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A highly efficient urea detection using flower-like zinc oxide nanostructures

Manvi Tak [a], Vinay Gupta [a], Monika Tomar [b,*]

[a] Department of Physics and Astrophysics, University of Delhi, Delhi 110007, India
[b] Department of Physics, Miranda House, University of Delhi, Delhi 110007, India

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ABSTRACT

A novel matrix based on flower-like zinc oxide nanostructures (ZnONF) has been fabricated using hydrothermal method and exploited successfully for the development of urea biosensor. Urease (Urs) is physically immobilized onto the ZnO nanostructure matrix synthesized over platinized silicon substrate. The surface morphology and crystallographic structure of the as-grown ZnONF have been characterized using a scanning electron microscope (SEM) and X-ray diffraction (XRD) techniques. The fabricated amperometric biosensor (Urs/ZnONF/Pt/Ti/Si) exhibits a linear sensing response towards urea over the concentration range 1.65 mM to 16.50 mM with an enhanced sensitivity (~132 μA/mM/cm^2) and a fast response time of 4 s. The relatively low value of Michaelis–Menten constant (K_m) of 0.19 mM confirms the high affinity of the immobilized urease on the nanostructured ZnONF surface towards its analyte (urea). The obtained results demonstrate that flower-like ZnO nanostructures serve as a promising matrix for the realization of efficient amperometric urea biosensor with enhanced response characteristics.

1. Introduction

Recently, need for a continuous real time monitoring of toxic chemicals present in the environment known as pollutants has drastically increased. A lot of pollutants have been found at various stages during recycling and industrial processes; at agricultural, urban and industrial sites and in waste waters and effluents. Hence, for environmental monitoring, there is demand for techniques possessing fast-response, portability, high sensitivity, robustness and high shelf-life, which can be exhibited by the electrochemical methods for detection. There are several reports found in literature related to the environmental monitoring using electrochemical analysis, for e.g., Parvan et al. have reported results related to detection of hydroxylamine in water samples using electrochemical detection [1]. Not only in environmental but also in pharmaceuticals and health care industries as well as in clinical diagnosis field, electrochemical detection method has gained considerable attention. There are reports available in literature related to the detection of important drugs and chemicals for human body such as norepinephrine, N-acetylcysteine, epinephrine, isoproterenol and levodopa using electrochemical method [2–9]. This method has also been used for detecting a number of important biomolecules such as glucose, uric acid, cholesterol and urea present in the human body.

Urea is one of the important biomolecules present in the human body. It is produced in the human body from ammonia as a result of urea cycle or by oxidation of amino acids and is excreted out in urine. The optimum amount of urea in human blood/serum is 8–20 mg dL^{-1} and any discrepancy in its normal level may be dangerous [10]. Hence, its continuous monitoring is of utmost importance for the clinical purpose. The detection of urea is crucial not only in clinical diagnosis but also in other fields including agriculture and food science [10]. Thus, there has been a growing demand for the estimation of urea. Various methods are available for urea detection such as gas chromatography, calorimetry, fluorimetry, conductometry, inductometry and ion sensitive field effective transistors [11–15]. However, these techniques suffer from complicated sample pre-treatment steps and involve expensive instrumentation [11–15]. Electrochemical biosensors serve as an efficient alternative to overcome some of these problems. Few reports are available in literature aiming towards the development of the potentiometric urea biosensor with application of pH sensitive electrode or an ion selective electrode [16]. For instance, Ansari et al. have studied the effect of various ZnO nanostructures prepared by sol–gel technique on urea detection [17]. Though the reported biosensing results show wide detection range of urea but the sensitivity is very low and is not suitable for practical applications. Furthermore, the stability and interference related concerns have not been considered which are amongst the most essential characteristics required in assessing the performance of the biosensor [17]. Hence, limited success has been obtained on the fabrication of potentiometric urea biosensor, where poor selectivity as

* Corresponding author.
E-mail address: monikatomar@gmail.com (M. Tomar).

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well as sensitivity has always been an issue of major concern. Alternatively, amperometric technique owing to its high sensitivity, selectivity and reproducibility eliminates such problems and so, has been the most preferred detection technique for various biomolecules [18].

A key parameter in fabrication of the amperometric biosensor is the identification of a suitable matrix which not only provides fast electron communication feature but also offers high surface area for high enzyme loading [19–24]. Amongst various matrices, nanostructured metal oxides are extensively exploited due to their unique properties including high surface to volume ratio, good stability, and favorable orientation for the immobilization of specific enzyme [24–28]. Recently, ZnO nanostructures have been utilized as an efficient matrix in the field of biosensing for detecting a number of biomolecules due to its good electron communication feature, wide band-gap, high chemical stability, good biocompatibility and most importantly high isoelectric point (IEP ~ 9.5) which is important for the immobilization of enzymes having low IEP such as urease (~4.0) by physical adsorption technique [21,29,30]. However, there have been very few reports in literature on the utilization of ZnO nanostructures for detecting urea using amperometric technique. For instance, there is a report by Ali et al. based on the fabrication of urea sensor using nanostructured zinc oxide film but the fabricated biosensor exhibits very low sensitivity (1.44 μA mM^{-1} cm^{-2}) as well as increased sensor cost due to utilization of double enzymes (i.e. urease and glutamate dehydrogenase) [31]. Another report by Palomera et al. is based on the fabrication of urea biosensor using ZnO nanorods which give a wide linear response for urea detection (i.e. 1–20 mM) and low detection limit (0.13 mM) but the sensitivity is very poor (0.40 μA mM^{-1} cm^{-2}) [32]. Hence, low sensitivity and stability are the main concerns which make these reported urea biosensors unsuitable for any practical commercial application thus giving a lot of scope for further improvement.

