A novel urea biosensor based on zirconia

G. Sumana [a], Maumita Das [a,b], Saurabh Srivastava [a], B.D. Malhotra [a,*]

[a] Department of Science and Technology Centre on Biomolecular Electronics, Biomedical Instrumentation Section, Materials Physics and Engineering Division, National Physical Laboratory (Council of Scientific and Industrial Research), Dr.K.S.Krishnan Marg, New Delhi 110012, India
[b] Department of Chemistry, University of Delhi, Delhi-110007, India

Keywords:
Biosensor
Urea
Nanostructured metal oxide
Zirconia
Electrochemical deposition
Glutamate dehydrogenase
Keto glutamate
Urease

A B S T R A C T

Electrochemically deposited biocompatible zirconia (ZrO_2) film on gold coated glass electrodes has been utilized for the fabrication of urea biosensor. The prepared ZrO_2 films and bioelectrodes have been characterized using Fourier transform infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM) and electrochemical techniques, respectively. The urea biosensor, fabricated by immobilizing mixed enzyme [urease (Urs) and glutamate dehydrogenase (GLDH)] on this nanobiomaterial, shows linearity up to 40 mg dL^{-1} of analyte (urea) and sensitivity of 0.071 μA/(mM cm^{-2}) with stability up to 4 months when stored at 4 °C. The low value of Michaelis–Menten constant (K_m) estimated using Hans plot as 0.5 mM indicates enhancement in the affinity and/or activity of enzyme attached to this nanostructured biocompatible matrix.

1. Introduction

Estimation of urea in serum/blood/urine is important for the diagnosis of renal and liver diseases. An increase in urea level [normal range in blood is 8–20 mg/dL] causes renal failure, urinary tract obstruction, dehydration, shock, burns, and gastrointestinal bleeding. Moreover, reduced urea level may cause hepatic failure, nephritic syndrome, and cachexia. Besides clinical diagnostics, urea estimation is an important parameter in pharmacy, food industry, environment monitoring and protection etc. In pharmaceutical industry, urea is used as a component of many ointments, its level in these products must be strictly controlled. In food industry, the control of food quality (mainly farm products) also includes determination of urea, for example in milk. The production of fertilizers and environmental protection are other areas for application of urea determinations. Urea levels in river or ground waters give the evidence of contamination with sewage. The high content of urea is one of the reasons for algae blooming [1–5].

Most urea biosensors (Urs), utilizing [Urs] are based on catalytic conversion of urea to hydrogen bicarbonate and ammonium. It has been observed that ammonium ions easily diffuse in solution. Thus, glutamate dehydrogenase (GLDH) has been used as an alternate since it catalyzes the reaction between ammonium ions, α-ketoglutarate (α-KG) and nicotinamide adenine dinucleotide (NADH) to produce L-glutamate and NAD^+.

The immobilization of Urs on a given matrix is a crucial step for the fabrication of urea biosensor. Extensive efforts have been made to utilize nanomaterials to immobilize Urs for urea detection. Nanostructured metal oxides such as cerium oxide, tin oxide and zirconia have been used for the fabrication of transducer surface because of their unique ability to promote faster electron transfer between electrode and active site of desired enzyme. Zirconia (ZrO_2) is an ideal material for immobilization of biomolecules with oxygen groups and it has significantly higher isoelectric point, pH stability and it plays an important role for optical, dielectric, corrosion-resistant coatings, and sensor applications [6–13]. Compared to sol gel method, electrodeposition of ZrO_2 offers several advantages like strong adhesion between the deposited film that may be advantageous for biosensor fabrication [14,15]. Therefore, in the present manuscript, studies related to the fabrication of urea biosensor using electrochemically deposited biocompatible nanostructured ZrO_2film deposited onto Au surface have been reported.

2. Experimental details

2.1. Materials

Zirconium oxychloride ($ZrOCl_2 \cdot 8H_2O$), potassium chloride (KCl), Urease (Urs), glutamate dehydrogenase (GLDH), nicotinamide adenine dinucleotide (NADH), α-ketoglutarate (α-KG) have been procured from Sigma-Aldrich (USA). All chemicals used are of molecular biology grade. Deionized water (Milli Q 10 TS) has been used for the preparation of reagents. All the solutions and glassware are autoclaved prior to being used.

