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A Bioanalytical Chemistry Experiment for Undergraduate Students: Biosensors Based on Metal Nanoparticles

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Nanotechnology is increasingly playing an important role in (bio)analytical chemistry. This has created the need for appropriate teaching modules that allow undergraduate students to gain the knowledge of using nanotechnology products for practical applications *(1)*. An example of such an application is the development of modern biosensors. Nanomaterials, and in particular gold nanoparticles, facilitate direct and fast electron transfer between the oxidoreduction species and the transducer *(2–5)*. Particles that have dimensions ≤100 nm are commonly referred to as nanoparticles. Their high surface area provides a large number of binding points for biomolecule attachment and an increase in activity. In addition, their size allows minimum diffusion of the substrate or product thus overcoming the problem of sensitivity *(6)*. These unique properties make nanoparticles an excellent choice as transducer material for the design of modern biological sensors.

The goal of this experiment is to expose students to the modern biosensor based on gold nanoparticles. The experiment involves electrochemical deposition of gold nanoparticles onto an electrode surface, enzyme immobilization to fabricate an enzyme sensor, characterization of the biosensor with respect to analytical performance, and its application for the quantitative analysis of phenol. The procedure is simple and versatile for adoption to a conventional instrumental laboratory; it requires inexpensive reagents and equipment (a simple galvanostat–potentiostat) and can be performed by third- or fourth-year students.

The experiment was built on our experience in enzyme sensor development *(7, 8)* and on published literature data *(4, 5)* adapted to undergraduate education. Essentially, the students prepare their own biosensor with simple tools. These include a glassy carbon electrode (GCE), solutions of enzyme, and $HAuCl_4$. The system uses tyrosinase (Tyr) as the biological recognition element. In the presence of molecular oxygen, this enzyme catalyzes the oxidation of phenol into a quinone in a two step-reaction (Figure 1). The quinone may be reduced back to catechol by applying an electrical potential. This results in a catalytically amplified signal that can be measured and quantified using electrochemical methods *(8)*. The magnitude of the signal is proportional to the quantity of phenol present in the reaction medium.

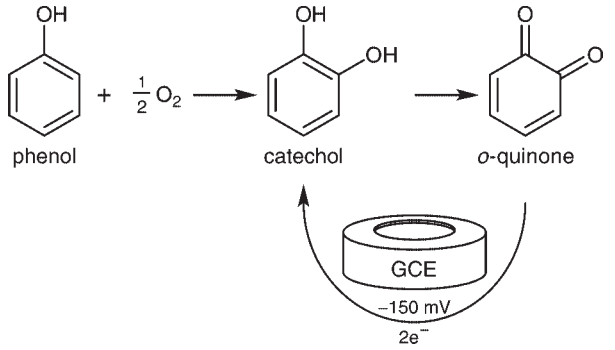

Figure 1. Reaction scheme showing the enzymatic catalysis of phenol to *o*-quinone by tyrosinase and electrochemical detection of *o*-quinone.

By involving knowledge of electrochemistry, biochemistry, and material chemistry, this experiment offers the possibility of highly interdisciplinary training for undergraduate students. It also represents an opportunity to introduce students to undergraduate research. To our knowledge, this is the first experiment involving nanomaterials for biosensing that has been adapted for integration into the undergraduate analytical chemistry laboratory. The experiment was developed for our instrumental laboratory intended for chemistry and biomolecular science majors. Several classical enzyme (mainly glucoseoxidase) sensors have already been introduced into the undergraduate curriculum *(9–11)*.

Experimental

In a typical undergraduate experiment, the procedure consists of the following steps: (i) modification of the GCE electrode with gold nanoparticles by applying a constant potential, (ii) characterization of the Au-modified GCE surface using cyclic voltammetry, (iii) immobilization of tyrosinase (This is accomplished using glutaraldehyde chemistry; Figure 2), (iv) calibration and characterization of the sensor using amperometry in stirred solutions, and (v) determination of phenol in unknown samples with the sensor. The experiment can be completed in two sessions of a typical three-hour laboratory.