The sensitivity may be enhanced by improving the nanostructure morphology (different shapes) of the matrix for effective loading of the enzyme. On the other side the stability of biosensor depends on the quality of the matrix which in turn depends on its growth kinetics.

In order to improve these parameters, it is important to note that the grown nanostructures should be crystalline as high crystallinity results in larger grain size and reduced grain boundaries which promotes fast communication of the electrons and also provides better stability to the nanostructured bioelectrodes. Hydrothermal method is a well-known method for the synthesis of ZnO nanostructures of very high crystallinity as well as purity. Besides this, it is a comparatively easier, quicker and economical method for the nanostructure fabrication. Hence, looking at the advantages offered by the amperometric electrochemical detection technique and the hydrothermally route for nanostructure synthesis, an effort has been made in the present work to realize a simple electrochemical urea biosensor using ZnO nanostructures possessing flower-like morphology. Hence, the main emphasis is towards the development of hydrothermal assisted flower-like ZnO nanostructure matrix for effective loading of enzyme with fast electron communication features. Such matrix is expected to give enhanced response characteristics towards urea. To the best of our knowledge no report yet has been available in literature based on the electrochemical detection of urea utilizing presently fabricated unique nanostructure morphology bearing flower-like ZnO nanostructures.

2. Experimental

2.1. Materials

Urea, Urease (Urs) (Type (iii) from Jack Beans) procured from Sigma, USA. Zinc acetate dihydrate ($Zn(C_2H_3O_2)_2 \cdot 2H_2O$, 99.0%), Monoethanolamine (MEA) ($CH_2(OH)CH_2NH_2$, 99.0%), Methanol (CH_3OH, 99.0%), Zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$, 99.0%) were purchased from Sigma-Aldrich, USA. Hexamine (HMTA) ($C_6H_{12}N_4$, 99.0%) was purchased from Titan Biotech Ltd., India. Sodium phosphate monobasic anhydrous and sodium phosphate dibasic dihydrate were obtained from Sisco Chemicals, India. All chemicals were used without further purification. 50 mM phosphate buffer saline (PBS), pH 7.0 (0.9% NaCl) solution was prepared by adjusting the proportion of monobasic sodium phosphate solution and dibasic sodium phosphate solution (Sisco Chemicals) and then adding 0.9% NaCl to the solution. Deionized (DI) water (resistivity ~ 18.2 MΩ-cm) was used for the preparation of aqueous solutions.

2.2. Synthesis of flower-like ZnO nanostructures

ZnO nanostructures have been synthesized by using a seeding layer of ZnO prepared by sol–gel followed by the hydrothermal growth process and is discussed in the following sections:

(i) Preparation of ZnO seeding layer: sol–gel method0.375 M zinc acetate solution was prepared in methanol at room temperature. MEA, a sol stabilizer, was added keeping 1:1 molar ratio of zinc acetate to MEA making the solution for seeding. The seed solution was stirred at 60 °C yielding a clear, transparent and homogeneous solution which was aged for 24 h. The solution, thus obtained, was spin coated onto Platinum (Pt) coated silicon substrate. A thin film of Pt (~100 nm) was coated on the Si substrate by RF sputtering technique using a metal Pt (99.99% pure) target in 100% Ar gas atmosphere at 10 mTorr sputtering pressure. A thin buffer layer of Ti (~20 nm) was sputter coated by sputtering prior to Pt deposition under the same deposition conditions to improve the adhesion of Pt on the Si substrate. Subsequently, the spin coated ZnO thin film was baked at 300 °C to remove residual solvents. The foregoing process of spin-coating and thereafter baking was repeated several times to obtain ZnO seeding layer of desired thickness.

(ii) Growth of flower-like ZnO nanostructures: hydrothermal processZnO nanostructures were synthesized on the seed layer using hydrothermal process at 90 °C for 4 h. For controlling the dimensions of the nanostructures, hydrothermal growth of ZnO nanostructures was carried out in various concentrations (0.005 M, 0.01 M and 0.02 M) of the zinc nitrate and HMTA equimolar aqueous solutions. The substrates were then removed from the solution followed by thorough washing with deionized water and then dried in air at room temperature.

2.3. Urea bioelectrode fabrication

20 μL of freshly prepared urease solution (1 mg/mL, in 50 mM PBS, pH 7.0) was immobilized onto the surface of ZnO nanostructure based electrode by physical adsorption and kept for 5–6 h for drying at room temperature. At the physiological pH of ~7.0, flower-like ZnO nanostructures having a relatively high isoelectric point (IEP ~ 9.5) behaves as a positively charged matrix which can have a strong interaction with molecules having low IEP like urase (IEP ~ 4.0) when immobilized on its surface. Urease is immobilized on the surface of ZnO nanostructured matrix by physical adsorption technique and a strong electrostatic interaction between them binds and the urease molecules on the surface of flower-like ZnO nanostructured matrix. Finally, the prepared bioelectrode was stored at 4 °C overnight followed by extensive washing with a PBS buffer to remove any unbound enzyme. The bioelectrode was stored at 4 °C when not in use. Different concentrations of urea solutions were prepared in DI water. The detailed schematic for the fabrication of the flower-like ZnO nanostructure based bioelectrode is shown in Scheme 1.

2.4. Biosensor characterizations and electrochemical measurements

The surface morphology and crystallographic structure of the prepared ZnONF matrix were characterized by scanning electron