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Construction of glutamate biosensor based on covalent immobilization of glutmate oxidase on polypyrrole nanoparticles/polyaniline modified gold electrode

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ABSTRACT

A method is described for construction of a highly sensitive electrochemical biosensor for detection of glutamate. The biosensor is based on covalent immobilization of glutamate oxidase (GluOx) onto polypyrrole nanoparticles and polyaniline composite film (PPyNPs/PANI) electrodeposited onto Au electrode. The enzyme electrode was characterized by cyclic voltammetry (CV), scanning electron microscopy (SEM), X-ray diffraction (XRD), transmission electron microscopy (TEM), Fourier transform infra-red spectroscopy (FTIR) and electrochemical impedance spectroscopy (EIS). The biosensor showed optimum response within 3 s at pH 7.5 (0.1 M sodium phosphate) and 35 °C, when operated at 50 mV s^{-1}. It exhibited excellent sensitivity (detection limit as 0.1 nM), fast response time and wider linear range (from 0.02 to 400 μM). Analytical recovery of added glutamate (5 mM and 10 mM) was 95.56 and 97%, while within batch and between batch coefficients of variation were 3.2% and 3.35% respectively. The enzyme electrode was used 100 times over a period of 60 days, when stored at 4 °C. The biosensor measured glutamate level in food stuff, which correlated well with a standard colorimetric method ($r = 0.99$).

1. Introduction

Glutamate (Glu) is one of the 22 amino acids, which are used to synthesize proteins and takes part in typical metabolic functions like energy production and ammonia detoxification. Glutamate is probably best known as "monosodium glutamate (monosodium salt)" or "MSG" which is employed as a flavor or taste enhancer in food. It is usually available together with other food additives and spices in most large food stores, to give a taste known as Umami. The excessive intake of it simulates glutamate receptors in CNS of vertebrates & thereby release glutamate from neurons which lead to neuronal degeneration & cell death beside several neurological disorders including stroke, epilepsy, Alzheimer's diseases & Parkinson's disease as well as learning & memory power [1–6]. It has also been linked to Chinese Restaurant Syndrome (CRS) [7], being a common ingredient of Chinese food [8]. The term "Chinese restaurant syndrome" is a sudden fall in blood pressure with subsequent fainting after ingestion of very spicy food which is rich in MSG. Hence, there is need to determine its presence in variety of foods. India's Prevention of Food Adulteration Act has set an upper limit of MSG in food, which is 1% [9]. Different techniques have been developed to determine Glu, e.g. potentiometric titration [10], chromatographic [11–15], spectrophotometric [16–18] and fluorimetric [19–22], but these methods require time consuming sample preparation, costly equipment and skilled persons to operate. Biosensors overcome these drawbacks, as these are simple, sensitive, rapid and specific. Recently biosensors have been improved using combination of nanomaterials and conducting polymers.

Polypyrrole, obtained by polymerization of pyrrole is a conducting polymer. The film of polypyrrole is of yellow in color but get darken in air due to oxidation. Although polypyrrole is an insulator, its oxidized derivatives are good electrical conductor with a conductivity range of 2–100 S cm^{-1} [23]. Nanoparticles of polypyrrole have shown the higher conductivity when doped with short alkyl chain than long alkyl chain [24] due to large surface area for reactions and highly porous in sol form [25,26]. PPyNPs sandwiched with core shell Fe_3O_4 nanoparticles have been recently used to improve the analytic performance of potentiometric glucose biosensor [27]. Similarly polyanilne (PANI) is also a polymer of aniline, which has been used in biosensor architecture as transducer, due to its electronic & biomolecular properties [28,29]. The present work describes a unique approach of immobilizing glutamate oxidase onto PPyNPs/PANI, modified Au electrode and its application in construction of an amperometric biosensor for the determination

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of L-glutamte. PPyNPs/PANI, based glutamate biosensor is expected to provide high sensitivity, high biocompatibility, high charge transfer rate and good stability.

2. Experimental

2.1. Materials

Glutamate oxidase (GluOx) from Sigma–Aldrich, St. Louis, USA, potassium ferrocynide ($K_2Fe_2CN_2$) & potassium ferricynide ($K_2(FeCN)_6{\cdot}3H_2O$), aniline, sodium dodecylsulfate (SDS) ($(NH_4)_2S_2O_8$), APS and polypyrrole, and potassium chloride (KCl), from SISCO Research Lab., Mumbai, India, Glutamic acid & Glutraldehyde from LOBA cheme. PVT. LTD. Mumbai, were used. Double distilled water (DW) was used throughout the experimental studies.

2.2. Apparatus used

Potentiostat/Galvanostat (Autolab, model: AUT83785, manufactured by Eco Chemie) with a three electrode system composed of a Pt wire as an auxillary electrode, an Ag/AgCl electrode as reference electrode and GluOx/PPyNPs/PANI/Au electrode as a working electrode. Transmission electron microscope (TEM) (JEOL 2100F) and Scanning electron microscope (SEM) (Zeiss EV040). UV Spectrophotometer (Shimadzu, Model 160A), X-ray diffractometer (XRD), (122 Rigaku, D/Max2550, Tokyo, Japan) Fourier transform infra-red spectrophotometer (FTIR) (Thermo Scientific, USA).

