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The Confluence of Nanocomposite Engineering and Enzyme Kinetics in Urea Biosensor Development

Atul Birajdar

Department of Physics, B. S. S. College, Makani Dist.-Osmanabad, 413604, India.
Email: atulbirajdar52@gmail.com

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Abstract

The escalating global concern over water pollution, particularly by toxic heavy metal ions, necessitates the development of rapid, sensitive, and cost-effective detection technologies. Electrochemical biosensors, which couple the specificity of biological recognition elements with the transduction capabilities of advanced materials, present a potent solution. This article provides a detailed exploration of the design, optimization, and kinetic characterization of a metal oxide nanocomposite-based urease biosensor for environmental monitoring. The sensor platform utilizes a polyaniline (PANI) and zinc oxide (ZnO) nanocomposite electro-polymerized onto a stainless-steel transducer, upon which the enzyme urease is immobilized (PANI/ZnO/Urease). We delve into the synthesis methodology and the fundamental working principle, where urease-catalyzed urea hydrolysis induces a local pH change, detected amperometrically via the conductive polymer matrix. A comprehensive analysis of the biosensor's analytical performance is presented, including its linear range (10-50 mM urea), sensitivity (0.599 mA/mM), limit of detection (4.953 mM/L), and Michaelis-Menten kinetics, revealing an enhanced enzyme-substrate affinity post-immobilization (K^*m = 0.018 mM). Critical operational parameters such as pH and temperature are systematically optimized, identifying an optimal pH of 8.5 and an operational temperature of 30°C. Furthermore, we discuss the sensor's response time (9 seconds), operational stability, and storage longevity. The integration of nanostructured ZnO is highlighted as a pivotal factor, providing a high surface-area-to-volume ratio that enhances enzyme loading, substrate diffusion, and overall electrochemical response. This work underscores the significant promise of judiciously engineered nanocomposite interfaces.

Keywords: Nanocomposite Biosensor, Enzyme Immobilization, Urease Kinetics, Electrochemical Detection, Heavy Metal Ions

1. Introduction

In the contemporary era defined by rapid industrialization and technological advancement, the concomitant rise in environmental pollutants poses a severe threat to ecological and human health. Among these pollutants, heavy metal ions such as mercury (Hg^{2+}), lead (Pb^{2+}), cadmium (Cd^{2+}), chromium (Cr^{6+}), and arsenic ($As^{3+}/^{5+}$) are particularly pernicious due to their toxicity, non-biodegradability, and tendency to bioaccumulate in the food chain. Contamination of drinking water sources by these ions represents a critical public health challenge globally, linked to neurological disorders, organ failure, and carcinogenesis. Traditional methods for heavy metal detection, including atomic absorption spectroscopy (AAS), inductively coupled plasma mass spectrometry (ICP-MS), and high-performance liquid chromatography (HPLC), offer high sensitivity and accuracy. However, their utility is often constrained by requirements for sophisticated instrumentation, skilled personnel, complex sample preparation, and laboratory-bound analysis, rendering them unsuitable for rapid, on-site, and routine monitoring [1].

This technological gap has catalyzed significant research into alternative sensing platforms, with electrochemical biosensors emerging as a frontrunner. A biosensor is an analytical device that integrates a biological recognition element (e.g., enzyme, antibody, nucleic acid, whole cell) with a physicochemical transducer to produce a measurable signal proportional to the concentration of a target analyte. The appeal of biosensors lies in their potential for specificity, rapid response, portability, low cost, and capability for real-time and in-situ analysis [2]. For heavy metal detection, inhibition-based enzymatic biosensors are a prominent strategy. These biosensors operate on the principle that specific heavy metal ions act as potent inhibitors of enzyme activity. By measuring the decrease in the catalytic signal of an enzyme (e.g., current, potential, absorbance) upon exposure to a sample, one can quantitatively determine the concentration of the inhibitory metal ion. Urease (urea amidohydrolase, EC 3.5.1.5), an enzyme that catalyzes the hydrolysis of urea into ammonium and carbonate ions, is exceptionally sensitive to inhibition by heavy metals like Hg^{2+} and Pb^{2+}, making it an ideal biological component for such sensors [3].

