



## Nanostructured Rutile Titanium Dioxide Based Platform for Application to Urease Biosensor

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### ABSTRACT

Studies relating to immobilization of Urease (Urs) sensor based on titanium dioxide-Poly Vinyl alcohol deposited on gold electrode. Synthesis of porous Nanostructured rutile Titanium Oxide (TiO<sub>2</sub>) nanoparticles (30 nm) by Sol-gel citrate method. XRD and Laser Raman studies show that TiO<sub>2</sub> is in rutile phase with tetragonal structure. Electrochemical studies shows decreased in Voltage–Current characteristics of Urs/TiO<sub>2</sub>-PVA bioelectrode after immobilization of Urease due to insulating characteristics of enzyme Urease. Surface characterization of TiO<sub>2</sub>-PVA nanocomposite gold electrode and Urs/TiO<sub>2</sub>-PVA nanocomposite gold bioelectrode by scanning electron microscopy (SEM) exhibit TiO<sub>2</sub> has active surface area for enzyme immobilization. Monitoring of Urease biosensor immobilization was performed using cyclic voltametry (CV) and EIS (Electrochemical impedance technique). Response study of Urs/TiO<sub>2</sub>-PVA gold bioelectrode at different urea concentration shows good linearity and also shows high sensitivity for urea detection within (5-50mg/dL), limit of detection was 5.8mg/dL with regression coefficient 0.99, Low value of Michaelis-Menten constant (K<sub>m</sub>, 4.08mg/ dL) indicate excellent enzyme affinity.

**Keywords:** Nanostructured-TiO<sub>2</sub>, Urease, Nanocomposite, Biosensor, Urea detection.

### INTRODUCTION

Biosensors built on electrochemistry are now attracting ample attention as potential successors to wide range of analytical technique due to unique properties.<sup>1,2</sup> Urea [(NH<sub>2</sub>)<sub>2</sub>CO] is a diamide of carbonic acid also known as Carbamide which is waste product of many living organism and major Organic component of human urine.<sup>3</sup> Urea plays valuable act in metabolism of nitrogen containing compounds, so its accumulation above critical level must be carefully determined. Urea is widely distributed in nature its analysis is considerable interested in clinical and agricultural chemistry<sup>4</sup>.

Increasing urea in blood and urine can cause urinary tract obstruction, dehydration, shock, burns and gastrointestinal bleeding, whereas reducing in urea level may be seen in nephritic syndrome, Cachexia and hepatic failure<sup>5</sup>. Hence it is maximum attraction to grown cost effective technique for real time watch of Urea in human serum, including Urine and blood sample. Numbers of transducer have been used for detection of Urea including Amperometric, Potentiometric, optical, thermal, and piezoelectric<sup>6,7</sup> amongst them amperometric biosensor based on Urease is promising method because of its effectiveness, simplicity, ease of calibration etc<sup>8</sup> Immobilization of enzyme is key step for the preparation of biosensor. Most of existing urea biosensor utilizes Urease as sensing element. Urease functionally belonging to super family of amidohydrolases and phosphotriesterase.<sup>9</sup> The Urease catalyzes the hydrolysis of Urea to carbonic acid and ammonia is final product<sup>10</sup> Nanostructure metal oxide have recently been used for fabrication of biosensing area, because of their ability to

promote faster electron transfer between electrode and active site of desired enzyme.<sup>11</sup> Some smart nanomaterial such as Zinc Oxide<sup>12,13</sup>, Cerium Oxide<sup>14</sup>, Titanium Oxide<sup>15</sup>, Iron Oxide<sup>16</sup> have been investigated for fabrication of enzyme based electrochemical biosensor.

