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Full Length Article

Electroenzymatic sensing of urea using CP/MWCNT-inulin-TiO_2 bioelectrode

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ABSTRACT

The present study is aimed at developing a sensitive biosensor for urea by the incorporation of MWCNT-Inulin-TiO_2 and urease (Ur) onto carbon paste matrix and to propose a suitable mechanism of interaction. Multi-Walled Carbon nanotubes (MWCNTs) were considered as a novel nanomaterial owing to their unique properties but their hydrophobicity limit their applications. A successful strategy to improve their hydrophilicity is to modify MWCNTs with biopolymers. Inulin (Inu), a versatile carbohydrate polymer was extracted from *Allium sativum* L. and used for anchoring TiO_2. The prepared Inu-TiO_2 composite was used to modify MWCNTs in order to synergistically improve the stability and the biocompatibility. The MWCNT-Inu-TiO_2 bio-nanocomposite was characterized by UV–vis, FT-IR, micro-Raman, XRD, SEM, TEM and TGA techniques. Differential Pulse Voltammetric (DPV) studies were carried out to show the sensitivity of the fabricated CP/MWCNT-Inu-TiO_2/Ur bioelectrode towards urea. The fabricated bioelectrode exhibited wide linear range (0.083 mM to 25 mM) with low detection limit (0.9 μM), good sensitivity (29.6 mA mM^{-1} cm^{-2}), high storage stability (120 days) and good selectivity (in presence of biointerferences like ascorbic acid, uric acid, glucose, lactose, Na^+ and K^+). The fabricated bioelectrode was also effectively applied for the sensing of urea as an adulterant in milk samples.

1. Introduction

In the present scenario, sensitive and selective determination of biologically important biomolecules such as glucose, urea, creatinine etc., is required for clinical diagnostics, environmental monitoring and food toxicology. Among them urea is widely distributed in nature and is present in blood and various other fluids like milk, urine etc. Milk is considered to be the major source of nutrition and is consumed by adults and children all over the world. As a regular practice, water is added to milk in order to increase the volume of milk. Adulteration of milk is quite common in both developed and developing countries. Some of the common adulterants in milk include ammonium sulphate, boric acid, caustic soda, formalin, hydrogen peroxide, melamine, sugar, urea etc., Such adulterants are harmful to human consumption and hence both qualitative and quantitative determination need to be carried out. In order to evaluate the quality of milk, certain common parameters viz., percentage of fat, percentage of solid-not-fat (SNF), protein content etc., will be tested. Normally adulterants are added to increase these parameters. Urea, although a natural constituent of milk, commercially available urea is added in order to enrich the milk with false protein content which results in lowering the nutritive value of milk [1].

According to Food Safety and Standards Authority of India (FSSAI) and Prevention of Food Adulteration (PFA) acts, the upper allowable limit of urea in milk is 70 mg/100 ml [2,3].

If this level exceeds, it causes adverse effects to human health. The excessive intake of milk adulterated with urea causes overburdening of kidneys as they need to filter excess urea from the body [4]. The normal concentration of urea in human serum is 1.7 to 8.3 mM (15–40 mg/dl) [5] and the level may rise up to 100 mM (602 mg/dl) under pathophysiological conditions [6]. The increased level of urea in blood causes burns, shock, urinary tract obstruction, renal failure etc., whereas a lower concentration of it leads to hepatic failure, nephrotic syndrome etc. [7]. The measurement of urea in blood and urine is the best way to determine the normal functioning of kidney and to detect kidney related diseases. Kidney related disorders are ranked third among the other life-threatening diseases, next to cancer and cardiac ailments. Hence accurate monitoring of urea in the biological samples becomes important [8].

Qualitative analysis of urea in milk samples are based on simple colour reactions with available chemical reagents. Such methods are valid only for a limited range of concentration and

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are not precise although they are simple, fast and easy to perform. Other quantitative methods like Near Infrared Raman (NIR) spectroscopy [9], Liquid Chromatography (LC) [10], GC/IDMS (Gas Chromatography/Isotope Dilution Mass Spectrometry [11], High Performance Liquid Chromatography (HPLC) [12], colorimetry and fluorimetry [13], potentiometry [14], spectrophotometry [15] etc, are employed for the determination of urea. Such methods suffer from serious disadvantages like complicated pretreatment steps, lack on-site monitoring etc. Therefore detection of urea through electrochemical method is highly adopted and versatile as it not only helps in the sensitive determination of urea but also provide an insight into the interaction of urea with the nickel (II) ions present in the active site of urease enzyme [16]. In the development of electrochemical urea biosensors, immobilization of urease (EC 3.5.1.5.) over modified electrodes is the key parameter which decides the sensitivity and reproducibility of the sensor. Recently, carbon based materials like Carbon nanotubes (CNTs) are extensively used since their discovery due to their remarkable electrical, chemical, mechanical and structural properties. In order to take advantages of these properties and to improve their solubility, the agglomeration of CNTs need to be prevented through suitable modification. At present composite materials that combine the properties of the individual components were emerged to improve the performance of CNTs thereby enhancing their utility as biosensing material for the sensitive determination of biologically active components [17–20].

In the present study, Inulin (Inu), a newly emerged electroactive carbohydrate polymer incorporated with TiO_2 (a biocompatible, non-toxic and a prospective interface for the immobilization of biomolecules) [21] was used to improve the solubility and the biocompatibility of Multi-Walled CNTs (MWCNTs). The MWCNT-Inu-TiO_2 bio-nanocomposite so prepared was suitably incorporated along with the urease enzyme into the carbon paste (CP) matrix and was used for the electrochemical sensing and quantification of urea in milk samples.