2.2. Electrochemical deposition of nanostructured zirconium oxide film on gold coated glass plates (ZrO_2/Au)

Zirconia films have been deposited onto bare gold electrode in an aqueous electrolyte of 5.0 mmol L^{-1} $ZrOCl_2$ and 0.1 mol L^{-1} KCl by cycling the potential between −1.5 and +0.7 V (versus Ag/AgCl) at a scan rate of 20 mV s^{-1} for 10 consecutive scans [9]. This cathodic electrodeposition is based on the electro synthesis of inorganic Zr–O^-particles. In $ZrOCl_2 \cdot 8H_2O$ solution, $ZrOCl_2$ may hydrolyze to tetramer $[Zr_4(OH)_8(H_2O)16]^{8+}$ and form colloidal particles under the basic conditions around electrode surface that might have formed from the cathodic reduction of water as indicated in the following equation:

$$2H_2O + 2e^- \rightarrow H_2 + 2OH^- \quad (1)$$

$$Zr - OH + OH^- \rightarrow Zr - O^- + H_2O \quad (2)$$

Therefore, the negatively charged Zr–O^- colloidal particles are formed around the Au electrode. The electro synthesis helps in the accumulation of colloidal particles at the electrode surface resulting in the formation of the ZrO_2 film.

2.3. Immobilization of Urs and GLDH onto ZrO_2/Au electrode

10 μL of bienzyme solution containing Urs (10 mg/mL) and GLDH (1 mg/mL) in 1:1 ratio [prepared in Tris buffer (5 mM)] is immobilized onto ZrO_2/Au electrode. The oxygen atom of the biomolecule covalently binds with the zirconium atom of zirconia, since both have the affinity to bind with each other. The Urs-GLDH/ ZrO_2/Au bioelectrodes are kept undisturbed for about 12 h at 4 °C. Finally, the dry bioelectrode is immersed in 50 mM phosphate buffer saline (PBS) (pH 7.0) in order to wash out any unbound enzymes from the electrode surface.

2.4. Characterization

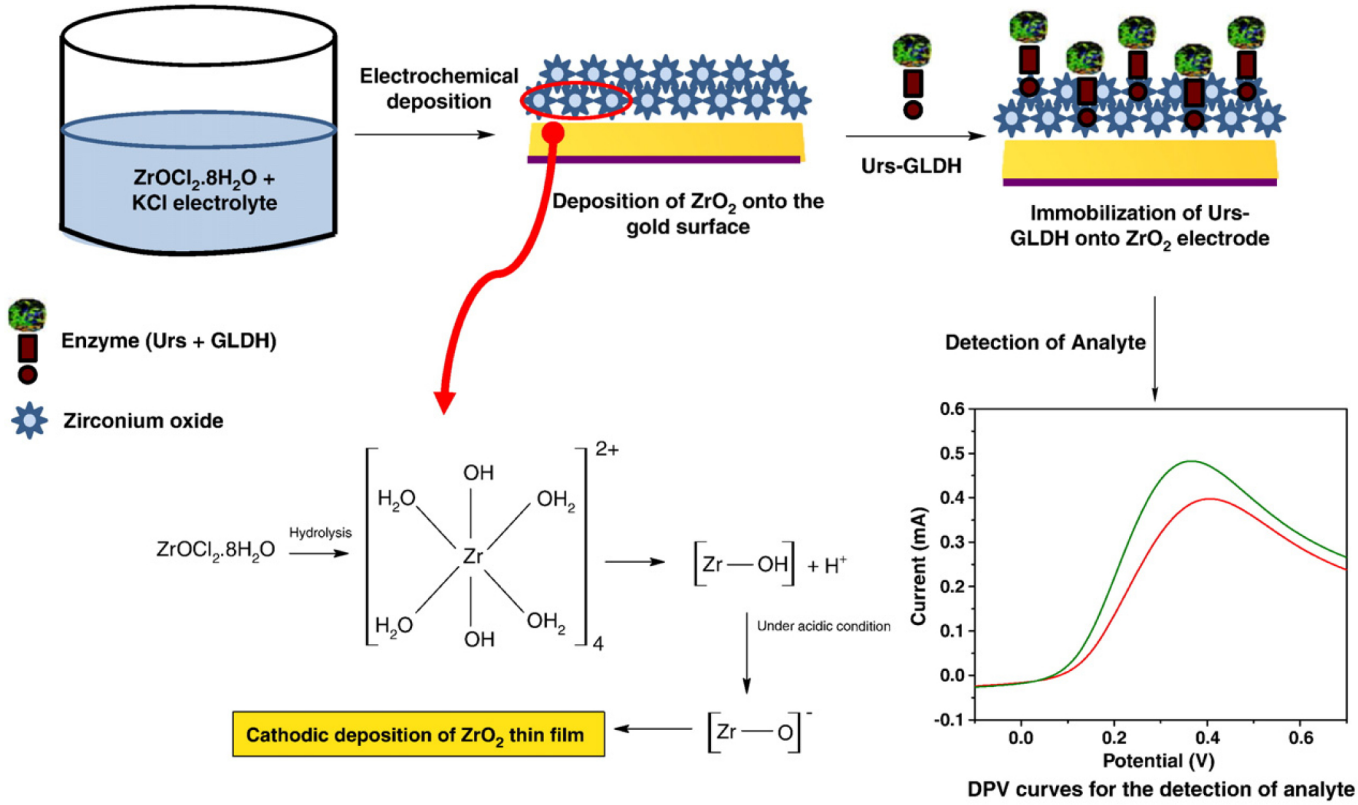
The morphological and structural characterization of the prepared electrodes have been carried out using Scanning electron microscopy (SEM, Leo 40) and Fourier transform infrared spectroscopy (FT-IR, Perkin-Elmer Model spectrum BX using ATR accessory), respectively. Electrochemical studies (cyclic voltammetry, differential pulse voltammetry and impedance measurements) have been carried out on Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands). These measurements are carried out using a three electrode cell with Urs-GLDH/ZrO_2/Au bioelectrode as the working electrode, platinum (Pt) as the counter electrode, and saturated Ag/AgCl electrode as a reference electrode in 50 mM phosphate buffer saline of pH 7.0 containing 5 mM of $[Fe(CN)_6^{-3/-4}]$.

