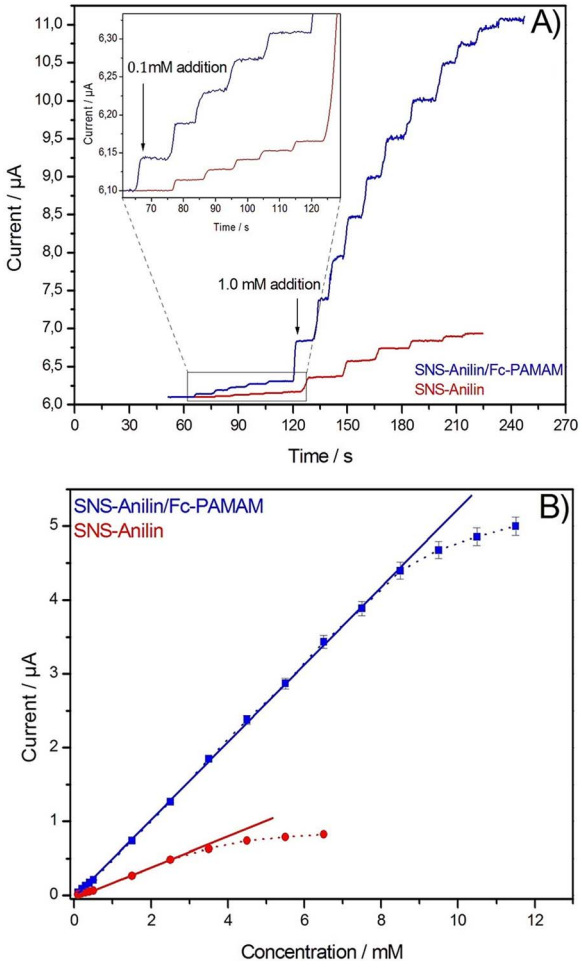

Fig. 4. A) Amperometric response of urea bio-sensing electrode on known amount of urea concentration at 10 mM (pH 7.4) PBS and applied potential of +0.45 V, B) Calibration curve of urea concentration obtained from the amperometric response of the urea bio-sensing electrode.

Table 1
Comparison of Analytical Performance of PGE/SNS-Anilin/DAFc/Urease electrode.

Electrode	L.R.	D.L.	R.T. (s)	Technique	Ref.
Rhodinized polymer membrane	0.1–2.6 mM	50 µM	15	AMP	[29]
Functionalized H40-Au	1.0–3.0 mM	11 µM	3	AMP	[30]
ZnO thin film	0.8–13.3 mM	2.24 mM	NR	CV	[31]
Polyaniline/Nafion	0.5 µM–1 mM	0.5 µM	40	AMP	[32]
Poly(N-3-aminopropyl pyrrole −co-pyrrole)	1.6–52 µM	1.6 µM	40	AMP	[33]
Nylon net	0.01–0.3 mM	10 µM	NR	AMP	[13]
Graphite and platinum composite	10–250 µM	10 µM	120	AMP	[34]
Fe_3O_4 chitosan nanobiocomposite	0.83–6.65 mM	0.83 mM	10	DPV	[35]
EG-Ag-Z-Epoxy	0.2–1.4 mM	0.05 mM	NR	CV	[36]
Pt-BDD	1–25 mM	1.79 mM	300	DPV	[37]
SNS-Anilin/DAFc	0.1–8.5 mM	12 µM	2	AMP	This work

L.R.-Linear Range; D.T.-Detection Limit; R.T.-Response Time; N.R.-Not Reported; AMP-Amperometric; CV-Cyclic Voltammogram.

systems and strong basic systems are reducing biosensor's response which might be due to enzyme deformation that leads to loss of catalytic capability of enzyme. Therefore pH 7.4 was selected as optimum working pH for urea measurements.

