Electrochemical Urea Biosensor Based on Sol-gel Derived Nanostructured Cerium Oxide

Anees A Ansari1*, Md. Azahar2 and B. D. Malhotra3*
1King Abdullah Institute for Nanotechnology, King Saud University, Riyadh, 11451 Kingdom of Saudi Arabia
2Department of Science & Technology Centre on Biomolecular Electronics, National Physical Laboratory, Dr. K S Krishnan Marg, New Delhi-110012, India
3Department of Biotechnology, Delhi Technological University, Shabad Daulatpur, Main Bawana Road, Delhi 11042, India
E-mail: aneesaansari@gmail.com ; bansi.malhotra@gmail.com

Abstract. Urease (Urs) and glutamate dehydrogenase (GLDH) have been co-immobilized onto a nanostructured–cerium oxide (Nano–CeO2) film deposited onto an indium-tin-oxide (ITO) coated glass substrate by dip-coating via sol-gel process for urea detection. This nanostructured film has characterized using X-ray diffraction (XRD), Fourier transform infrared (FTIR), Scanning electron microscope (SEM) and electrochemical techniques, respectively. The particle size of the Nano–CeO2 film has been found to be 23 nm. Electrochemical response (CV) studies show that Ur-GLDH/Nano–CeO2/ITO bioelectrode is found to be sensitive in the 10–80 mg/dL urea concentration range and can detect urea concentration up to 0.1 mg/dL level. The value of Michaelis–Menten constant (Km) estimated using Lineweaver–Burke plot found as 6.09 mg/dL indicates enhancement in the affinity and/or activity of enzyme attached to their nanobiocomposite. This bioelectrode retained 95% of enzyme activity after 6 months at 4°C.

1. Introduction
Novel analytical devices based on nanostructured metal oxides are known to be cost-effective and highly sensitive due to the large surface-to-volume ratio, show excellent selectivity and their optical and electrical properties arising from electron and phonon confinement. These nanostructured materials can be used as sensing biomolecules[1-18]. Besides these nanostructured metal oxides have unique properties such as non-toxicity, biocompatibility, high chemical and thermal stability, high isoelectric point (IEP ~9.2) and negligible swelling in both aqueous and non-aqueous solvents[1-6]. A large number of nanomaterials such as carbon nanotubes[7], conducting polymers[8], graphene[9], nanowires[10], zinc oxide nanoparticles and chitosan composite[11] film and nanoporous materials have been used for fabrication of various biosensors. Many nanostructured metal oxide such as zirconium oxide (ZrO2), tin oxide (SnO2), cerium oxide (CeO2) and zinc oxide (ZnO) have been utilized for immobilization of proteins, enzymes and antigens for accelerated electron transfer between active sites of protein and electrode[12-13]. On the other hand, MnO2 and TiO2 having low IEP values are suitable for the immobilization of high IEP proteins.

* To whom any correspondence should be addressed.
Recent times have been significant advances towards the production of nanocrystalline metal oxides using a variety of physical and chemical processes, such as sputtering, sol–gel, hydrothermal, spray pyrolysis etc. Among the various methods sol-gel technique has been considered as very attractive for the preparation of metal oxide particles due to easy preparation under ambient conditions, tunable porosity, high thermal stability, chemical inertness and negligible swelling in aqueous and non-aqueous solutions[14-15]. Besides this, due to inherent low temperature process, the sol-gel technology provides an attractive method for the immobilization of heat-sensitive biomolecules such as enzymes, proteins and antibodies[16]. The positively charged sol–gel cerium oxide not only provides a friendly environment for the enzyme to retain its activity but also encapsulate the negatively charged electron transfer mediator within the sol-gel film via electrostatic interaction[17]. The sensitivity and stability of the biosensors can perhaps be improved by controlling charge, porosity and electronic conductivity of a given sol-gel film. These attractive features have led to an increased research towards developing the electrochemical sensors and biosensors[18].