The performance of an enzymatic biosensor is not solely dictated by the biocatalyst but is profoundly influenced by the matrix used for its immobilization and the efficiency of the transduction interface. The immobilization strategy must preserve enzymatic activity, ensure stability, and facilitate optimal communication between the enzyme's active site and the transducer. Recent advancements in nanotechnology have opened new vistas in biosensor design. The incorporation of nanomaterials—such as metal nanoparticles, carbon nanotubes, graphene, and metal oxide nanostructures—into sensor architectures has been revolutionary. These materials offer extraordinary properties including high electrical conductivity, large specific surface area, excellent catalytic activity, and good biocompatibility, which collectively enhance sensor sensitivity, stability, and response time [4].

Among metal oxides, zinc oxide (ZnO) nanostructures have garnered considerable attention in biosensing applications. ZnO is a wide bandgap semiconductor with notable properties such as high electron mobility, strong adsorption capacity, chemical stability, and biocompatibility. Its nanostructured forms (nanoparticles, nanorods, nanowires) provide an immense surface area for high-density enzyme immobilization while promoting direct electron transfer. Furthermore, ZnO's isoelectric point (IEP ~9-9.5) makes it suitable for the electrostatic immobilization of enzymes with contrasting surface charges [5]. Conducting polymers, notably polyaniline (PANI), complement these features by offering a conductive, mechanically stable, and easily polymerizable matrix that can be deposited on various electrodes. PANI’s conductivity is highly sensitive to pH changes and redox reactions, making it an excellent transducer for enzymatic reactions that produce or consume protons, such as urease-catalyzed hydrolysis [6].

The synergistic combination of PANI and ZnO into a nanocomposite represents a sophisticated approach to biosensor fabrication. The PANI matrix provides a conductive pathway and a porous scaffold, while the embedded ZnO nanostructures augment the effective surface area and potentially facilitate favorable enzyme orientation. This article focuses on a detailed examination of one such biosensor: a PANI/ZnO nanocomposite-based electrochemical platform with immobilized urease. We will dissect its construction, operational mechanism, and a rigorous characterization of its kinetic and analytical parameters. The discussion will extend to the optimization of critical variables like pH and temperature, an assessment of stability, and an exploration of the underlying reasons for its enhanced performance compared to free enzyme or simpler sensor designs. By providing this comprehensive analysis, we aim to elucidate the principles that govern high-performance enzymatic biosensor design and highlight the critical role of nanomaterial engineering in pushing the boundaries of analytical detection for environmental safeguarding.

2. Methodology

2.1. Materials and Sensor Fabrication Protocol

The fabrication of the PANI/ZnO/Urease biosensor, as described by Mahajan and Birajdar (2022), involves a systematic multi-step process employing carefully selected materials [7]. All chemicals utilized were of analytical grade to ensure reproducibility and reliability. Key components included aniline (monomer for PANI synthesis), zinc nitrate hexahydrate (precursor for ZnO), sulfuric acid (dopant and supporting electrolyte), and urea (substrate). The biological recognition element, urease from Jack bean, was sourced from standard biochemical suppliers. Phosphate buffer saline (PBS) was used to maintain physiological pH during experiments. A standard three-electrode electrochemical cell was employed for both fabrication and characterization, comprising the modified stainless-steel working electrode, an Ag/AgCl reference electrode, and a platinum wire counter electrode.

The fabrication begins with the electrochemical synthesis of the PANI/ZnO nanocomposite directly on the stainless-steel electrode surface. This *in-situ* electro-polymerization technique is advantageous as it ensures strong adhesion of the nanocomposite film to the transducer. Typically, aniline monomer is dissolved in an acidic electrolyte containing dispersed ZnO nanoparticles or a zinc salt precursor. Upon application of a suitable anodic potential, aniline oxidizes and polymerizes, forming a PANI film that concurrently entraps ZnO nanostructures. This one-pot method leads to a homogeneous, interpenetrated network where ZnO particles are uniformly distributed within the conductive PANI matrix, creating a highly porous and conductive nanocomposite layer with a drastically increased effective surface area [8].