3. Results and discussion

3.1. Optical properties

Fourier transform infrared (FT-IR) spectra of ZrO_2/Au electrode exhibits characteristic infrared absorption peaks (Fig. 1a) at 555 cm^{-1} and 668 cm^{-1} for the symmetric stretching of Zr–OZr species indicating the formation of zirconia (ZrO_2) on the gold surface. The additional band observed at 1560 cm^{-1} and 1628 cm^{-1} are due to the vibration of Zr–O–C species. In the FT−IR spectrum of Urs−GLDH/ZrO_2/Au bioelectrode (Fig. 1b), Urs−GLDH binding is indicated by the appearance of additional absorption bands at 1652 cm^{-1} assigned to the carbonyl stretch and also, a broad band seen around 3370 cm^{-1} is attributed to amide bond present in enzyme.

The results of SEM studies carried out on ZrO_2/Au and Urs−GLDH/ZrO_2/Au electrodes are shown in Fig. 2. Uniform distribution of zirconium oxide nanoparticles with regular and patterned morphology is observed in the case of ZrO_2/Au (image a). The presence of aggregated structure (image b) can be attributed to covalently bound Urs-GLDH molecules, indicating effective immobilization of enzyme.



Schematic of probable reaction pathway.

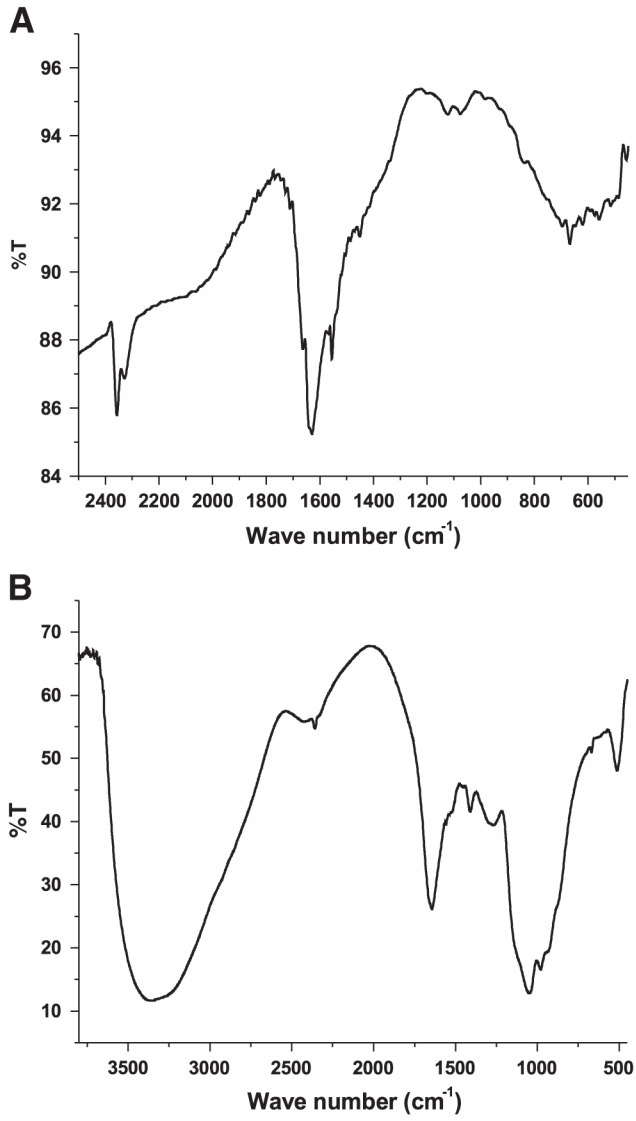


Fig. 1. FT-IR spectra of (A) ZrO_2/Au and (B) Urs-GLDH/ZrO_2/Au electrodes.

3.2. Electrochemical studies

Cyclic voltammetric (CV) and electrochemical impedance spectroscopic (EIS) studies have been carried out in phosphate buffer saline (PBS, 50 mM, pH, 7.0, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and in the potential range, −0.6 to 0.6 V at 50 mV/s rate and in the frequency range, 0.01–105 Hz, respectively with ZrO_2/Au and Urs-GLDH/ ZrO_2/Au electrodes. In Fig. 3A, ZrO_2/Au shows a well-defined redox behavior (curve a) at 0.288 V (E_{pc}) and the cathodic peak current at 5.45×10^{-4}A. But the redox peak current decreases to 2.35×10^{-4}A after immobilization of Urs-GLDH (curve b). This may be due to the insulating nature of Urs-GLDH enzyme that may perturb the electron transfer between the medium and the electrode resulting in the slowing down of redox process during the biochemical reaction. In EIS (Fig. 3B), the semicircle part corresponds to electron transfer limited process and its diameter is equal to the electron transfer resistance, R_{CT} that controls electron transfer kinetics of the redox probe at the electrode interface. It can be seen that the value of R_{CT} obtained as 183 Ω for ZrO_2/Au electrode (curve a) increases to 890 Ω for Urs-GLDH/ZrO_2/Au bioelectrodes (curve b). This increase in R_{CT} is attributed to the fact that most biological molecules, including enzymes, are poor electrical conductors at low frequencies (at least <10 kHz) and cause hindrance to the electron transfer. These results indicate binding of Urs-GLDH onto ZrO_2/Au electrode.

Fig. 3C shows CVs of Urs-GLDH/ZrO_2/Au bioelectrodes as a function of scan rate from 50 to 300 mV/s. The proportional increase of redox current with respect to scan rate is observed indicating diffusion-controlled system. The surface concentration of Urs-GLDH/ZrO_2/Au bioelectrode estimated from plot of i_p versus scan rate, using Brown–Anson model has been found to be 2.34×10^{-7} mol m^{-2}. The diffusion coefficient has been estimated using Sandel–Sevcik equation.

$$I_p = \left(2.69\times10^5\right)n^{3/2}AD^{1/2}Cv^{1/2} \quad (3)$$

where I_p is peak current, n is electron stoichiometry, A is electrode area (0.05 m^2), C is surface concentration (2.34×10^{-7} mol m^{-2}), and ν is scan rate (50 V/s). The D value has been obtained as $8.32\times10^{-5}m^2s^{-1}$.

4. Electrochemical response studies

Electrochemical response studies of Urs-GLDH/ZrO_2/Au bioelectrode have been carried out as a function of urea concentration in the presence of 30 μL of nicotinamide adenine dinucleotide (NADH, 3.7 mg/dL) and 70 μL of α-Keto glutarate (α-KG, 47.5 mg/dL) using differential pulse voltammetry in PBS solution {50 mMPBS (pH 7, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$}. It is observed that the magnitude of current obtained for the Urs-GLDH/ZrO_2/Au bioelectrode increases on addition of urea (Fig. 4A). The response time of the