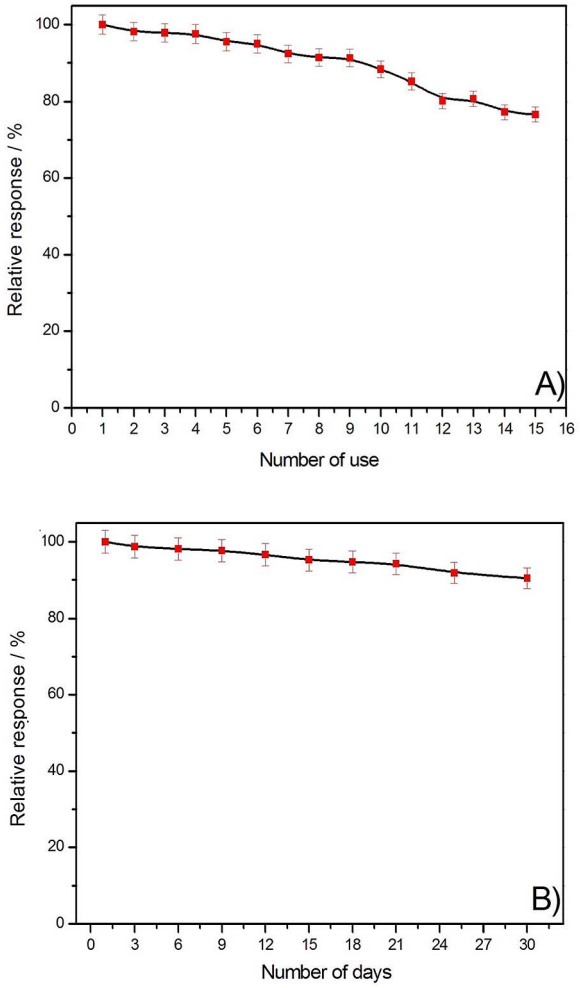

Fig. 5. A) Operational stability of bio-sensing electrode in 10 mM PBS pH 7.4 with applied potential of 0.45 V (n = 3), B) Storage stability of enzyme electrode at 10 mM PBS pH 7.4 with applied potential of 0.45 V (n = 3).

Table 2
Analytical performance of proposed bio-sensing electrode (n = 3).

Urea added (mM)	Urea found (mM)	RSD (%)	Recovery (%)
0.5	*0.49*	*2.4*	*98%*
1.5	*1.41*	*3.1*	*94%*
3.0	*2.88*	*2.95*	*96%*
5.0	*5.24*	*2.67*	*104.8%*

3.3. Amperometric response of enzyme electrode

Fig. 4A shows amperometric current response of bio-sensing electrode to 0.1 mM and 1.0 mM urea additions in electrochemical cell containing 10 mM PBS, pH 7.4 under 0.45 V applied potential. Comparison of SNS-Aniline and SNS-Aniline/DAFc electrodes demonstrated that ferrocene cored dendrimer provided almost 3 times higher current response than SNS-Aniline electrode which can be attributed to mediator usage providing faster electron transfer. It can be clearly seen that electrodes' performance in detection of big and small amounts of urea concentrations is excellent with sensitivity of 0.54 µA/mM. PGE/SNS-Aniline/DAFc based electrode showed low response time reaching steady-state within 2 s for 96% response.

Calibration plots of bio-sensing electrodes are illustrated in Fig. 4B where amperometric response was obtained from continues additions of 0.1 mM and 1.0 mM urea. SNS-Aniline/DAFc based electrode demon-

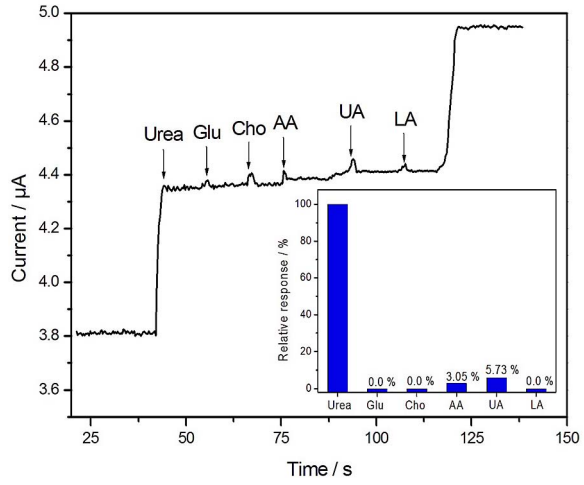

Fig. 6. Effect of interferents (Glucose (Glu), Cholesterol (Cho), Ascorbic acid (AA), Uricacid (UA) acnd Lactic acid (LA)) on amperometric response of urea bio-sensing electrode.