Among those, nanostructured TiO<sub>2</sub> has received much attention as an immobilization matrix for the design of desired biosensors because of its one-dimensional nanostructured, electronic conductivity, and larger specific surface area. Various Wet chemical methods have been reported for the preparation of nano-sized TiO<sub>2</sub>. Sol gel route has been regarded as an excellent method to synthesized nano-sized metallic oxide<sup>17</sup> and has been widely employed for the preparation of TiO<sub>2</sub> nanoparticles.<sup>18,19</sup>

Problem of aggregation and rapid biodegradation of TiO<sub>2</sub> nanoparticles on given matrix containing biomolecule can perhaps be overcome by modified this nanoparticles using Poly vinyl alcohol (PVA) by preparing hybrid nanocomposite. PVA is non toxic and biocompatible synthetic polymer with good film forming ability.<sup>20</sup> PVA is indeed well-known for its wide range of potential application in pharmaceutical, medical and membrane fields.<sup>21</sup> PVA as water soluble organic additive was applied to achieve polymer nanostructured hybrid film.<sup>22</sup>

The response time of such biosensor is directly associated with hydrolysis rate of urea electrode surface, therefore rapid production of NH<sub>4</sub> ions on electrode will lead to highly sensitive biosensor. The NH<sub>4</sub> ions are not electroactive and they are oxidized two approaches using second enzyme or catalytically active enzyme<sup>23</sup> have developed amperometric sensor for urea using two enzyme Urease and Isocitrate dehydrogenase. Some

researcher have develop amperometric route by oxidation of ammonia using enzyme glutamate dehydrogenase.<sup>24</sup> The main disadvantage of this electrode is saturation and fast poisoning of metal catalytic surface. Based on second approach, the biosensing element was immobilized on modified polymer membrane.<sup>25</sup>

In our case the biosensing element Urease immobilized on the surface of polymer membrane with TiO<sub>2</sub>. Both component TiO<sub>2</sub> and Urease contribute to the decreasing of production cost of biosensor by avoiding use of second enzyme an amperometric measuring system has been develop so that to omit the used of second enzyme.

## MATERIALS AND METHODS

### Material

Urease enzyme (Urs), Poly vinyl alcohol (PVA), Urea (M.Wt-60.60gr/mol), Titanium n-butoxide, [Ti (O-Bu)<sub>4</sub>]<sub>n</sub>, citric acid, Ethanol, potassium ferricyanide [Fe(CN)<sub>6</sub>]<sup>3-/4-</sup>, potassium chloride, potassium chloride [KCl], phosphate buffer [PBS], All chemicals and solvents were of analytical grade. The deionized water has been used for preparation of reagents.

### Preparations of solution

Phosphate buffer [50Mm] (ph.-7.0): solution prepared by adjusting the proportion of monobasic sodium phosphate [NaH<sub>2</sub>PO<sub>4</sub>] and dibasic sodium phosphate [Na<sub>2</sub>HPO<sub>4</sub>] solution. Urease enzyme (Urs): Urease enzyme (1mg/ml) prepared in PBS (Ph.7.0), Different Concentration of Urea [5-50mg/dL] were prepared in deionized water.

### Preparation of TiO<sub>2</sub> nanoparticles

Porous TiO<sub>2</sub> nanoparticles (30 nm) prepared by using sol-gel citrate method.<sup>25</sup> Titanium (IV) butoxide [Ti (O-Bu)<sub>4</sub>]<sub>n</sub> is dissolved in ethanol stirred with citric acid and ethanol at 80° C for 3 hrs to get homogeneous and transferent solution. The solution was further heated at about 120° for 12 hrs in pressure vessel to form the gel precursor. The prepared product was subjected to 3hrs heat treatment (Calcinated) at 350°C in muffle furnace and then milled to a fine powder. The dried powder then calcinated at 650°C in order to improve the crystallinity and sensitivity of material.

### Preparation of TiO<sub>2</sub>-PVA nanocomposite gold electrode

Poly vinyl alcohol (PVA) structure (Fig.1B) consists of carbon chain backbone attached with hydroxyl group. PVA prepared by heating 1g of PVA with 10 mL distilled water at 80°C for an hour with continuous stirring. Then, mixture was left to cool down to room temperature; Sticky slurry of PVA is obtained. To prepare TiO<sub>2</sub>-PVA nanocomposite gold electrode sol-gel derived TiO<sub>2</sub> nanoparticles dispersed in this cool viscous solution of PVA, then this solution is stirred at room temperature. Then this solution was deposited on gold electrode by dip coating technique to obtain TiO<sub>2</sub>-PVA nanocomposite

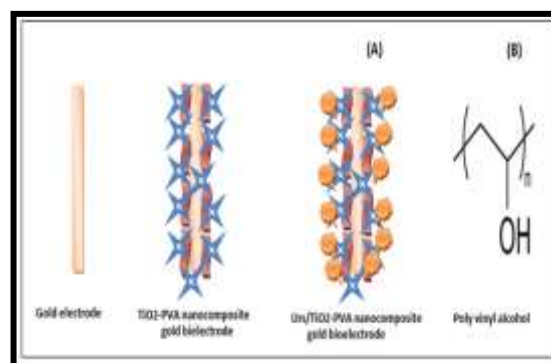
gold electrode then dry this prepared electrode at room temperature.