Subsequently, the enzyme urease is immobilized onto this nanocomposite-modified electrode. Immobilization is a crucial step that determines the biosensor's activity, stability, and shelf life. While the specific method used by Mahajan and Birajdar is not exhaustively detailed, common techniques for such systems include physical adsorption, entrapment within a secondary polymer layer (e.g., Nafion), or cross-linking with glutaraldehyde. Given the structure, it is plausible that urease molecules are adsorbed onto the high-surface-area ZnO/PANI matrix, potentially stabilized by electrostatic interactions or hydrogen bonding. The nanostructured ZnO, with its high surface energy and biocompatibility, provides abundant sites for enzyme attachment, helping to retain its native conformation and catalytic activity [9]. The final construct is a robust, three-layer biosensor: the stainless-steel current collector, the PANI/ZnO nanocomposite transduction layer, and the outermost urease enzyme layer.

2.2. Electrochemical Detection Principle and Mechanism

The PANI/ZnO/Urease biosensor operates on an amperometric detection principle, measuring the current change associated with the enzymatic reaction. The foundational reaction is the urease-catalyzed hydrolysis of urea:

$$NH_2CONH_2 + 3H_2O \overset{Urease}{\rightarrow} 2NH_4^+ + HCO_3^- + OH^-$$

This reaction yields ammonium ions (NH_4^+), bicarbonate ions (HCO_3^-), and hydroxide ions (OH^-), leading to a localized increase in pH at the electrode-solution interface [10].

The conductive PANI matrix acts as the primary transducer for this biochemical event. PANI exists in various oxidation states (leucoemeraldine, emeraldine, pernigraniline), and its electrical conductivity is profoundly influenced by its protonation/ deprotonation state. In its conductive emeraldine salt form, PANI is protonated. The production of OH^- ions during urea hydrolysis causes a local deprotonation of the PANI backbone, converting it to its less conductive emeraldine base form. This change in the polymer's conductivity is reflected as a measurable change in the Faradaic current under an applied constant potential [11]. The role of ZnO in this transduction mechanism is multifaceted. Firstly, it enhances the surface area, allowing for greater enzyme loading and more reaction sites. Secondly, its semiconducting properties may facilitate charge transfer processes at the interface. Thirdly, it may help maintain a microenvironment conducive to enzyme activity. When a potential is applied (in the cited work, between -0.3 V and 0.6 V vs. Ag/AgCl), the current response is modulated by the conductivity state of PANI, which in turn is dictated by the rate of urea hydrolysis. Therefore, the steady-state current is directly proportional to the urea concentration in the sample.

For heavy metal detection, the biosensor functions in an inhibition mode. A baseline current response (I_0) is first established for a fixed concentration of urea in a clean buffer. The sample potentially containing heavy metal ions is then introduced. If inhibitors like Hg^{2+} or Pb^{2+} are present, they bind to the active site or crucial thiol groups of urease, partially deactivating the enzyme. This results in a decreased rate of urea hydrolysis, a smaller local pH change, and a consequent reduction in the measured current response (I_i). The percentage of inhibition can be calculated as:

$$\% \text{ Inhibition} = \frac{I_0 - I_i}{I_0} \text{ X } 100$$

This percentage correlates with the concentration of the inhibiting metal ion, enabling quantitative analysis [12].

3. Results

3.1. Analytical Performance: Sensitivity, Linearity, and Limit of Detection

The analytical efficacy of the PANI/ZnO/Urease biosensor was rigorously evaluated through amperometric measurements. Figure 1 in the original study presented the cyclic voltammetric response of the biosensor upon successive additions of urea (10 to 50 mM) in phosphate buffer (pH 7.2). The current was observed to increase linearly with each addition until reaching a saturation point, indicative of the enzyme kinetics governing the process [7].

A calibration curve was constructed by plotting the steady-state current against urea concentration (Figure 3 in the source). Linear regression analysis yielded the equation: $I \text{ (mA)} = 0.599 \times [\text{Urea}] + 7.235$, with a high correlation coefficient (R^2) of 0.989. This confirms excellent linearity within the tested range of 10-50 mM. The slope of this calibration curve defines the biosensor's sensitivity, which was determined to be 0.599 mA/mM. This value represents the current change per unit change in substrate concentration and is a direct metric of the sensor's responsiveness [13].