Table 3
Real sample application of enzyme electrode in human blood serum and human urine measurements of urea (n = 3).

Blood Sample	Urea added (mM)	Urea found (mM)	RSD (%)	Recovery%
Sample 1	*0.0*	*3.21*	*2.3*	–
Sample 2	*1.0*	*4.21*	*3.23*	*102%*
Sample 3	*3.0*	*6.49*	*3.57*	*109.3%*
Sample 4	*6.0*	*8.72*	*2.97*	*91.83%*
Urine Sample	Urea added (mM)	Urea found (mM)	RSD (%)	Recovery%
Sample 1	*0.0*	*2.93*	*3.64*	–
Sample 2	*0.5*	*3.45*	*2.58*	*104.0%*
Sample 3	*1.5*	*4.36*	*4.1*	*95.3%*
Sample 4	*3.0*	*5.88*	*3.23*	*98.3%*

strated best performance in urea detection with linear range of 0.1 mM–8.5 mM and detection limit of 12 μM where SNS-Aniline linear range is almost two times narrower being from 0.1 mM to 3.5 mM. Detection limit being the smallest amount of detectable analyte by bio-sensing electrode is determined by LOD = standard deviation of background signal (std)/sensitivity (S) [38]. Calculated regression equation for SNS-Aniline/DAFc (G1) electrode was $\Delta I(\mu A) = 0.5252[\text{Urea (mM)}] - 0.0261$ with regression coefficient (R) of 0.9997 where for SNS-Aniline based electrode regression equations was $\Delta I(\mu A) = 1883[\text{Urea (mM)}] - 0.0146$ with R coefficient of 0.9968. Comparison of analytical performance of SNS-Aniline/DAFc based electrode with reported studies in literature is demonstrated in Table 1. As it can be seen, fabricated electrode has excellent performance in urea detection with fast response of 2 s and wide linear range which is competitive with the most of the reported urea biosensors.

3.4. Operational and storage stability

Fig. 5A, illustrates operational stability of amperometric urea bio-sensing electrode which is performed by recording current response of 15 consequent measurements carried out in 10 mM PBS with pH 7.4 at applied potential of 0.45 V. In first nine measurements, electrode retained 90% of its initial response after which current response started to decrease eventually losing 20% of its initial response at the end of 15 measurements.

The long-term stability of bio-sensing electrode has been performed in period of 30 days where electrode was stored in 10 mM PBS with pH 7.4 at 4 °C. As illustrated in Fig. 5B, proposed electrode showed excellent performance and it successfully retained 90% of its initial response after 30 days of storage. Fabricated electrodes' durability can be attributed to simple and stable modification process.

3.5. Analytical recovery and interference

In order to verify the effectiveness and reliability of proposed bio-sensing electrode, it has been subjected to analytical recovery experiments that were carried out in 10 mM PBS with pH 7.4 at applied potential of 0.45 V with successful additions of urea in concentrations of 0.5, 1.5, 3.0, and 5.0 mM (n = 3). As shown in Table 2, enzyme electrode showed outstanding performance with recovery range of 94% to 104.8% and RSD values of 2.4–3.1% demonstrating possession of high reliability and reproducibility properties.

Before application of bio-sensing electrode to urea measurement from real samples, interference study has been conducted using glucose (Glu), cholesterol (Cho), ascorbic acid (AA), uric acid (UA), and lactic acid probably being present in the sampling environment. The amperometric response to a standard urea concentration was compared with the current obtained in the presence of the variable concentrations of physiological interfering species. Obtained results (Fig. 6) show that AA caused 3.05% and UA 5.73% current increment when added to the electrochemical cell operated under 0.45 V leading to the conclusion that they do have insignificant interference effect on the bio-electrode urea sensing performance. Furthermore, glucose, cholesterol, and lactic acid did not have any effect on amperometric response of enzyme electrode. In conclusion, enzyme electrode is highly reliable to be applied in real samples.