### Urease immobilization

An important part in biosensor construction is the immobilization of biomolecule on the transducer. Various enzyme immobilization techniques are in used includes encapsulation, entrapment, covalent binding, physical adsorption and cross linking.<sup>26</sup>

In the present work Urs/TiO<sub>2</sub>-PVA nanocomposite gold bioelectrode prepared by physical adsorption method. Urease (1mg/ml solution freshly prepared in Phosphate buffer (ph-7.0) was immobilized onto the surface of PVA-TiO<sub>2</sub>/gold electrode. Prior to immobilization, the Urs/PVA-TiO<sub>2</sub>/Au bioelectrode washed with PBS to ensure uniform spread of urease over the bioelectrode and left to remove any unbound urease and kept at 4°C when not in used.

Schematic diagram for immobilization technique used for development of TiO<sub>2</sub>-PVA nanocomposite gold bioelectrode and Urs/TiO<sub>2</sub>-PVA gold bioelectrode shown in Fig.1 [A].



**Figure 1:** [A] Schematic diagram for immobilization technique used for development of urea biosensor [B] Poly vinyl alcohol (PVA) structure

## RESULTS AND DISCUSSION

### Structural Studies of TiO<sub>2</sub> nanoparticles

#### X-Ray study

Fig.2.[A].shows X-Ray diffraction (XRD) pattern of synthesized TiO<sub>2</sub> nanoparticles reveals reflection planes that are consistent with TiO<sub>2</sub>. Titania samples exist only in rutile phase with tetragonal structure with their characteristics peak of 2θ value 27.492, 36.15, 39.27, 41.32, 44.136, 54.43, 62.88, 64.19 which corresponding Planes (110), (101), (200), (111), (210), (220), (002), (002), (310) respectively were observed which confirmed the formation of rutile phase of TiO<sub>2</sub> (JCPDS-87-0710,21-1276). The crystalline size of TiO<sub>2</sub> calculated using Debye Scherer's formula,

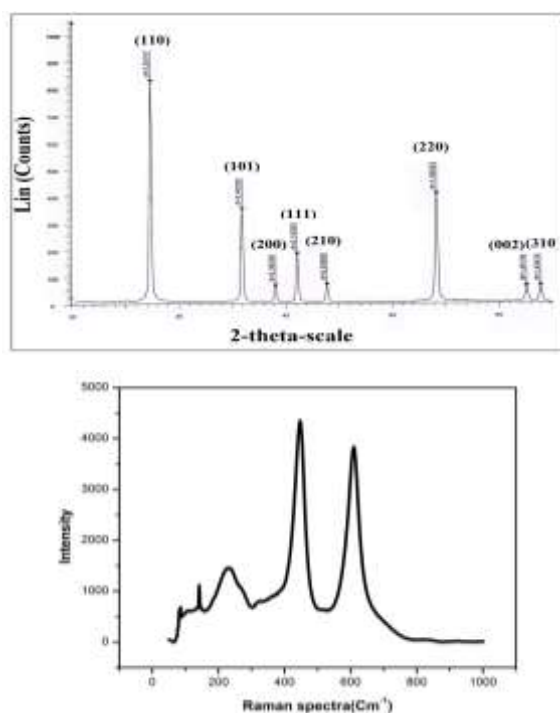
$$D = \frac{K \lambda}{\beta \cos \theta}$$

Where, D is crystalline size, K is usually taken as 0.94, known as Brags constant usually taken as 0.89, λ is wavelength of X-Ray radiation (0.15418nm) for Cu-Kα, β is

full width half maximum of diffraction peak measured at  $2\theta$ . The average particle size of synthesized  $\text{TiO}_2$  nanoparticles was found to be 30 nm.