The limit of detection (LOD), defined as the minimum concentration of analyte that can be reliably distinguished from the background noise, is a critical parameter. It was calculated using the formula $LOD = 3 \times (SD/m)$, where SD is the standard deviation of the blank (background) signal and m is the sensitivity (slope). The reported LOD for urea was 4.953 mM/L [7]. While this LOD is in the millimolar range, suitable for certain applications like blood urea monitoring, the primary application here is as an inhibition sensor for metals. For such sensors, the relevant figure of merit is often the IC_{50} (concentration causing 50% inhibition) or the lowest detectable concentration of the inhibitor. The low LOD for urea indicates a sensitive underlying enzymatic platform, which is prerequisite for developing a sensitive inhibition assay for metals, as even small amounts of inhibitor will cause a measurable change in a sensitive system.

3.2. Michaelis-Menten Kinetics and Enzyme-Substrate Affinity

Enzyme kinetics provide deep insight into the catalytic efficiency and the impact of immobilization. The Michaelis-Menten constant (K*m) and the maximum current (I_max) are derived from the electrochemical data.* Km represents the substrate concentration at which the reaction rate is half of V_max and is inversely related to the enzyme's affinity for its substrate; a lower K*m indicates higher affinity.

A Lineweaver-Burk plot (double reciprocal plot of 1/I vs. 1/[S]) was used to determine these parameters (Figure 4 in the source). For the immobilized urease in the PANI/ZnO matrix, K*m was found to be 0.018 mM, and I_max was 83.33 mA. This* Km value is significantly lower than the value reported for free urease in solution (typically around 1.30 mM) [14]. This notable decrease in K*m post-immobilization suggests a substantial increase in the enzyme's apparent affinity for urea. This phenomenon can be attributed to several factors intrinsic to the nanocomposite support. The nanostructured environment may cause favorable conformational changes in the enzyme, positioning its active site more accessibly. More importantly, the highly porous and hydrophilic nature of the PANI/ZnO matrix likely enhances the diffusion and preconcentration of urea molecules towards the immobilized enzyme sites, effectively increasing the local substrate concentration at the active site. This "concentrating effect" leads to a faster saturation of the enzyme at lower bulk concentrations, manifesting as a lower apparent* Km [15]. The high I_max value further corroborates successful immobilization of a large amount of active enzyme due to the high surface area of the nanocomposite.

4. Discussion

4.1. Effect and Optimization of pH

The activity of enzyme-based biosensors is intrinsically linked to the pH of the medium, as enzymes possess optimal pH ranges for maximum activity. Deviations can lead to protonation/deprotonation of critical amino acid residues in the active site, altering the enzyme's three-dimensional structure and reducing catalytic efficiency [16]. For the PANI/ZnO/Urease biosensor, the effect of pH was studied from 4.0 to 10.0 (Figure 5 in the source). The current response increased nearly linearly from pH 4.0 to 8.5, beyond which it declined. Therefore, the optimum pH for the biosensor operation was identified as 8.5 [7].

This optimum is a compromise between the native activity of urease (which often has a broad optimum around neutral to slightly alkaline pH) and the properties of the transducer materials. Notably, the isoelectric point (IEP) of nanostructured ZnO is reported to be between 8.5 and 9.5 [17]. At pH values near or above the IEP, the ZnO surface becomes negatively charged. Urease, depending on its source and purification, often has an IEP around 5.0-5.1, making it negatively charged at pH 8.5 as well. While electrostatic repulsion might be expected, the complex nanocomposite environment with PANI likely involves multiple modes of interaction beyond simple electrostatics. The observed pH optimum of 8.5 likely represents the point where the enzyme's intrinsic activity and the charge state of the nanocomposite surface conspire to create the most favorable microenvironment for both catalysis and efficient transduction via PANI's pH-dependent conductivity.

4.2. Effect and Optimization of Temperature

Temperature influences enzymatic reactions by increasing kinetic energy and reaction rates, but excessive heat causes protein denaturation. The biosensor's response was evaluated between 20°C and 45°C (Figure 6 in the source). The current increased with temperature up to a maximum at 30°C, after which it decreased due to the onset of thermal denaturation of urease [18]. This optimum temperature of 30°C is slightly lower than the typical optimum for free urease (often around 37-45°C), which is common upon immobilization. The confinement within a polymeric matrix can impose diffusional constraints and slightly alter the enzyme's thermal stability profile.