The estimation of urea is of clinical interest since decreased urea concentration (normal range is 15-40 mg/dl) causes hepatic failure, nephritic syndrome and cachexia and increased urea level in blood and urine causes renal failure, urinary tract obstruction, dehydration, shock, burns and gastrointestinal bleeding[19-21]. Therefore, development of rapid, cheap and reliable methods for urea determination is important. Among the numerous analytical methods reported in literature, enzymatic methods offer an improvement in terms of specificity, response time, miniature size, reproducibility and selectivity. Saha et. al. fabricated cerium oxide (CeO$_2$) film deposited onto platinum (Pt) electrode using pulsed laser deposition (PLD) has been utilized for immobilization of glucose oxidase (GOx)[22]. Feng et. al. have prepared a nanoporous CeO$_2$/chitosan composite matrix for the immobilization of single-stranded DNA probes for the detection of cancer genes[23]. Khan et. al. ZnO-chitosan nanocomposite has recently been used for application to urea and cholesterol biosensors[11].

We have used a facile sol-gel chemical route to prepare Nano-CeO$_2$ film onto indium-tin-oxide glass substrate (ITO) to immobilize a mixture of ureas and GLDH enzymes for application to urea detection using electrochemical spectroscopy technique.

2. Experimental procedure

2.1. Chemicals and reagents

Urease (Urs), glutamate dehydrogenase (GLDH), nicotinamide adenine dinucleotide (NADH) α-keto glutarate (α-KG), Na$_2$HPO$_4$ and NaH$_2$PO$_4$ were procured from Sigma Aldrich (USA). Ammonium ceric nitrate, urea, NaCl, NH$_4$OH and HNO$_3$ reagents were procured from Merck India Ltd, Mumbai, India. All these chemicals were of analytical grade and used without further purification. Indium-tin-oxide (ITO) coated glass plates were obtained from Balzers, UK. The deionized water obtained from Millipore water purification system (Milli Q 10 TS) was used for preparation of solutions and buffers.

2.2. Fabrication of sol-gel derived nano-structured CeO$_2$ film/electrode

1 gm of ceric ammonium nitrate [(NH$_4$)$_2$Ce(NO$_3$)$_6$] was dissolved in deionised water (20 ml). Then 5 ml (1 M) solution of ammonium hydroxide (NH$_3$OH) was added drop wise in this solution with constant stirring for 4 h at 25 °C to maintain pH 10. A pale yellow precipitate of Ce(OH)$_4$ thus obtained was washed several times with deionized water until a neutral pH was achieved$^{13}$. Subsequently, dilute HNO$_3$ (1M) was added to the precipitate at 60 °C to obtain a solution of pH 1. A transparent yellow solution thus obtained was further concentrated by heating. The resulting sol was used to fabricate thin film on ITO coated glass plate via dip coating technique and was allowed to dry at 500 °C.

2.3. Electrode modification and immobilization Urease-GLDH
10 µL of 1:1 molar mixture of urease and GLDH (1.0 mg/mL, in PBS, 50 mM, pH 7.0) was immobilized onto sol-gel derived Nano-CeO$_2$ electrode by physisorption method. The biochemical reaction using mixed enzyme system is shown in Eq.1. Prior to being used, Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode was allowed to dry overnight under desiccated conditions and then washed with phosphate buffer saline (PBS, 50 mM, pH 7.0, 0.9 % NaCl) to remove any unabsorbed enzymes (Urs-GLDH) and stored in a desiccator at 25°C when not in use.

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\begin{align*}
\text{Urea} & \xrightarrow{\text{urease}} 2\text{NH}_4^+ + \text{HCO}_3^- \\
2\text{NH}_4^+ + \alpha\text{-ketoglutarate} + \text{NADH} & \xrightarrow{\text{GLDH}} 2\text{-Glutamate} + \text{NAD}^+ \\
\text{NADH} & \xrightarrow{2\text{e}^-} \text{NAD}^+ + 2\text{e}^-
\end{align*}
\] (Eq. 1)

2.4. Characterization of sol-gel derived Nano-CeO$_2$/ITO electrode and Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode

X-ray diffraction (XRD, Cu Ka radiation (Rigaku)) study was performed to identify the crystal structure of sol-gel derived Nano-CeO$_2$ film. Scanning electron microscopy (SEM, LEO-440) studies were conducted to examine the surface morphology. FTIR spectra of sol-gel derived Nano-CeO$_2$ films were recorded using FTIR (Perkin-Elmer) spectrophotometer to investigate binding of ureas enzyme onto sol-gel derived Nano-CeO$_2$/ITO film. Electrochemical measurements were conducted on an Autolab Potentiostat/Galvanostat (Eco Chemie, Netherlands) using a three-electrode cell containing ITO act as a working electrode, Ag/AgCl as reference electrode and platinum (Pt) wire serve as a counter electrode in phosphate buffer saline (PBS, 50 mM, pH 7.0, 0.9% NaCl) containing 5 mM [Fe(CN)$_6$]$_{3-4-}$.