### Raman spectra of $\text{TiO}_2$ nanoparticles

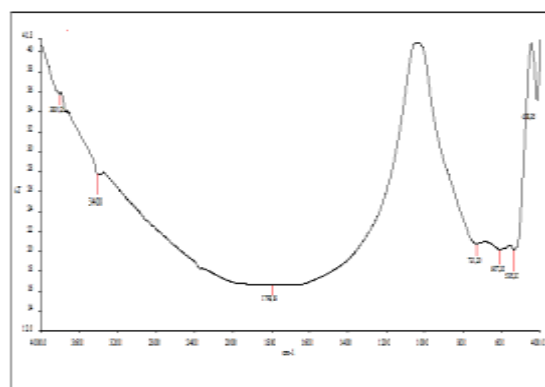
Raman spectroscopy can be thought of as a means to measure the inelastic light scattering which results from the excitation of the vibrations in molecular and crystalline materials all as a function of wave number. Raman scattering is usually from a laser in the visible, near infrared or near ultraviolet region. Excitations in the system are absorbed or emitted by the laser light, resulting in the energy of the laser photons being shifted up or down. The shift in energy gives information about the photon modes in the system. The vibration mode symmetries of the rutile are indicated in Figure.2. [B]. Raman peaks at 144, 235, 443, 610  $\text{cm}^{-1}$  that come from the rutile  $\text{TiO}_2$  are also observed, which means that the coatings were composed of rutile structures at this temperature.



**Figure 2:** A] X-Ray diffraction pattern of  $\text{TiO}_2$  nanoparticles B] Raman spectra diffraction pattern of  $\text{TiO}_2$  nanoparticles

### Fourier transmission infrared spectroscopy (FTIR) spectroscopy

FTIR analysis was used to determine the functional groups of titanium dioxide nanoparticles. In Fig.3. Shows FTIR spectrum of titanium dioxide nanoparticles at wave number range  $4000\text{--}400\text{ cm}^{-1}$ , in which the peaks at  $3400\text{ cm}^{-1}$  due to stretching vibration of the  $\text{--OH}$  group. Another band of around  $1600\text{ cm}^{-1}$  is attributed to the O-H bending mode. The peaks at  $500\text{ cm}^{-1}$ ,  $700\text{ cm}^{-1}$  shows bending and stretching mode of Ti-O-Ti. Peak at  $535.20\text{ cm}^{-1}$  corresponds to vibration bending of Ti-O band in finger print region.

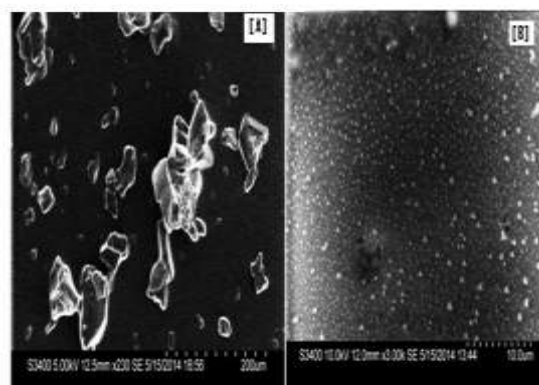


**Figure 3:** FTIR Spectra of  $\text{TiO}_2$  nanoparticles

### Characterization of $\text{TiO}_2$ -PVA nanocomposite gold electrode and Urs/ $\text{TiO}_2$ -PVA/gold bioelectrode

#### Scanning Electron Microscopy

Fig.4.[A],[B] shows results of Scanning electron microscopy (SEM) studies carried out On  $\text{TiO}_2$ -PVA/gold electrode and Urs/ $\text{TiO}_2$ -PVA/gold bioelectrode.  $\text{TiO}_2$ -PVA/gold electrode shows nanoparticles uniformly embedded in porous Polymer network, and after immobilization of Urease  $\text{TiO}_2$ -PVA/gold electrode shows globular homogenous morphology (small ball or clumps) due to presence of enzyme immobilization). This shows that SEM exhibit  $\text{TiO}_2$  has active surface area for enzyme immobilization.