3.6. Real sample application

After analytical recovery and interference experiments proved reliability and selectivity of fabricated enzyme electrode, it was tested in real samples. Real sample experiments were conducted using human blood sample which was obtained from 36-year-old healthy male and urea sample which was obtained from 26-year-old healthy male. Non-diluted blood sample was spiked with 1.0, 3.0, and 6.0 mM urea after which prepared samples were analyzed by enzyme electrode. As it can be seen from Table 3, urea concentration in blood sample was found to be 3.21 mM which is normal concentration level since in healthy patients' blood average urea level vary in the range of 3.3–6.7 mM [1]. Enzyme electrode current response in blood samples is illustrated in terms of recovery being in the range of 91.83%–109.3% with RSD of 2.3–3.57% for n = 3, making bio-sensing electrode accurate and reliable in measuring urea in blood samples. Urine sample was diluted for 100 times before urea concentration analyses were performed aiming to make the results fitting into the linear range of enzyme electrode. Urine samples were spiked with concentrations of 0.5, 1.0, and 1.5 mM urea. As demonstrated in Table 3, urea concentration in urine was found to be 2.93 mM which is similarly found by Tiwari et al., [30]. The bio-sensing electrode's current response represented in terms of recovery was found to be in the range of 95.3%–104.0% with RSD of 2.58–4.1% for n = 3 showing successful application of fabricated enzyme electrode in measuring urea in urine samples. Overall, fabricated amperometric urea biosensor was successfully applied in real sample analysis giving reliable and accurate results, which are supported by interference and analytical recovery studies promising for the possibility of exploiting biosensor in urea measurements.

4. Conclusions

SNS-NH_2 monomer was electrochemically polymerized onto pencil graphite electrode after which being functionalized with ferrocene cored PAMAM first generation dendrimers for the immobilization of

urease enzyme through glutaraldehyde crosslinking. Fabricated biosensing electrode displayed a linear range within 0.1–8.5 mM urea having detection limit of 12 μM and sensitivity value of 0.54 μA/mM. Short response time of 2 s, negligible interference current response, and high reproducibility properties of fabricated bio-electrode promise the accurate and reliable application in the urea determination for the diagnosis of renal, hepatic, and circulatory system abnormalities in addition to the urea monitoring in the dialysis patients.