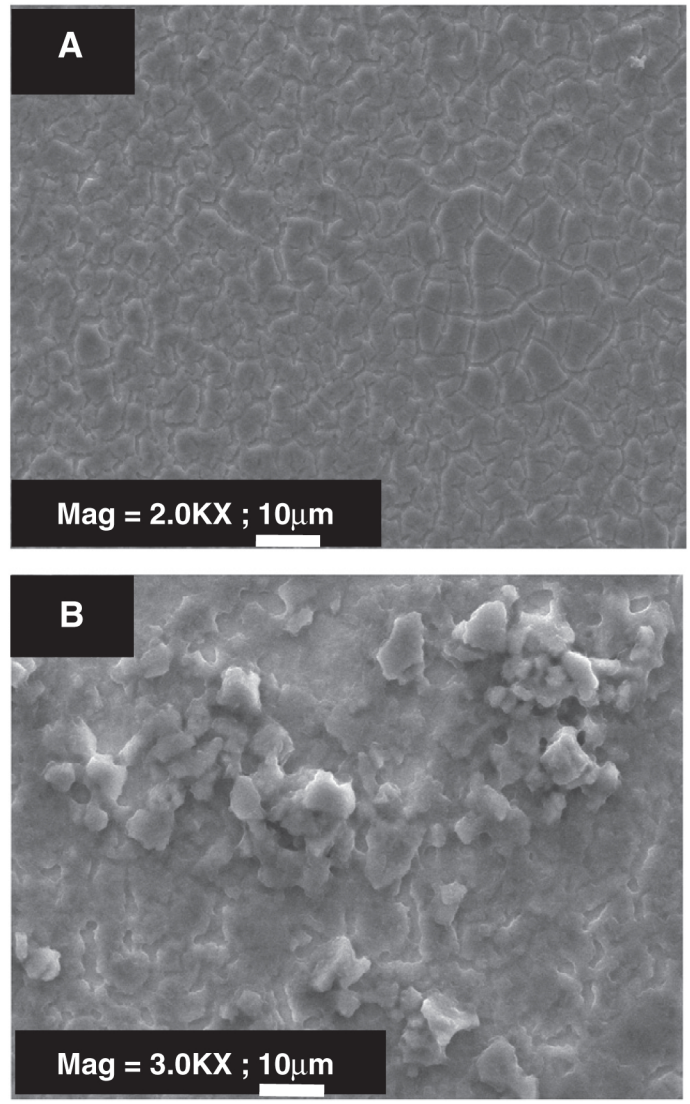


Fig. 2. SEM images of (A) ZrO_2/Au and (B) Urs-GLDH/ ZrO_2/Au electrodes.