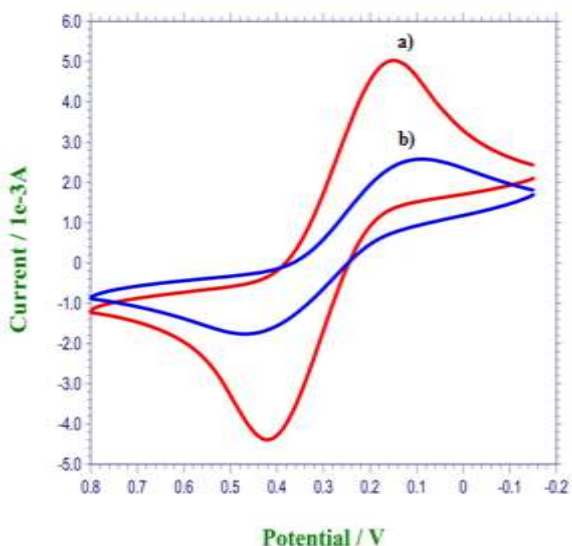
**Figure 4:** Scanning electron microscopy of (A)  $\text{TiO}_2$ -PVA nanocomposite gold electrode (B) Urs/ $\text{TiO}_2$ -PVA gold bioelectrode

### Electrochemical study

#### Cyclic voltametric study

Cyclic voltametric studies have been conducted a)  $\text{TiO}_2$ -PVA nanocomposite gold electrode b) Urs/ $\text{TiO}_2$ -PVA gold bioelectrode at scan rate of  $0.1\text{ V/s}$  using three electrode cell in  $0.1\text{ M KCl}$  solution containing  $5\text{ Mm } [\text{Fe}(\text{CN})_6]^{3-/4-}$  as a mediator is shown in Fig.5, this study carried out at fixed potential range- $0.2$  to  $0.8\text{ V}$ . Well defined Oxidation-Reduction curve are observed. Due to semiconducting behavior of nanostructured  $\text{TiO}_2$  nanoparticles,  $\text{TiO}_2$ -PVA nanocomposite gold electrode gives higher value of current [curve a]. After immobilization of enzyme

(Urease) the current response of Urs/TiO<sub>2</sub>-PVA gold bioelectrode [curve b] becomes decreases this may due to insulating behavior of enzyme on electrode surface which obstructs the electron acceleration produced due to ferro and ferri molecule in 0.1M KCl electrolyte.



**Figure 5:** Cyclic voltamogram of (A) TiO<sub>2</sub>- PVA nanocomposite gold electrode (B) Urs/TiO<sub>2</sub>- PVA gold bioelectrode

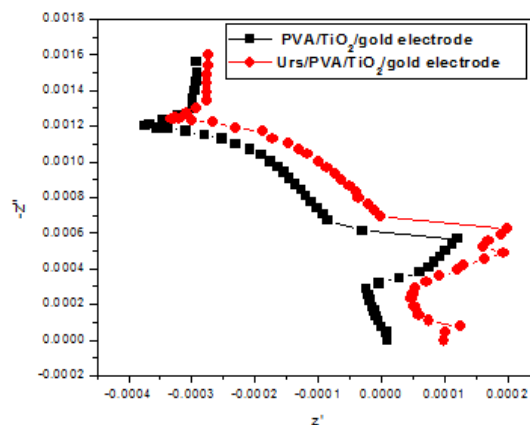
**Impedimetric study**

Electrochemical impedance spectra (EIS) of the various electrodes in KCl (0.1M) containing 5Mm [Fe (CN)<sub>6</sub>]<sup>3-/4</sup> frequency range [42Hz-5MHz] have been studied to evaluate the charge-transfer resistance (Rct) Fig.6. In the EIS semicircular part corresponds to electron transfer limited process and diameter is equal to Rct that controls electron transfer kinetics of redox probe at electrode. The Rct depends on dielectrics and insulating features. The Rct value of TiO<sub>2</sub>-PVA nanocomposite gold electrode and Urs/TiO<sub>2</sub>-PVA gold bioelectrode found to be 5×10<sup>-5</sup>kΩ and 1.4×10<sup>-4</sup> kΩ. The Rct value increases due to enzyme’s intrinsic insulating property which increases film resistance. This is due to insulating nature of enzyme that impedes the charge transfer process and inhibit the fast flow of redox species. Heterogeneous electron transfer (Ke) values of modified electrode can be calculating using equation,