2.3. Assay of free GluOx

The assay of free GluOx was carried out as described by Satyapal and Pundir (1993) [30] with modification. The assay was based on quantification of H_2O_2, generated from oxidation of glutamic acid catalyzed by GluOx, using a color reaction consisting of 4-aminophenazone, phenol and peroxidase as chromogenic system [31]. The reaction mixture containing 1.8 ml of sodium phosphate buffer pH 7.5 (0.1 M), 0.1 ml of L-glutamate solution (1 mM), 0.1 ml of Glu Ox solution (5 U/ml) was incubated at 37 °C for 10 min. One ml of color reagent was added and incubated it in dark at 37 °C for 20 min to develop the color, A_{520} was read and H_2O_2 concentration was extrapolated from its standard curve. One unit of enzyme is defined as amount of enzyme required to catalyze the formation of 1.0 nmol of H_2O_2 from oxidation of glutamate per min/ml under standard assay conditions.

Preparation of color reagent: the color reagent was prepared according to the method of Bais et al. (1980) [30] consisted of 50 mg of 4-aminophenazone, 100 mg solid phenol and 1 mg horseradish peroxidase per 100 ml of 0.4 M sodium phosphate buffer, pH 7.0. It was stored in amber colored bottle at 4 °C and discarded after one week.

2.4. Preparation of PPyNPs

PPyNPs were prepared by microemulsion method [24]. An aqueous solution of 0.08 M SDS was prepared & stirred vigorously for 20 min, then pyrrole was added & stirred vigorously for 30 min. Alcohol was added. After 24 h, excessive methanol was added to terminate the reaction. The precipitate was centrifuged at 5000 rpm for 15–20 min, washed it with methanol, DW & finally with acetone. The PpyNPs were generated, filtered, dried & kept in dessicator.

2.5. TEM of PPyNPs

The morphological characterization of the PPy nanoparticles was carried out in a Transmission electron microscope, at J.N. University, New Delhi.

2.6. Electrodeposition of PPyNPs/PANI onto gold electrode

The surface of Au electrode (2 cm × 1 mm) was polished manually by alumina slurry (diameter 0.05 μm) with a polishing cloth, followed by thorough washing with DW and then placed into ethanol, sonicated to remove adsorbed particles and finally washed with DW for 3–4 times. Aniline (200 μl) and PPyNPs suspension (200 μl) was added into 25 ml of 1 M H_2SO_4 and co-electropolymerized them onto surface of Au electrode through cyclic voltammetry in a potentiostat–galvanostat by applying 50 successive polymerization cycles at −0.3 to 0.7 V at a scan rate of 20 mV s^{-1} (Fig. 1). The resulting PPyNPs/PANI modified Au electrode was washed thoroughly with DW to remove unbound matter and kept in a dry Petri-plate at 4 °C.

2.7. Immobilization of GluOx onto PPyNPs/PANI modified Au electrode

PPyNPs/PANI/Au electrode was immersed into 1 ml of 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.5) for 30 min & then washed thoroughly in 0.05 M sodium phosphate buffer pH 7.5. The glutaraldehyde activated PPyNPs/PANI/Au electrode was dipped into 1.5 ml of GluOx solution (5 U/ml) in 0.1 M sodium phosphate buffer (pH 7.5) and kept overnight at room temperature for immobilization. The resulting electrode with immobilized GluOx was washed 3–4 times with 0.1 M sodium phosphate buffer, pH 7.5 to remove residual unbound protein. The resulting GluOx/PPyNPs/PANI/Au electrode was used as working electrode and stored at 4 °C when not in use. This working electrode was characterized by SEM at different stages of its construction.

2.8. Scanning electron microscopy of Gluox/PPyNPs/PANI/AuE

The SEM images of bare Au electrode, PpyNPs/PANI/Au and GluOx/PPyNPs/PANI/Au electrode were taken in a scanning electron microscope (Zeiss EV040) at J.N. University, New Delhi on commercial basis.

2.9. Construction and testing of glutamate biosensor

An amperometric L-glutamate biosensor was constructed by connecting with Ag/AgCl as reference electrode, GluOx/PPyNPs/PANI/AuE (working electrode) and Pt wire as counter electrode through potentiostat. The CV of this biosensor was recorded in a 25 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 100 μl of 1 mM glutamate in the range of 0.05–0.5 V at a scan rate of 50 mV s^{-1}.

2.10. Optimization of glutamate biosensor

To optimize working conditions of the biosensor, effects of pH, incubation temperature, time and substrate (Glutamic acid)

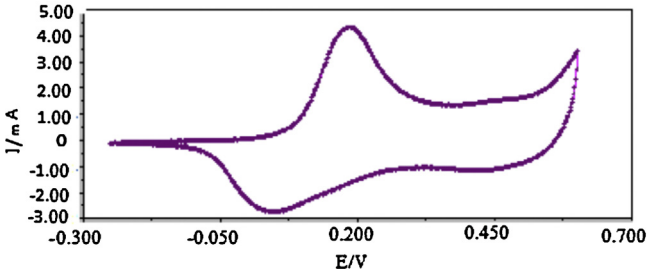

Fig. 1. Cyclic voltamogram for electrodeposition of PPyNPs/PANI composite film. Supporting electrolyte: 1 M H_2SO_4 solution; scan rate: 20 mV s^{-1}.