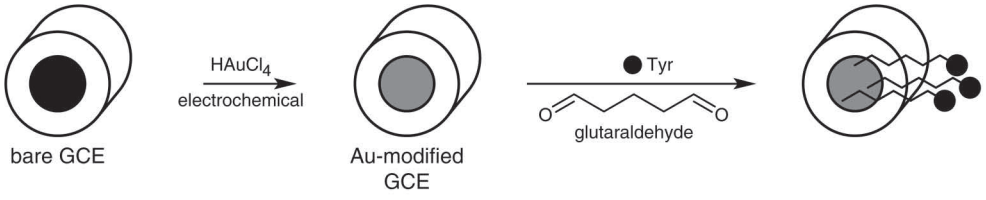

Figure 2. Schematic diagram of the biosensor fabrication. Tyr is the tyrosinase enzyme.

Equipment and Reagents

An Epsilon potentiostat–galvanostat, a classical 10 mL electrochemical cell, and electrodes (glassy carbon working electrode, Ag/AgCl reference, and platinum wire as auxiliary electrode) from Bioanalytical Systems were used for the electrochemical experiments. The chemicals and reagents used are as follows: tyrosinase (catechol oxidase monophenol, dihydroxyphenylalanine) T3824, 50,000 units and phenol (analytical grade 99%) from Sigma; glutaraldehyde (25%), chlorauric(III) acid trihydrate, potassium hexacyanoferrate(III), potassium phosphate monobasic, potassium nitrate, and high purity methanol (HPLC) from Fisher Scientific; and sodium phosphate dibasic anhydrous from J. T. Baker.

Procedure

In the first laboratory period, the students prepare the electrode and fabricate the biosensor. In the second session, the students characterize the biosensor in terms of analytical performance (sensitivity, linear range, detection limit, and response time) and use it for the determination of unknowns.

Gold nanoparticles were electrodeposited by applying a constant potential of −200 mV for 1 min in a solution of 167 mg L^{-1} $HAuCl_4$. The solution was prepared in distilled water and deoxygenated by purging with N_2 for about 15 min. Prior to deposition, the GCE electrode was cleaned and dried according to the following sequence: polish with 0.3 μm alumina powder, wash ultrasonically for 10 min in distilled water, rinse with distilled water and methanol, and dry under a nitrogen stream. The surface of the electrode prior to and after deposition was characterized by cyclic voltammetry using the model reversible redox couple $Fe^{II}(CN)_6^{4-}$–$Fe^{III}(CN)_6^{3-}$ *(12)*. For this purpose, the three electrodes were immersed in a classical electrochemical cell containing 4 mL $K_3Fe(CN)_6$ (2 mM prepared in 1 M KNO_3) and the potential was scanned between −200 and 700 mV versus Ag/AgCl reference electrode at a scan rate of 100 mV/s.

The enzyme immobilization procedure consists of the deposition of 5 μL Tyr (100 IU μL^{-1}) onto the gold-modified GCE. The electrode was allowed to dry for ~30 min at room temperature and then immersed in a solution of 25% glutaraldehyde for 30 min to allow crosslinking. Finally, it was rinsed with distilled water and stored in phosphate buffer solution (0.1 M) at pH 6.5 when not in use. Calibration of the biosensor and phenol detection was performed using dc potential amperometry at a constant applied potential of −150 mV. All the electrochemical experiments were carried out in a standard three electrode cell configuration with a Ag/AgCl reference electrode, a Pt wire auxiliary electrode, and the gold-modified GCE/enzyme electrode working electrode.

