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ORIGINAL PAPER

Amperometric enzyme electrode for glucose determination based on poly(pyrrole-2-aminobenzoic acid)

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Abstract Polymerization of pyrrole and 2-aminobenzoic acid has been investigated, and a functionalized stable film of poly(pyrrole-2-aminobenzoic acid) (PP2ABA) has been obtained electrochemically onto platinum electrode. Different cyclic voltammetric behavior is obtained for polypyrrole and PP2ABA during electrosynthesis. Fourier-transformed infrared spectrometry and surface-enhanced Raman spectrometry measurements on the two films have confirmed the presence of carboxylate group in the films. The enzyme, glucose oxidase, was covalently immobilized on a conducting PP2ABA film, and amperometric response was measured as a function of concentration of glucose at a potential of 0.7 V vs Ag/AgCl in 0.1 M phosphate buffer at pH 6.2. The effect of polymeric film thickness, pH, and possible interferents were investigated. The linear range of the calibration curve is from 3 to 40 mM with a sensitivity of 0.058 μA mM^{-1} cm^{-2} and a limit of detection of 0.5 mM. The apperent Mishaelis–Menten constant K_M is calculated to be 1×10^{-2} mM, and the response time is 5 s.

Keywords Poly(pyrrole-2-aminobenzoic acid) · Conducting polymer · Enzyme electrode · SERS

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Introduction

As a new class of electrode material, the properties of conducting polymer electrodes have led to their use in bioelectrochemistry applications [1–5]. Conducting polymer electrodes can mediate electron transport between biological macromolecules and the electrodes [6]. However, the stability of the enzyme is one of the critical parameters regarding the rate of loss in activity of enzyme and mechanical behavior of the conducting polymer film. Among the conducting polymers, a very stable polymer film of polypyrrole can be easily formed on electro-oxidation of pyrrole on an electrode surface [7–11]. Electropolymerization is generally preferred because it provides a better control of film thickness and morphology.

The use of pristine polyaniline polymer is limited in bioelectrochemistry applications due to the pH dependence of its conductivity. The pH values above 4, polyaniline becomes electrochemically inactive [12]. Incorporation of the carboxylic or sulfonic acid as ring substituents in the polymer backbone could charge the acidity constant of the amine groups and obtained modified polyaniline showed its electroactivity in less acidic medium and conductivity does not fall off with increase in the case of polyaniline [13]. Research on the synthesis and chemical and electrochemical properties of substituted anilines have been reported [14, 15]. The main objective for the synthesis of the functionalized of polyaniline was to construct sensors based on conductivity changes [16] and increase in the solubility to obtain better processability [17, 18]. It is also worth mentioning that the insertion of carboxylic substituents results in the modification of the polymer film via covalent binding.

The majority of the research was reported dealing with the synthesis and characterization of *o*-aminobenzoic acid and either chemical or electrochemical copolymerization of aniline and 2-aminobenzoic acid [19–26]. The electro-

polymerization of carboxylate-substituted anilines (2-3 and 4-aminobenzoic acids) in sulfuric acid solution has been investigated [19]. It is reported that the ortho-substituted aniline is oxidized at lower potentials seems to reflect a higher stability of the meta- and para-substituted anilines compared to 2-aminobenzoic acid. The polymer obtained from the 2-aminobenzoic acid oxidation reacted higher thickness, and the oxidation charge recorded under the voltammetric curves decreases in the order ortho>para>meta. Mixture of aniline and carboxylate-substituted aniline in different ratios was also polymerized on glassy carbon electrode using cyclic voltammetry [25]. Copolymer of aniline and carboxylate-substituted aniline show differences in morphology compared to polyaniline. The behavior at neutral pH is dominated by aniline and use of substituted aniline electropolymerization without polymerization of mixture with aniline is not preferable due to the synthesis for a longer period of time in order to archive the necessary film thickness [25].

Up to now, polymer films of PP2ABA were prepared electrochemically on gold and platinum surface [27, 28]. The electroanalysis of copper ion in this conducting polymer electrode was achieved by differential pulse stripping voltammetry. Polymerization of pyrrole and 2-aminobenzoic acid might have potential advantages in electrochemical biosensors, for example as electrode materials not subject to fouling or as substrates for enzyme immobilization. In the present work, we report for the first time on the use of a PP2ABA film for constructing a new amperometric sensor for the determination of glucose and detailed spectroscopic characterization of PP2ABA film. Pyrrole has been polymerized electrochemically with 2-aminobenzoic acid so as to take the advantages of polypyrrole stability and carboxylate-substituted aniline for interference rejection. Covalent coupling of enzyme was performed to the resulting electrode film by glutaraldehyde. The polymer film is characterized by cyclic voltammetry, Fourier-transformed infrared spectroscopy (FTIR) and surface-enhanced Raman spectroscopy (SERS). The conductivity of material is tested showing an improvement with respect to the behavior of poly-2-aminobenzoic acid in the same conditions. For the determination of optimum working conditions of the enzyme electrode, the effect of polymeric film thickness, pH, and type covalent coupling reagent on the measured current values were investigated. The stability of the enzyme electrode and the influence of possible interferents were also examined.

Experimental

Chemicals

Pyrrole (97%, Aldrich) and 2-aminobenzoic acid (Aldrich) were used as received. Glutaraldehyde and glucose oxidase were purchased from Sigma, and all other chemicals were analytical grades and used as received. All solutions were prepared using deionized water (18.2 MΩ cm) free from organic matter, obtained from a Millipore purification system, and deaerated with argon to remove the oxygen present.