$$k_e = \frac{RT}{n^2 F^2 A R_{ct} C}$$

Where, R is the gas constant (8.314Jmol<sup>-1</sup>k<sup>-1</sup>), T is temperature(298 K), n is the number of electron transfer (1), F is faraday constant (96485 J mol<sup>-1</sup>K<sup>-1</sup>), A is area of electrode (1cm<sup>2</sup>) and C is the concentration of redox species in the electrolyte solution (5Mm), Heterogeneous electron transfer (Ke) values for TiO<sub>2</sub>-PVA nanocomposite gold electrode and Urs/TiO<sub>2</sub>-PVA gold bioelectrode found to be 1.026×10<sup>-8</sup>Cm.s<sup>-1</sup> and 3.66×10<sup>-7</sup> Cm.s<sup>-1</sup>.

High value for bioelectrode shows indicating a faster electron exchange between the redox species.



**Figure 6:** Electrochemical impedance spectra of TiO<sub>2</sub>-PVA nanocomposite gold electrode and Urs/TiO<sub>2</sub>-PVA gold bioelectrode

**Scan rate**

Figure 7 demonstrates typical CV of Urs/TiO<sub>2</sub>-PVA nanobiocomposite gold bioelectrode with scan rate varying from 0.1-0.8 v/s in KCl [0.1M].

With increase in the scan rate, there is increase in both the cathodic and anodic peak currents accompanied with small shift and increased peak-to-peak separation.

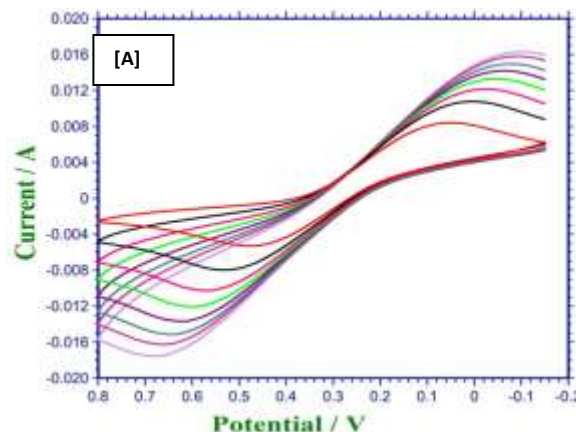
A Proportional increases in redox current with respect to square root of scan rate [Fig.7.B] is observed including diffusion controlled system. Fig inside 7. The surface concentration of Urs/TiO<sub>2</sub>-PVA gold bioelectrode (5.16×10<sup>-4</sup> mol/cm<sup>2</sup>) estimated from plot of I<sub>p</sub> Vs Scan rate (√1/2) using Brown-Anson model equation,

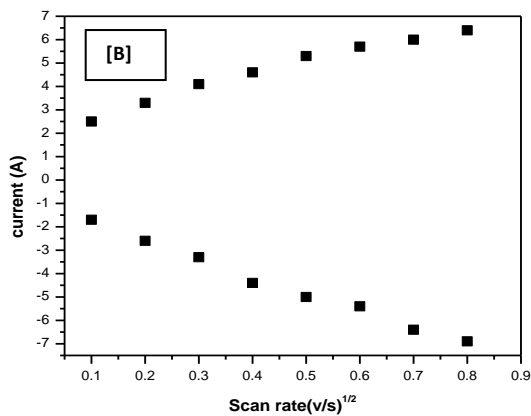
$$I_p = \frac{n^2 F^2 I^* A V}{4RT}$$

Where, n is number of electron transfer (1), F is faraday constant (96485 C ml-1), I\* is surface concentration, A is area of electrode (1cm<sup>2</sup>), R is gas constant (8.314Jmol<sup>-1</sup>k<sup>-1</sup>) is T is temperature (298K), I<sub>p</sub>/V<sup>1/2</sup> is slope of calibration curve. The diffusivity of ions [Fe (CN)<sub>6</sub>]<sup>3-/4</sup> calculated using Randles Sevcik equation

$$I_p = (2.69 * 10^5) n^3 / 2 AD^{1/2} CV^{1/2}$$

The diffusion coefficient of Urs/TiO<sub>2</sub>-PVA nanobiocomposite gold bioelectrode was found to be 1.34 ×10<sup>-3</sup> cm<sup>2</sup>/s.