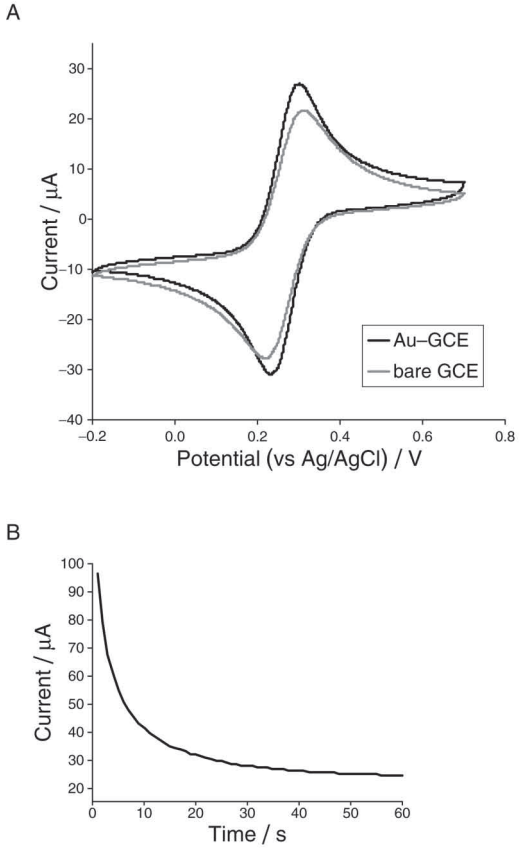

Figure 3. (A) Cyclic voltammograms of bare GCE and Au-modified GCE in 0.002 M potassium hexacyanoferrate(III) at 100 mV s^{-1}. (B) Electrochemical signal recorded during the electrodeposition of gold nanoparticles onto the GCE.

Hazards

Phenol and glutaraldehyde are harmful if swallowed and irritating to the eyes, respiratory tract, and skin. Chlorauric acid is a strong oxidizing agent. Potassium hexacyanoferrate (III) is a slight skin and eye irritant and nonhazardous in case of ingestion. Tyrosinase may be harmful if it comes in contact with skin, eyes, or inhaled.

Results and Discussion

Due to their extraordinary electrocatalytic activity, electrodes modified with gold-nanoparticles have attracted a growing interest for many electroanalytical applications *(13–15)*. In this experiment, gold nanoparticles were electrodeposited onto the GCE from a solution of $HAuCl_4$ according to a published literature procedure *(4, 5)*. This resulted in a deposition of a smooth layer of aggregates of gold nanoparticles that was confirmed by the change in the color of the working GCE surface from gray to yellow. The particles have an average size of ~50 nm, which was confirmed by scanning electron microsopy (SEM). This is in agreement with published literature data *(4)*. The electrochemical signal recorded during the electrodeposition is shown in Figure 3B The modified Au–GCE was further characterized by cyclic voltammetry using $Fe^{II}(CN)_6^{4-}$–$Fe^{III}(CN)_6^{3-}$ as a model redox probe (Figure 3A). Compared to the bare GCE, the peak potential separation ΔE_p was reduced from 93 mV to 70 mV when Au–GCE was used. Concurrently, a current amplification of both anodic and cathodic peaks was observed, suggesting that the presence of Au promoted the electron transfer at the surface of the electrode.

Crosslinking of the enzyme using glutaraldehyde resulted in the stable attachment of Tyr onto the electrode. The presence of gold nanoparticles plays an important role in enhancing enzyme activity and promotes easy accessibility of substrate molecules to the catalytically active site, resulting