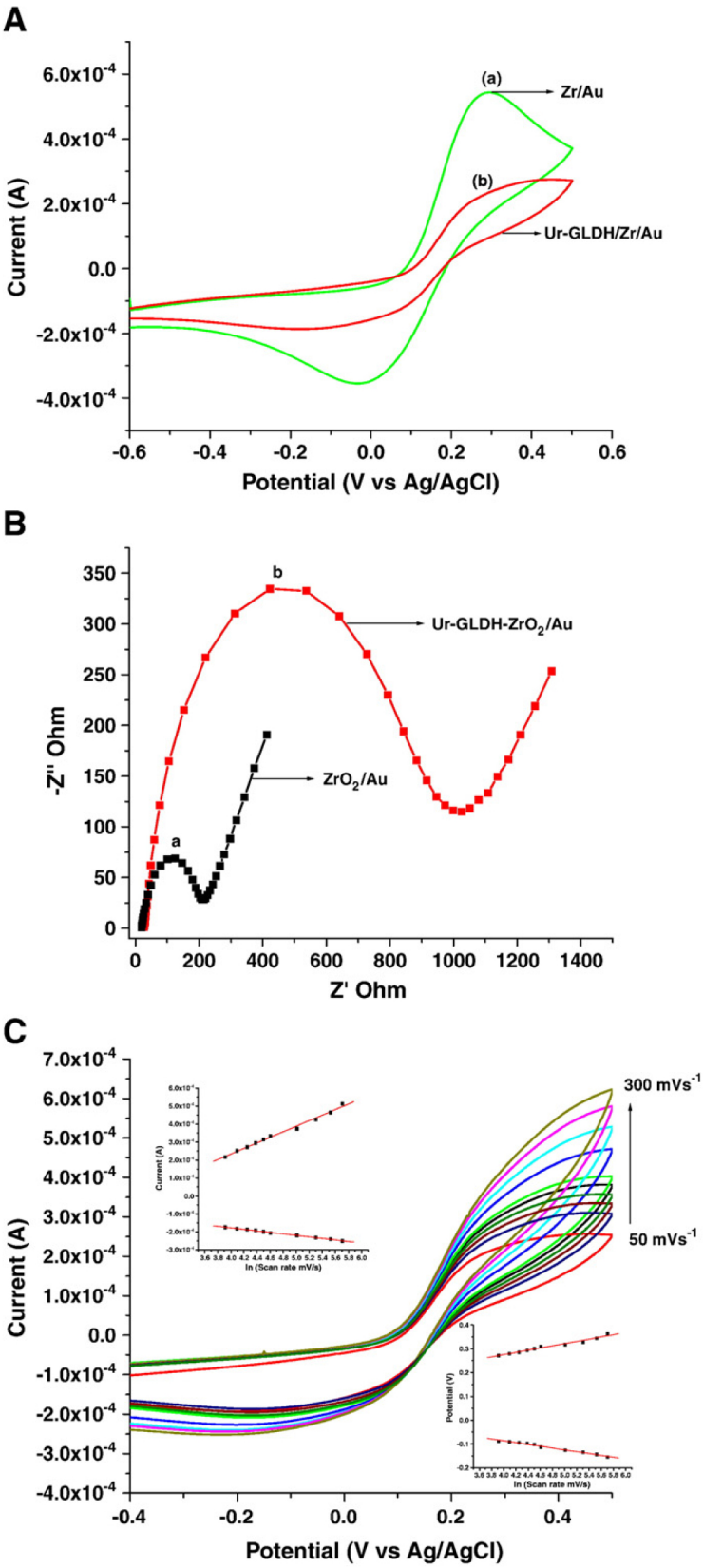


Fig. 3. (A) Cyclic voltammograms and (B) EIS studies of (a) ZrO_2/Au and (b) Urs-GLDH/ZrO_2/Au electrodes; (C) CVs of Urs-GLDH/ZrO_2/Au bioelectrode at different scan rates (50–300 mV/s) in PBS (50 mM, pH 7.0, 0.9% NaCl) containing 5 mM $[Fe(CN)_6]^{3-/4-}$.

Urs-GLDH/ZrO_2/Au bioelectrode found to be about 10s and the quick response is attributed to faster electron communication feature of ZrO_2/Au electrode. It is revealed that Urs-GLDH/ZrO_2/Au bioelectrode can be used to estimate urea from 5 to 100 mg/dL. The sensitivity of the Urs-GLDH/ZrO_2/Au bioelectrode calculated from the slope of the curve has been found to be 0.071 μA/(mM cm^{-2}) (inset Fig. 4A). Urs-GLDH/ZrO_2/Au bioelectrode shows a good linearity in the concentration range (5–100 mg dL^{-1}) and the current varies as

$$I(A) = 3.93 \times 10^{-5} + 7.09 \times 10^{-8} \times Urea\ concentration(\text{mg / dL}) \quad (4)$$

with the value of correlation coefficient as 0.99 (inset Fig. 4A) and standard deviation of 0.023 μA/mg/dL. The detection limit of Urs-GLDH/ZrO_2/Au bioelectrode is estimated to be about 5 mg/dL with reproducibility of 5–6 times. This bioelectrode achieves 95% of steady state current in less than 10s indicating fast electron exchange between Urs-GLDH and ZrO_2/Au electrode.

The value of the apparent Michaelis–Menten constant (K_m) has been calculated to check the suitability of matrix for urea biosensor fabrication using Hanes plot ([S] versus [S]/[I], where S = analyte concentration and I = corresponding current; Fig. 4B). K_m value has been found to be 0.5 mM for the immobilized Ur-GLDH indicating the high catalytic activity of the enzyme at low substrate concentration. It is observed that this bioelectrode retains about 85% of enzyme (Ur-GLDH) activity even after about 8 weeks when stored in refrigerated conditions (4 °C).

The proposed biochemical reaction during the urea detection is shown in Fig. 4C. Urs catalyzes hydrolysis of urea to carbamine acid that gets hydrolyzed to ammonia (NH_3) and carbon dioxide (CO_2). GLDH catalyzes the reversible reaction between α-KG and NH_3 to NAD^+ and linked oxidative deamination of L-glutamate in two steps. The first step involves a Schiff base intermediate being formed between NH_3 and α-KG. The second step involves the Schiff base intermediate being protonated due to the transfer of the hydride ion from NADH resulting in L-glutamate. NAD^+ is utilized in the forward reaction of α-KG and free NH_3 that are converted to L-glutamate via hydride transfer from NADH to glutamate. NAD^+ is utilized in the reverse reaction, involving L-glutamate being converted to α-KG and free (NH_3) via deamination reaction. The electrons generated from the biochemical reactions are transferred to the ZrO_2/Au electrode through the Fe(III)/Fe(IV) couples that help in amplifying the electrochemical signal resulting in increased sensitivity of the sensor.