3. Results and discussion

3.1. Characterization of nano- CeO$_2$ film

The XRD diffraction pattern (Figure 1) shows a crystallographic phase present in the sol–gel derived CeO$_2$ film deposited on the glass substrate via dip-coating technique. A high degree of preferential orientation is evident, giving rise to spectra resembling a single crystal diffraction pattern. XRD pattern of the deposited film reveals all reflection planes of CeO$_2$, corresponding to the (111), (200), (220) and (311) crystallographic plane cubic fluorite structure (space group Fm3m (225)) as identified using the standard data JCPDS Card No. 34-0394. The intensities and positions of the peaks are in perfect agreement with the literature values. No peak of any other phase is detected indicating the high purity of the material. The broadening of the reflections in the diffractogram distinctly indicates the formation of nano-dimensional crystals. The average crystallite size of CeO$_2$ nanoparticles was estimated by analysis of the broadening of (111) and (220) reflections via Scherrer formula found to be 10-15 nm.

3.2. Characterization of nano- CeO$_2$ film

The infrared absorption spectra of Nano-CeO$_2$/ITO (a) and Urs-GLDH/Nano-CeO$_2$/ITO (b) shows the characteristic absorption bands of the deposited films (Figure 2). A diffused band at 3404 cm$^{-1}$ and two weak bands at 1414 and 1010 cm$^{-1}$, corresponding to the stretching and bending vibrations of the hydroxyl (O–H) groups, respectively, are observed. The appearance of these bands suggests the adsorption of moisture on the surface of nanostructured film. The FTIR spectrum shows a sharp and intense band at around 426 cm$^{-1}$ assigned to the Ce-O stretching band and indicates the deposition of CeO$_2$ film on the ITO surface. These observed bands are shifted and some new bands are appearing at 1740, 1380 and 1050 cm$^{-1}$ corresponding to the stretching and bending vibrations of the amide, the functional groups. This indicates that the Urs-GLDH mixed enzymes solution has been successfully immobilized on the Nano-CeO$_2$ matrix. A sharp band at 450 cm$^{-1}$ is observed due to the electrostatic interaction and hydrogen bonding of the Nano-CeO$_2$ with the enzymes (Urs-GLDH).
3.3. Scanning electron microscopy studies

Figure 3 shows results of the SEM studies carried out on Nano-CeO$_2$/ITO film and of Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode, respectively. The SEM micrograph shows that the film surface is free of cracks, uniform in thickness porous in nature and composed of very small fine grains that are homogenously distributed (image 3a). The adsorption or immobilization of urease enzyme onto the surface of sol-gel derived Nano-CeO$_2$/ITO electrode is dependent on the morphology of the substrate. It can be seen that after immobilization of Urs-GLDH onto Nano-CeO$_2$/ITO electrode, displays flower like structure with high porosity, inter-connectivity and significant free volume favoring the effective immobilization of a large number of enzymes (image 3b).