References

[1] Food and Drug Administration (FDA), Investigations Operations Manual. Appendix C, (2015) pp. 443.
[2] D. Aronson, M.A. Mittleman, A.J. Burger, Elevated blood urea nitrogen level as a predictor of mortality in patients admitted for decompensated heart failure, Am. J. Med. 116 (2004) 466–473.
[3] J.W. Schrocka, M. Glasenappb, K. Drogell, Elevated blood urea nitrogen/creatinine ratio is associated with poor outcome in patients with ischemic stroke, Clin. Neurol. Neurosurg. 114 (2012) 881–884.
[4] O.O. Soldatkin, I.S. Kucherenko, S.V. Marchenko, B. Ozansoy Kasap, B. Akata, A.P. Soldatkin, S.V. Dzyadevych, Application of enzyme/zeolite sensor for urea analysis in serum, Mater. Sci. Eng. C 42 (2014) 155–160.
[5] A.R. Butler, D. Walsh, Colorimetric non-enzymic methods for the determination of urea: consideration of the chemical principles upon which an analytical method is based is an essential step in the optimization of that method. It can also contribute to the simplification of procedures used and hence cut the costs of many routine analyses, Trends Anal. Chem. 1 (1982) 120–124.
[6] D.M. Sullivan, J.L. Havlin, Flow injection analysis of urea nitrogen in soil extracts, Soil Sci. Soc. Am. J. 55 (1991) 109–113.
[7] P. Dallet, L. Labat, E. Kummer, J.P. Dubost, Determination of urea, allantoin and lysine pyroglutamate in cosmetic samples by hydrophilic interaction chromatography, J. Chromatogr. B: Biomed. Sci. Appl. 742 (2000) 447–452.
[8] M. Tabata, T.J. Murachi, A chemiluminometric method for the determination of urea in serum using a three enzyme bioreactor, Biolumin. Chemilumin. 2 (1988) 63–67.
[9] X. Hu, N. Takenaka, M. Kitano, H. Bandow, Y. Maeda, M. Hattori, Determination of trace amounts of urea by using flow injection with chemiluminescence detection, Analyst 119 (1994) 1829–1833.
[10] F. Roch-Ramel, An enzymic and fluorophotometric method for estimating urea concentrations in nanoliter specimens, Anal. Biochem. 21 (1967) 372–381.
[11] M. Czauderna, J. Kowalczyk, Simple, selective, and sensitive measurement of urea in body fluids of mammals by reversed-phase ultra-fast liquid chromatography, Czech J. Anim. Sci. 57 (2012) 19–27.
[12] S. Kodama, T. Suzuki, Highly sensitive method for urea detection in wine, J. Food Sci. 60 (1995) 1097–1099.
[13] P. Bertocchi, D. Compagnone, G. Palleschi, Amperometric ammonium ion and urea determination with enzyme based probe, Biosens. Bioelectron. 11 (1996) 1–10.
[14] T. Osaka, S. Komaba, M. Seyama, K. Tanabe, High-sensitivity urea sensor based on the composite film of electroinactive polypyrrole with polyion complex, Sens. Actuators B Chem. 35/36 (1996) 463–469.
[15] J.C. Chen, J.C. Chou, T.P. Sun, S.K. Hsiung, Potable urea biosensor based on extended gate field effect transistor, Sens. Actuators B Chem. 91 (2003) 180–186.
[16] E.F. Renny, D.K. Daniel, A.I. Krastanov, C.A. Zachariah, R. Elizabeth, Enzyme based biosensor for detection of urea in milk, Biotechnol. Biotechnol. Equip. 19 (2005) 198–201.
[17] H. Yoon, Current trends in sensors based on conducting polymer nanomaterials, Nanomaterials 3 (2013) 524–549.
[18] M. Gerard, A. Chaubey, B.D. Malhotra, Application of conducting polymers to biosensors, Biosens. Bioelectron. 17 (2002) 345–359.
[19] G. Dhawan, G. Sumana, B.D. Malhotra, Recent developments in electrochemical urea biosensors, Biochem. Eng. J. 44 (2009) 42–52.
[20] M. Karadag, C. Geyik, D.O. Demirkol, F.N. Ertas, S. Timur, Modified gold surfaces by 6-(ferrocenyl)hexanethiol/dendrimer/gold nanoparticles as a platform for the mediated biosensing applications, Mater. Sci. Eng. C 33 (2013) 634–640.
[21] M. Şenel, Construction of reagentless glucose biosensor based on ferrocene conjugated polypyrrole, Synth. Met. 161 (2011) 1861–1868.