5. Conclusions

We have successfully fabricated urea biosensor based on zirconium oxide films deposited electrochemically onto gold coated glass substrates from an aqueous electrolyte of $ZrOCl_2$ and KCl by cycling the potential between −1.5 and +0.7 V (versus Ag/AgCl) at a scan rate of 20 mV s^{-1} for 10 consecutive scans. Mixture of Urs and GLDH has been covalently immobilized onto the ZrO_2/Au electrode using the affinity interactions between the oxygen atom of the biomolecule and the zirconium atom (Zr) of zirconia (ZrO_2). These nanostructured ZrO_2/Au electrodes exhibit linearity up to 100 mg dL^{-1} of analyte (urea) and sensitivity of 0.071 μA/(mM cm^{-2}). Besides this, the value of the apparent Michaelis–Menten constant (K_m), indicative of enzyme substrate interactions, has been found to be 0.5 mM. This low value of K_m for Urs-GLDH/ZrO_2/Au bioelectrode reveals increased enzyme (urease-GLDH)-substrate (urea) interactions indicating distinct advantage of this matrix over other matrices used for urea biosensor fabrication. Efforts should be made to utilize these electrodes for other bioanalytes such as cholesterol [16,17], glucose [18], lactate [19] and *in vivo* sensing.

Acknowledgments

We thank Professor R. C. Budhani, Director, National Physical Laboratory, India for facilities. M.D. thanks CSIR, India for award of a Senior Research Fellowship. Financial support received from DST, DBT (Grant No. GAP070832) and Ministry of Education, Science and Technology (Grant No. R32-20026) of Korea is gratefully acknowledged.