3.4. Electrochemical impedance spectroscopy

EIS measurements carried out on Nano-CeO$_2$/ITO electrode and Urs-GLDH/Nano–CeO$_2$/ITO bioelectrode at frequency range 0.0–5x10$^3$ Ω. It is an effective and powerful tool for characterizing the interfacial features of surface-modified electrodes. The modified electrode impedance can be presented as the sum of the real ($Z'$), and imaginary ($-Z''$) components that originate mainly from the resistance and capacitance of the cell, respectively. The general electronic equivalent circuit (Randles and Ershler model), includes the ohmic resistance of the electrolyte solution ($R_s$), the Warburg impedance ($D$), resulting from the diffusion of ions from the bulk electrolyte to the electrode interface. The double layer capacitance ($C_{dl}$) and charge-transfer resistance ($R_{ct}$) exists, if a redox probe is present in the electrolyte solution, where $R_s$ and $D$ denote bulk properties of the electrolyte solution and diffusion features of the redox probe in solution, respectively. The other two components $C_{dl}$ and $R_{ct}$, depend on the dielectric and insulating features at the electrode/electrolyte interface. Figure 4 shows the Faradaic impedance spectra, presented as Nyquist plots obtained from real ($Z'$) and imaginary ($-Z''$) of blank ITO (curve a), Nano-CeO$_2$/ITO electrode (curve b) and Urs-GLDH/Nano-CeO$_2$/ITO (curve c) bioelectrode. The values of $R_{ct}$ derived from the diameter of semicircle of impedance spectra are obtained as 2.3 kΩ for blank ITO electrode (curve a), 2.77 kΩ (curve b) for the Nano-CeO$_2$/ITO electrode and 2.92 kΩ for Urs-GLDH/Nano–CeO$_2$/ITO bioelectrode, respectively. The increase of $R_{ct}$ (2.92 kΩ) value and the shift of semicircle to higher frequency after deposition of the Nano–CeO$_2$ layer on the ITO electrode is attributed to the low electrical conductivity of the Nano–CeO$_2$ particles that provides slow electron transfer between Nano-CeO$_2$ particles and electrode.
result suggests that solution resistance ($R_s$) is slightly increased, but interfacial impedance (double layer capacitance, $C_{dl}$ and the charge transfer resistance, $R_{ct}$) is greatly increased after the deposition of Nano-CeO$_2$ film on the ITO electrode surface. It is observed that after the immobilization of mixed Urs-GLDH enzyme on the matrix the $R_{ct}$ value is increases dramatically due to insulating characteristics of the mixed Urs-GLDH enzyme molecules. It reveals that Warburg impedance ($D$), which represents the mass transfer, is enhanced after the immobilization of these enzymes (Urs-GLDH) on the Nano–CeO$_2$/ITO electrode surface, implying that enzyme molecules are indeed absorbed on Nano–CeO$_2$/ITO surfaces and induce impedance effects on the interface of the electrode surface. The double-layer capacitance ($C_{dl}$) is decreased, indicating that a higher enzyme (Urs-GLDH) concentration enhances the adsorption of enzyme on the Nano–CeO$_2$/ITO modified electrode.

**3.5. Electrochemical impedance spectroscopy**

The changes of electrode behavior after surface modification with enzymes (Urs and GLDH) were studied by cyclic voltammetry (CV) in the presence of ferricyanide mediator. When the electrode surface is modified by the biocatalytic material, the change in the electron transfer kinetics of $[\text{Fe(CN)}_6]^{3/4-}$ gives indication of the enzyme attachment. Figure 5 shows the cyclic voltammograms of blank ITO (a), Nano-CeO$_2$/ITO (b) and Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode (c) at a scan rate of 50 mVs$^{-1}$. As shown in Figure 5 well-defined redox couple of $[\text{Fe(CN)}_6]^{3/4-}$ is observed on the bare ITO electrode (curve a). When the Nano–CeO$_2$/ITO electrode layer is deposited on the ITO electrode, the peak current gradually decreases (curve b). The decrease in the peak potential after deposition of Nano–CeO$_2$ may be attributed to the hindrance of the electrons flow as a result of reduction in electrical conductivity of the nanostructured Nano–CeO$_2$/ITO electrode. Further, redox current of Urs-GLDH/Nano–CeO$_2$/ITO bioelectrode is found to decrease due to insulating characteristics of Urs-GLDH indicating slow down of redox process during the biochemical reaction. The reason is that an insulating layer of non-conducting enzyme had been assembled on electrode surface, which act as a electron transfer barrier.