Nevertheless, 30°C is a practical operating temperature, close to ambient conditions for many applications.

The activation energy (Ea) for the immobilized enzyme-catalyzed reaction was determined using an Arrhenius plot (plot of ln(I) vs. 1/T). The slope of the linear region yielded an Ea of 2.621 kcal mol^{-1} [7]. This value is higher than the activation energy reported for the PANI/ZnO composite alone (1.38 kcal mol^{-1}) [19]. The increase in Ea upon enzyme immobilization confirms that the rate-limiting step is indeed the biocatalytic reaction itself, not merely the charge transport in the nanocomposite. The immobilized urease acts as an effective catalyst, lowering the energy barrier for the reaction compared to an uncatalyzed pathway, but the measured Ea reflects the kinetics of the enzyme-substrate complex formation and turnover within the immobilized state.

4.3. Response Time and Stability Assessment

Response Time: The dynamic characteristic of a biosensor is gauged by its response time—the time required to reach 90% of the steady-state signal after analyte introduction. The PANI/ZnO/Urease biosensor exhibited a rapid response time of approximately 9 seconds for 10 mM urea (Figure 8 in the source) [7]. This swift response is a direct benefit of the nanocomposite architecture. The porous matrix minimizes diffusional barriers for the substrate (urea and products), allowing rapid equilibration. The high conductivity of PANI ensures fast electron transfer, translating the biochemical event quickly into an electrical signal. This rapid response is advantageous for real-time monitoring.

Operational and Storage Stability: Stability dictates the practical usability and cost-effectiveness of a biosensor. Operational stability, tested through repeated measurements on the same day, showed the biosensor could be used 4-5 times with consistent readings when measuring urea [7]. The decay in response thereafter is attributed to the gradual leaching or denaturation of the physically adsorbed enzyme. Storage stability was evaluated by storing the biosensor in PBS (pH 7.2) at 4°C and testing it over consecutive days. The sensor retained effective responsiveness up to the 5th day (Figure 9 in the source), after which activity declined significantly due to long-term denaturation of the immobilized enzyme [20, 21]. These stability figures, while indicative of a prototype, highlight areas for future improvement, such as employing covalent immobilization or better encapsulation techniques to enhance shelf life.

5. Conclusion

The PANI/ZnO/Urease biosensor represents a sophisticated integration of nanomaterials science, enzyme technology, and electrochemistry. The systematic investigation presented by Mahajan and Birajdar demonstrates that the engineered nanocomposite interface successfully addresses key challenges in biosensor design: enhancing enzyme loading via high surface area (ZnO), facilitating efficient signal transduction (PANI), and improving enzyme-substrate affinity through favorable microenvironments. The resultant biosensor exhibits commendable analytical performance—good sensitivity, a wide linear range, rapid response, and reasonable stability—for urea detection. As an inhibition platform, it holds strong promise for the detection of heavy metal ions like Hg^{2+} and Pb^{2+} in water.

Future work should focus on several fronts to transition this technology from a laboratory prototype to a field-deployable device. Firstly, the immobilization strategy could be advanced from physical adsorption to covalent attachment or cross-linking, potentially using the functional groups on ZnO or modified PANI, to drastically improve operational and storage stability. Secondly, while the study characterizes urea detection, a full validation of the inhibition assay for heavy metals is necessary, including determination of selectivity (interference from other ions), IC_{50} values, and detection limits in real water matrices (tap water, river water). Thirdly, miniaturization of the electrochemical cell and integration with portable potentiostat electronics would be essential for on-site applications. Exploring other nanocomposites or hybrid materials (e.g., with graphene or other metal oxides) could further push the limits of sensitivity and stability. In conclusion, the PANI/ZnO/Urease system stands as a compelling testament to the power of nanomaterial-enabled biosensor design, offering a viable pathway toward low-cost, rapid, and efficient tools for environmental monitoring and public health protection.

Conflicts of interest: The author stated that no conflicts of interest.

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