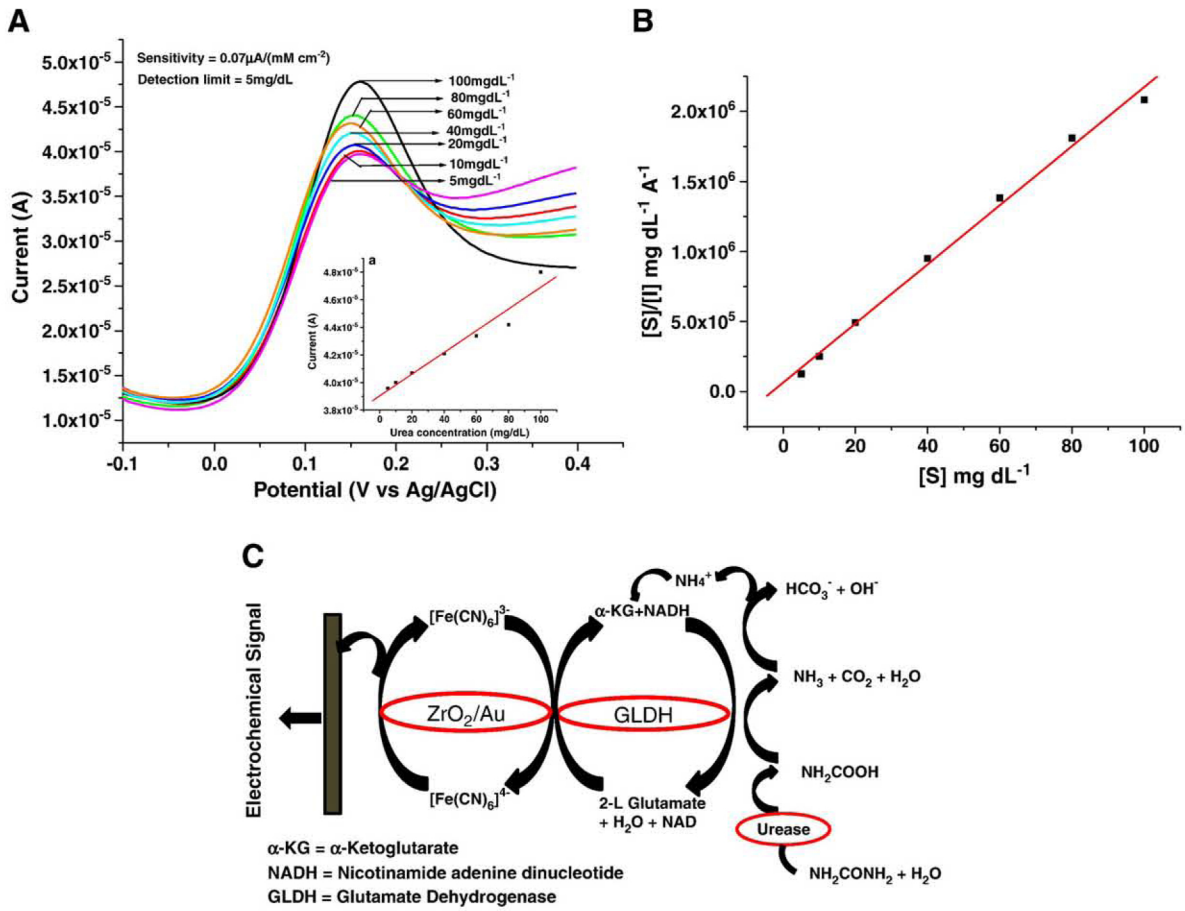


Fig. 4. (A)Electrochemical response of Urs-GLDH/ZrO_2/Au bioelectrode as a function of urea concentration (5–100 mg/dL)(inset figure shows the sensitivity of this bioelectrode); (B) Hans plot for K_m values; (C) Biochemical reaction during electrochemical detection of urea using Urs-GLDH/ZrO_2/Au bioelectrode.

References

[1] F. Kuralay, H. Özyörük, A. Yýldýz, Sens. Actu. B 114 (2006) 500–506.
[2] B. Lakard, G. Herlem, S. Lakard, A. Antoniou, B. Fahys, Biosens. Bioelectron. 19 (2004) 1641–1647.
[3] A. Sehitogullari, A.H. Uslan, Talanta 57 (2002) 1039–1044.
[4] M. Gutí´errez, S. Alegret, M.D. Valle, Biosens. Bioelectron. 22 (2007) 2171–2178.
[5] S. Karakus, ule Pekyardýmcý, and Esma Kýlýc, Artif. Cells, Blood Substit. Biotechnol. 33 (2005) 329–341.
[6] G. Sumana, M.K. Pandey, B.D. Malhotra, J. Mater. Res. 24 (2009) 1667–1673.
[7] Pratima Solanki, Ajeet Kaushik, Anees A. Ansari, G. Sumana, B.D. Malhotra, Appl. Phys. Lett. 93 (2008) 163903.
[8] Gunjan Dhawan, G. Sumana, B.D. Malhotra, Biochem. Eng. J. 44 (2009) 42–52.
[9] M. Fang, D.M. Kaschak, A.C. Sutorik, T.E. Mallouk, J. Am. Chem. Soc. 119 (1997) 12184–12191.