Figure 6 demonstrates typical CV of Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode with scan rate varying from 10 to 100 mVs$^{-1}$. With increase in the scan rate, there is increase in both the cathodic and anodic peak current accompanied with small shift and increased peak-to-peak separation. The cathodic and anodic peak currents increase linearly with scan rate varying from 10 to 100mVs$^{-1}$ indicating a surface controlled electrode process. This reveals that the electron transfer between enzyme and electrode could be easily performed and it was a surface confined electrochemical process.
3.6. Electrochemical impedance spectroscopy

Figure 4. Electrochemical Impedance spectroscopy of (a) bare ITO electrode (b) sol-gel derived Nano-CeO$_2$/ITO electrode and (c) Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode in PBS solution (50 mM, pH 7.0, 0.9% NaCl) containing 5 mM [Fe(CN)$_6^{3-/4-}$]

Figure 5. Cyclic voltammogram of (a) bare ITO electrode (b) sol-gel derived Nano-CeO$_2$/ITO electrode and (c) Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode in PBS solution (50 mM, pH 7.0, 0.9% NaCl) containing 5 mM [Fe(CN)$_6^{3-/4-}$]

Figure 6. CV of Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode as a function of scan rate (10-100 mV/s$^{-1}$) in ascending order in PBS solution containing 5 mM [Fe(CN)$_6^{3-/4-}$]

Figure 7 (inset) illustrate the CV response studies of the Urs-GLDH/Nano-CeO$_2$/ITO bioelectrode in PBS solution {pH 7, 0.9%NaCl, containing 5mM [Fe(CN)$_6^{3-/4-}$] in the presence of 30 µL of nicotinamide adenine dinucleotide (NADH, 3.7 mg/dL) and 70 µL of α-Keto glutamate (α-KG, 47.5
mg/dL) and a successive addition of urea at an applied 50 mV/s scan rate. The peak current rises sharply with increased concentration of urea, with the maximum response approach at the concentration of 80 mg/dL. This increase in electrochemical current response can be attributed to pH sensitive behavior of the Ur-GLDH/Nano-CeO$_2$/ITO bioelectrode. The sensor achieves 95% of the steady-state current in less than 5s indicating fast electron exchange between the Urs-GLDH and Nano–CeO$_2$/ITO electrode. Figure 7 shows the amperometric current of the fabricated biosensor increases linearly within the concentration range 10-80 mg/dL, with increasing urea concentration the correlation coefficient ($R$) as 0.991 indicating good electro-catalytic behavior of Urs-GLDH/Nano–CeO$_2$/ITO bioelectrode. The sensitivity of the Urs-GLDH/Nano–CeO$_2$/ITO bioelectrode calculated from the slope of curve was found to be 0.64 µA/mg/dL and low detection limit 0.1 mg/dL.

The value of $K_m$, which is a reflection of enzymatic affinity, was found to be 6.09 mg/dL. The lower $K_m$ value with respect to glutaraldehyde-cross-linked urease-albumin gel (6.5 mM) and TMOS-based sol-gel (4.95 mM) indicates easier diffusion of substrate and product molecules into and out of the Nano–CeO$_2$ film and the feasible configuration of the enzyme in the nanomaterial matrix. The high affinity of Urs-GLDH to urea can be attributed to Nano–CeO$_2$ nanoparticles because of its biocompatibility, large surface area, and high electron communication capability. The value of ionization electron potential (IEP) of CeO$_2$ and urease are known to be 9.0 and 5.3, respectively. Therefore, the Nano–CeO$_2$ matrix is positively charged in contrast to the enzymes that are negatively charged. Thus, immobilization of Urs-GLDH on the Nano–CeO$_2$ nanoparticles matrix is highly favored via electrostatic interaction.

3.7. Effect of pH onto Urs-GLDH/nano-CeO$_2$/ITO biosensor

The influence of pH of buffer on the response of the biosensor studied over the range 6.0–8.0 indicates that the current response increases with increase of pH of the substrate solution up to 7.0, after which it decreases. In view of the prime pH of the enzyme, the neutral pH was selected throughout this work (Figure 8).
4. Conclusions
Nanostructured CeO$_2$ film was prepared by sol gel technique and used to co-immobilization of Urs and GLDH enzymes via physical adsorption for urea detection. This biosensor exhibits excellent performance characteristics, such as sensitivity (0.64µA/mg/dL) and reproducibility, wide linear range (10–80 mg/dL), low detection limit (0.10 mM), and long-term stability of about 6 months. A relatively low value of the Michaelis–Menten constant obtained as 6.09 mg/dL indicates enhanced enzyme affinity of Urs to urea. The wide range of detection and high sensitivity may be assigned to amplification of the magnitude of current due to the alignment of Nano–CeO$_2$ nanoparticles to the matrix. Efforts should be made to use this electrode for the detection of urea in blood serum.

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