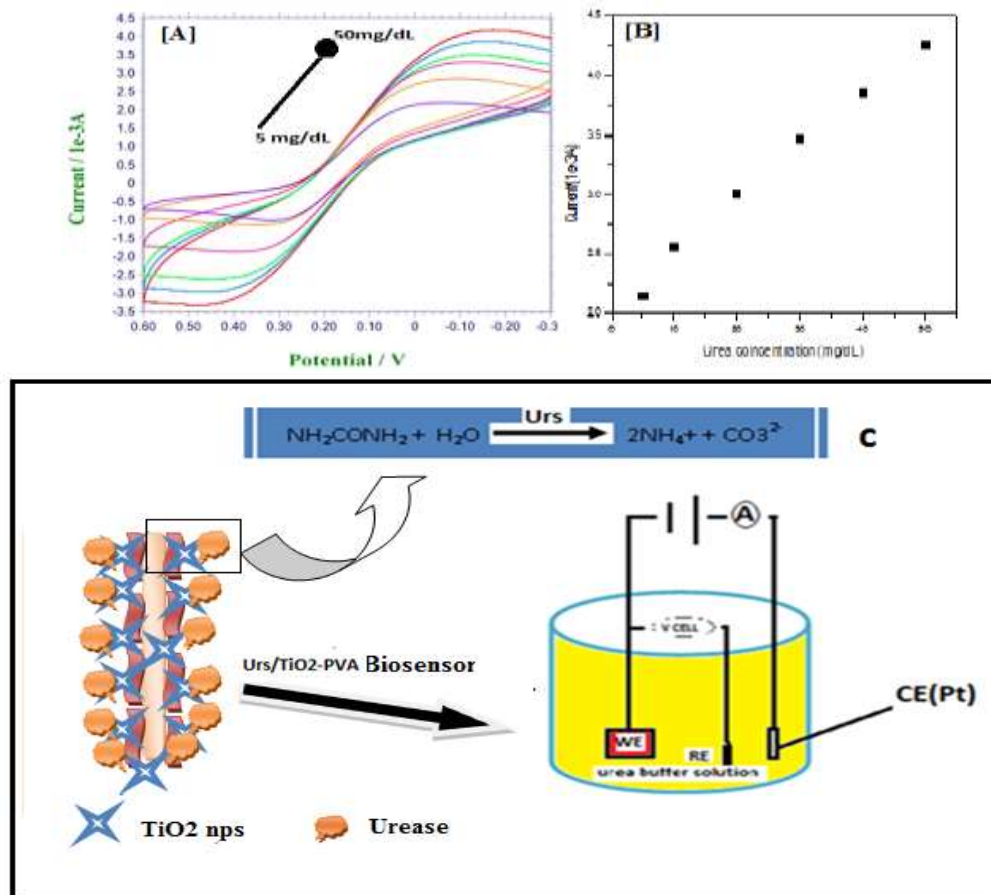


**Figure 7:** [A] CV of Urs/TiO<sub>2</sub>- PVA gold bioelectrode at different scan rates (0.1-0.9V/s) in KCl (0.1M) containing 5mM [Fe (CN)<sub>6</sub>]<sup>3-/4-</sup> [B] Redox current with respect to square root of scan rate

**Response study**

Fig.8. [A] shows CV response of Urs/TiO<sub>2</sub>-PVA nanobiocomposite gold bioelectrode in KCl containing [Fe (CN)<sub>6</sub>]<sup>3-/4-</sup> mediator for varying concentration of urea. It may be seen that peak cathodic current kept on increasing with successive addition of urea and shows good linearity in concentration range [5-50mg/dl] of urea shown in Fig.8. [B]. Upon exposing urea to Urs/TiO<sub>2</sub>-