[10] Qinyuan Zhang, Xiaoli Li, Jun Shen, Wu. Guangming, Jue Wang, Lingyan Chen, Mater. Lett. 45 (2000) 311–314.
[11] Shou-Qing Liu, Jing-Juan Xu, Hong-Yuan Chen, Bioelectrochemistry 57 (2002) 149–154.
[12] Yunhui Yang, Zhijie Wang, Minghui Yang, Jishan Li, Fang Zheng, Guoli Shen, Ruqin Yu, Anal. Chim. Acta 584 (2007) 268–274.
[13] C.T. Buscher, D. McBranch, D.Q. Li, J. Am. Chem. Soc. 118 (1996) 2950–2953.
[14] Maumita Das, Gajjala Sumana, R. Nagarajan, B.D. Malhotra, Appl. Phys. Lett. 96 (2010) 133703.
[15] N.N. Zhu, A.P. Zhang, Q.J. Wang, P.A. He, Y.Z. Fang, Anaytica Chimica Acta 510 (2004) 163–168.
[16] A. Kumar, Rajesh, A. Chaubey, S.K. Grover, B.D. Malhotra, Journal of Applied Polymer Science 82 (2001) 3486–3491.
[17] S.K. Arya, P.R. Solanki, Ravindra P. Singh, M.K. Pandey, M. Datta, B.D. Malhotra, Talanta 69 (2006) 918–926.
[18] Ajeet Kaushik, Raju Khan, Pratima Solanki, Pratibha Pandey, Javed, Sharif Ahmad, B.D. Malhotra, Biosensors & Bioelectronics 24 (2008) 676–683.
[19] M. Gerard, K. Ramanathan, A. Chaubey, B.D. Malhotra, Electroanalysis 11 (1999) 45.