[22] A. Fatoni, A. Numnuam, P. Kanatharana, W. Limbut, C. Thammakhet, P. Thavarungkul, A highly stable oxygen-independent glucose biosensor based on a chitosan-albumin cryogel incorporated with carbon nanotubes and ferrocene, Sens. Actuators B Chem. 185 (2013) 725–734.
[23] B. Zribi, E. Roy, A. Pallandre, S. Chebil, M. Koubaa, N. Mejri, H. Magdinier Gomez, C. Sola, H. Korri-Youssoufi, A.M. Haghiri-Gosnet, A microfluidic electrochemical biosensor based on multiwall carbon nanotube/ferrocene for genomic DNA detection of Mycobacterium tuberculosis in clinical isolates, Biomicrofluidics 10 (2016) 014115.
[24] F. Xiao, M. Gu, Y. Liang, M. Dong, Z. Zhao, D. Zhi, Synthesis, characterization and electrochemical behavior of ferrocene-containing highly branched polyurethane and its application in biosensing, J. Organomet. Chem. 772–773 (2014) 122–130.
[25] M. Senel, C. Nergis, E. Cevik, Novel reagentless glucose biosensor based on ferrocene cored asymmetric pamam dendrimers, Sens. Actuators B: Chem. 176 (2013) 299–306.
[26] E. Yildiz, P. Camurlu, C. Tanyeli, I. Akhmedov, L. Toppare, A soluble conducting polymer of 4-(2,5-di(thiophen-2-yl)-1H-pyrrol-1-yl)benzenamine and its multichromic copolymer with EDOT, J. Electroanal. Chem. 612 (2008) 247–256.
[27] M. Dervisevic, E. Çevik, Z. Durmuş, M. Şenel, Electrochemical sensing platforms based on the different carbon derivative incorporated interface, Mater. Sci. Eng. C 58 (2016) 790–798.
[28] M. Dervisevic, E. Dervisevic, H. Azak, E. Cevik, M. Senel, H.B. Yildiz, Novel amperometric xanthine biosensor based on xanthine oxidase immobilized on electrochemically polymerized 10-[4H-dithieno(3,2-b:2,3-d)pyrrole-4- yl]decane-1-amine film, Sens. Actuators B: Chem. 225 (2016) 181–187.
[29] Y. Velichkova, Y. Ivanov, I. Marinov, R. Ramesh, N.R. Kamini, N. Dimcheva, E. Horozova, T. Godjevargova, Amperometric electrode for determination of urea using electrodeposited rhodium and immobilized urease, J. Mol. Catal. B: Enzym. 69 (2011) 168–175.
[30] A. Tiwari, S. Aryal, S. Pilla, S. Gong, An amperometric urea biosensor based on covalently immobilized urease on an electrode made of hyperbranched polyester functionalized gold nanoparticles, Talanta 78 (2009) 1401–1407.
[31] A. Ali, A.A. Ansari, A. Kaushik, P.R. Solanki, A. Barik, M.K. Pandey, B.D. Malhotra, Nanostructured zinc oxide film for urea sensor, Mater. Lett. 63 (2009) 2473–2475.
[32] W.J. Cho, H.J. Huang, An amperometric urea biosensor based on a polyaniline-perfluorosulfonated ionomer, Comp. Electrode Anal. Chem. 70 (1998) 3946–3951.
[33] Rajesh V. Bisht, W. Takashima, K. Kaneto, 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 (2005) 3683–3690.
[34] A. Pizzariello, M. Stredansky, S. Stredanska, S. Miertus, Urea biosensor based on amperometric pH-sensing with hematein as a pH-sensitive redox mediator, Talanta 54 (2001) 763–772.
[35] A. Kaushik, P.R. Solanki, A. Ansari, G. Sumana, S. Ahmad, B.D. Malhotra, Iron oxide-chitosan nanobiocomposite for urea sensor, Sens. Actuators B Chem. 138 (2009) 572–580.
[36] F. Manea, A. Pop, C. Radovan, P. Malchev, A. Bebeselea, G. Burtica, S. Picken, J. Schoonman, Voltammetric detection of urea on an Ag-modified zeolite expanded graphite-epoxy composite electrode, Sensors 8 (2008) 5806–5819.
[37] E. Nicolau, J.J. Fonseca, C.R. Cabrera, Development of a urea bioprobe based on platinized Boron doped diamond electrodes, Electroanalysis 24 (2012) 2102–2108.
[38] M. Dervisevic, E. Dervisevic, E. Cevik, M. Senel, Novel Electrochemical Xanthine Biosensor Based on Chitosanepolypyrroleegold Nanoparticles Hybrid Bio-nanocomposite Platform, J. Food. Drug. Anal. (2017), http://dx.doi.org/10.1016/j.jfda.2016.12.005.
[39] I.G. David, D.-E. Popa, M. Buleandra, Pencil graphite electrodes: aa versatile tool in electroanalysis, J. Anal. Methods Chem. 2017 (2017) 1–22, http://dx.doi.org/10.1155/2017/1905968.