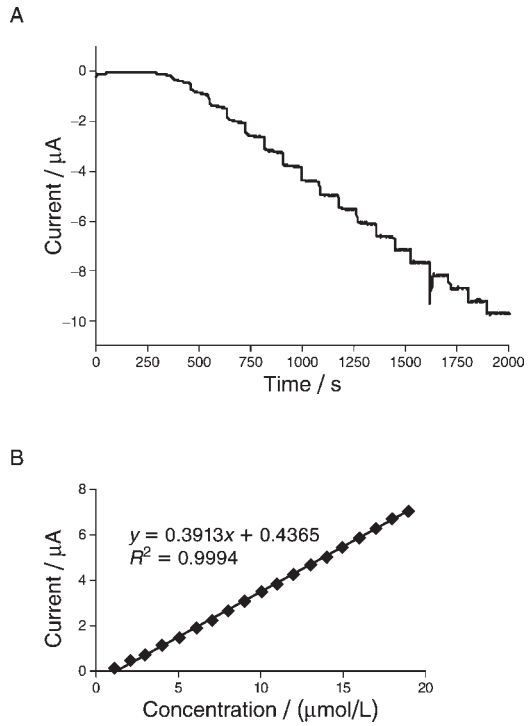

Figure 4. (A) Typical steady-state current–time response for increasing phenol concentrations (1 μM steps) at the Tyr–Au–GCE working electrode. (B) The calibration curve of the biosensor.

in a high sensitivity and a lower detection limit of the substrate. The Tyr–Au–GCE biosensor was characterized in terms of analytical performances using phenol as a substrate. The values of the current were plotted against substrate concentration to generate the calibration curve of the sensor, which enables determination of unknown samples. The biosensor attains a steady-state current within 12 s upon addition of substrate. The current increased linearly with the concentration of phenol in the range between 4×10^{-6} M and 1×10^{-4} M phenol with a correlation coefficient of 0.9994 (Figure 4B). The detection limit calculated at 3σ (where σ is an estimation of the standard deviation of the background signal) was 1.12×10^{-6} M phenol. The sensitivity of the sensor was 0.3913 A M^{-1}. Thereafter, the Tyr–Au–GCE electrode was used as the working electrode to detect phenol in an unknown sample. Students were required to perform triplicate analysis of the unknown. This gave them an indication of the reproducibility of measurements using the enzyme sensor. We found the variability between groups in calibration curves and determination of the unknown to be less than 10%, which shows the reproducibility and the robustness of the experiment for teaching purposes (average results obtained by 20 students working in groups of 2 or 3). When the same students repeated the analysis for the unknown, the relative standard deviation was less than 1% (see the Supplemental Material[W]).

The students were asked to evaluate and compare the kinetic constants of the enzyme in the free and immobilized state, which illustrates the effect of the immobilization on the enzymatic activity. The apparent Michaelis kinetic constants (K_M^{app} and V_m^{app}) were calculated according to the Lineweaver–Burk plot using phenol as the substrate. The apparent Michaelis constants of the immobilized enzyme decreased at least six times compared to free enzyme which suggests changes in enzyme activity and affinity for the substrate during immobilization. This could be attributed to steric and conformational changes of the enzyme when crosslinking with glutaraldehyde.

Conclusion

This experiment introduces and reinforces the use of electrochemical methods as a tool for nanosynthesis and bioanalytical applications. Owing to its reasonable stability and robustness in handling, Tyr was used as a model to demonstrate and apply the principles of enzyme kinetics and immobilization. The same experiment could be adapted for other enzymes and biocatalytic systems. In a single experiment, the students use three different electrochemical techniques (electrodeposition, cyclic voltammetry, and amperometry), apply biochemical principles (determination of enzyme kinetic constants, study of the effect of immobilization on the enzyme activity), and gain exposure to nanomaterials and biosensors. In addition to biosensor development and electrochemistry, the students are trained in basic analytical chemistry principles (calibration, determination of performance characteristics of an analytical instrument, determination of an unknown). In the undergraduate chemistry curriculum, this laboratory experiment could complement or replace the classical cyclic voltammetry experiment *(12)* used as a model example in electrochemistry. It could also serve as an introduction to electrochemistry and biosensors and give students the opportunity to perform interdisciplinary laboratory experiments at the undergraduate level and make connections between disciplines.

[W]Supplemental Material

Instructions for the students and notes for the instructor are available in this issue of *JCE Online*.

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