PVA/gold bioelectrode to urea, an enzymatic reaction takes place between Urs and Urea which can be represented as in Fig. 8. [C]. It results in production of NH<sub>4</sub><sup>+</sup> ion, bicarbonate, and hydroxide ion. The result obtains Cyclic Voltametry supports above sensing mechanism which clearly indicates oxidation reduction process. Fig.8.[B] shows calibration plot between cathodic current and urea Concentration(5-50mg/dL). Sensitivity of prepared Urs/TiO<sub>2</sub>-PVA gold bioelectrode towards urea is found to be 0.045 mA (mg/dL) which is calculated from the slope of plot of cathodic current verses Current. The obtained sensitivity is much higher comparison to corresponding value reported for other nanobiocomposite electrode<sup>27,28,21,29</sup>. The high sensitivity of prepared bioelectrode towards urea is attributed to excellent electron communication property. The limit of detection for Urs/TiO<sub>2</sub>-PVA/gold bioelectrode for urea has been calculated and is found to be 5.8mg/dl, with regression coefficient 0.99. The shelf life of bioelectrode has been monitored by measuring electrochemical current response with regular interval of one week; it is observed that current response decreases to 85% in about 4 weeks. Michalis-Menten constant (K<sub>m</sub>) calculated by Using Line weaver–Burke plot (1/I versus 1/[C]) and K<sub>m</sub> value has been found to be 4.08 mg/dL. This low value shows that a strong affinity between enzyme and substrate.



**Figure 8:** [A] Electrochemical response of Urs-TiO<sub>2</sub>-PVA nanobiocomposite gold bioelectrode at different urea concentration (5-50mg/ dL) [B] Calibration Curve between urea concentration (5-50mg/dl Concentration) [C] Detection mechanism of Urs-TiO<sub>2</sub>-PVA nanobiocomposite gold bioelectrode

**Table 1:** Comparing sensing performance of the TiO<sub>2</sub> nanocomposite based on urea biosensor using with some those reported literature

Bionanocomposite electrode	Sensitivity	Response time	Detection limit	Stability (life time)	references
Urs-PANi-Nafion/Au	4.2 mA Mm <sup>-1</sup> cm <sup>-2</sup>	-	1–10 Mm		<b>27</b>
Urs-GLDH/ZrO <sub>2</sub> -CH-ITO	0.07 (uAmM <sup>-1</sup> CM <sup>-2</sup> )	10s	0.8	-	<b>28</b>
Electrodeposited ZnO-PVA hybrid film	0.144 (kΩ/mgdl <sup>-1</sup> )	3s	3mg/dL	2month	<b>21</b>
Urs-GLDH/Fe <sub>3</sub> O <sub>4</sub> -CH-ITO	12.5 (uAmM <sup>-1</sup> CM <sup>-2</sup> )	10s	2 mg/dL	10 weeks	<b>29</b>
Urs/TiO <sub>2</sub> -PVA/gold electrode	0.045 mA (mg/dL)	2S	5.8mg/dL	1month	<b>Present work</b>

## CONCLUSION

As a result, the performance of biosensor depends on the amount of immobilized enzyme. Nanostructured TiO<sub>2</sub> (30 nm) was prepared by sol-gel technique. XRD and Laser Raman studies show that nanostructured TiO<sub>2</sub> is in rutile phase. Urease has been immobilized on TiO<sub>2</sub>-PVA nanocomposite electrode via physical adsorption method. Surface characterization shows perfect immobilization of urease enzyme and TiO<sub>2</sub> has active surface area for enzyme immobilization. Decreased in voltage-current characteristics of Urs/TiO<sub>2</sub>-PVA gold bioelectrode due to insulating characteristics of enzyme. Response study of Urs/TiO<sub>2</sub>-PVA gold bioelectrode at different urea concentration (5-50mg/dL) shows high sensitivity 0.045 mA (mg/dL), detection limit 5.8mg/dL with regression coefficient 0.99. Michaelis-Menten constant (K<sub>m</sub>) calculated by Using Line weaver–Burke plot (1/I versus 1/[C]) and K<sub>m</sub> value has been found to be 4.08 mg/dL. This is attributed to good electrochemistry behavior of bioelectrode film with excellent sensitivity. This suggests that TiO<sub>2</sub>-PVA matrix provides friendly environment for enzyme.

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