2. Experimental

2.1. Chemicals

MWCNT (SRL) with O.D 8–15 nm and length 10–30 μm, rutile-TiO_2 (SRL) (ultra-pure nanopowder, APS 250 nm), urease (Ur) (50,000–1,00,000 units) (SRL) from Jack beans, urea (Merck), dextrose (Merck), lactose (Merck), ascorbic acid (Merck) and uric acid (Loba Chemie) used for the present study were of analytical grade. Graphite fine powder (Loba Chemie) and high viscosity paraffin oil (density = 0.88 g/cm^3, Merck) was used as the binding agent for the preparation of the carbon paste electrodes. Phosphate buffer solutions were prepared from KH_2PO_4 (Loba Chemie) and K_2HPO_4 (Fischer Scientific) in the pH range 4.0–9.0.

The stock solutions of urea (1M), dextrose (0.1 M), lactose (0.1 M) ascorbic acid (0.1 M) and uric acid (0.1 M), NaCl (0.1 M) and KCl (0.1 M) were prepared by using millipore water. Urease (5 mg/ml) was prepared in phosphate buffer (pH 7) solution.

2.2. Instrumental techniques

The absorption spectra of the samples were recorded using Thermo Scientific Helious Alpha UV–vis spectrophotometer in the wavelength range of 200–800 nm. Fourier transform infrared spectra were obtained using IR Affinity- I Fourier transform Infrared spectrophotometer, Shimadzu in % transmittance mode covering the wave number between 400 and 4000 cm^{-1}. Spectroscopy grade KBr was used as the window material. The prepared samples were mixed with dried KBr, and pelletized. The surface morphology of the samples were examined by Scanning Electron Microscopy (SEM) (VEGA 3 TESCAN). Lyophilizer (Christ Alpha 1–2 LD plus, Fisher Bioblock Scientific, Germany) was used to freeze dry the sample to get the dry powder. The electrochemical measurements were performed with an electrochemical workstation (CHI 6063 C Electrochemical analyzer). A three-electrode cell was constructed, including platinum wire as counter electrode, Ag/AgCl (with saturated KCl) as reference electrode and the modified electrode as the working electrode. A digital pH meter was used to measure the pH of the solutions. All experiments were carried out at room temperature. Differential pulse voltammetric studies were carried out in the potential range from −0.8 V to 0.5 V.

2.3. Preparation of MWCNT- inu- TiO_2 bio-nanocomposite

The protocol reported by the authors [21] was employed for the extraction of inulin from garlic and the detailed procedure was described as follows. About 100 g of *Allium sativum* L. (Garlic) bulbs were crushed and blended well. The crushed garlic was extracted with 150 ml of hot millipore water and filtered through muslin cloth. The pH of the filtrate was increased to 8 by adding 0.1 M calcium hydroxide. The solution was allowed to stand for an hour. The residue formed at this stage was removed and to the resulting filtrate 0.8 M HCl was added drop wise to decrease the pH to 7. The filtrate was then concentrated. The yield of the concentrate was found to be 16%. It was then refrigerated and lyophilized to get the dry powder. The lyophilized Inu (yield: 14.2%) was then stored at 4 °C for further use.

Inu-TiO_2 bio-nanocomposite was prepared by solvent casting method [21]. 1g of Inu in 20 ml of deionized water was magnetically stirred (10 min) to ensure complete dissolution. 0.5 g of TiO_2 (33 wt % with an APS of 250 nm) was added to the Inu solution and magnetically stirred for 15 min. Subsequently the mixture was sonicated for 15 min by ultra-waving in a water bath to improve the dispersion of TiO_2 nanoparticles in the polymer matrix. Equal volume of ethanol was added to the homogenous mixture and the formed composite was separated by using Buchner funnel. It was dried and refrigerated for future use. The yield of the composite was found to be 96%.

Modification of MWCNTs was done by solution processing wherein MWCNTs are generally dispersed in a solvent and then mixed with polymer solution by ultrasonication. In the present study ultrasonication technique is employed for the modification of MWCNTs as it causes effective dispersion of the MWCNTs. The choice of solvent is generally made based on the solubility of the polymer. As the biopolymer Inu is water soluble, the solvent used for the dispersion of MWCNTs is water. 1 g of MWCNTs and 0.5 g Inu-TiO_2 were separately mixed with 5 ml of millipore water and subjected to ultrasonication for about 5 min. The Inu-TiO_2 suspension was added slowly to the MWCNT dispersion and ultrasonication was continued for 15 min. MWCNT-Inu-TiO_2 bio-nanocomposite can then be obtained by evaporating the solvent.

2.4. Fabrication of the biosensor

The CP/MWCNT-Inu-TiO_2/Ur electrode was prepared by hand mixing 0.03 g of MWCNT-Inu-TiO_2 with 0.67 g graphite powder, 0.3 g of paraffin and 20 μl of Ur with a mortar and pestle for 10 min until a uniformly wetted paste was obtained. The paste was then packed into the end of a glass tube (3 mm diameter and 7 cm length). A copper wire was inserted for providing electrical contact. Bare CP, CP/Inu, CP/TiO_2, CP/Inu-TiO_2, CP/MWCNT, CP/MWCNT-TiO_2, CP/MWCNT-Inu, CP/MWCNT-Inu-TiO_2 electrodes were also prepared by the same procedure and used for comparison of electrocatalytic activity of the modified electrode.

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