Instrumentation

Electrochemical experiments were performed with a Gamry potentiostat (model Reference 600, USA). Polymer film formation was achieved in a conventional one compartment three electrode cell under argon atmosphere. The FTIR spectra of the polymer were obtained using Bruker, Vector 600, FTIR spectrophotometer. SERS experiments were performed with a Jobin Yvon LABRAM Raman Spectrometer. In SERS expriments, before the electropolymerization of PP2ABA, gold electrodes were roughened by oxidation–reduction cycles in a separate cell. Then, the electrochemical synthesis of polypyrrole and PP2ABA films was carried out on the roughened gold substrates. The charge used in depositing polypyrrole and pyrrole-2-aminobenzoic acid films was 100 mC/cm^2. Before SERS experiments, the fully reduced polypyrrole and PP2ABA films were prepared by reducing the oxidized films in an acetonitrile solution containing 0.1 M $LiClO_4$ at a constant cathodic potential of −0.4 V vs Ag/AgCl until the current leveled off.

The dry conductivity values were measured using a four probe technique at room temperature. Gold-plated probes were used to avoid any errors that might arise from the ohmic contacts. At least ten different current values were used in the measurements. Acetonitrile and monomer solutions were kept in dark under nitrogen atmosphere. The working electrode which was cleaned by polishing with Al_2O_3 slurry for the cyclic voltammetric studies was a platinum disk (0.022 cm^2) The macro samples of polymer films were prepared on a Pt macroelectrode (1.0 cm^2) which was cleaned by holding it in a flame for a few minutes. The microelectrode and macroelectrode were rinsed with acetonitrile and dry before use. All data points reported in this work represent the average of three replicates. All experiments were run at room temperature.

Electrochemical polymerization

In order to obtain poly(pyrrole 2-aminobenzoic acid) film, varying amounts of pyrrole were added to acetonitrile solution containing 0.1 M 2-aminobenzoic acid and 0.1 M $LiClO_4$. The electropolymerization was carried out applying five potential cycles between 0 and 1.6 V vs Ag|AgCl. Obtained films were deposited on platinum working electrodes (0.022 cm^2) by applying a constant deposition potential or a program of successive voltammetric cycles. A platinum wire (0.2 mm i.d.×2.0 cm) was used as the

Table 1 Conductivities of poly(pyrrole-2-aminobenzoic acid) films prepared using various amount of monomer ratio

Pyrrole: 2-aminobenzoic acid monomer ratio (M)	(1:1)	(2:1)	(3:1)	(4:1)	(1:0)
Conductivity (S cm^{-1})	5.5×10^{-3}	3.5×10^{-3}	0.091	0.15	2.5

counter electrode and Ag|AgCl electrode was implied as the reference electrode. Following the synthesis process, the electrodes coated with the polymeric films were rinsed water and immersed in the background electrolyte free of monomer.

Immobilization of glucose oxidase

Covalent coupling of glucose oxidase to the conducting polymer electrode was performed using glutaraldehyde in phosphate buffer solution with a pH of 7.4. For immobilizing the glucose oxidase enzyme, first 10 μL of 0.1% glutaraldehyde was spread on a PP2ABA film and was allowed to dry. Then the glucose oxidase was prepared by dissolving 3 mg glucose oxidase in 1 mL of 0.1 mol/L phosphate buffer and 30 μL of this solution is added to the corresponding film electrode and kept for 4 h. After that, the poly(pyrrole 2-aminobenzoic acid) film electrode was rinsed with deionized water to remove nonreacted enzyme and was stored at 4 °C when not in use.

Results and discussion

The electrochemical behavior of the PP2ABA film was investigated between 0 and 1.6 V vs Ag|AgCl potential range. The use of very high potentials results in decreasing electroactivity and overoxidation of the polymer film. The potential limit of +1.6 V vs Ag|AgCl generates an electroactive film and there is no overoxidation current for the PP2ABA film up to +1.6 V vs Ag|AgCl.

Dry conductivity values of PP2ABA films synthesized at different amount of monomer were also measured. Table 1 lists the changes in the dry conductivity values with increasing pyrrole concentration in electrolysis solution. The conductivities of PP2ABA film observed between 0.15 and 5.5×10^{-3} S cm^{-1} depending on initial monomer ratio. Since the contribution to the conductivity from polypyrrole is far more than that of poly(2-aminobenzoic acid), the conductivity values increase with incorporation of pyrrole ring units into the polymeric structure. The conductivity of the PP2ABA film is higher than the conductivity of the poly (2-aminobenzoic acid) homopolymers at about 10^{-8} S cm^{-1} [20]. Although polypyrrole shows higher conductivity than the films formed from PP2ABA film in acidic solution, there are good evidences for incorporation of polymer chains of benzoic acid monomer with polypyrrole chains [29, 30].

FTIR spectral characteristics of PP2ABA film were found to be different from those of homopolymer films. FTIR spectroscopy was also used to determine the presence of carboxylate groups in the PP2ABA film. In the FTIR spectrum of the PP2ABA (monomer ratio 1:1) film, the carboxylate group in the chemical structure is demonstrated by the presence of the 1,630 and 1,730 cm^{-1} bands as shown in Fig. 1. The band at 1,452 cm^{-1} is assigned to the typical polypyrrole ring vibrations. The C—H in-plane vibration and C—N stretching vibration appeared at 1,040 cm^{-1} [31]. The band at 1,140 cm^{-1} may be assigned for N—C stretching band [32].

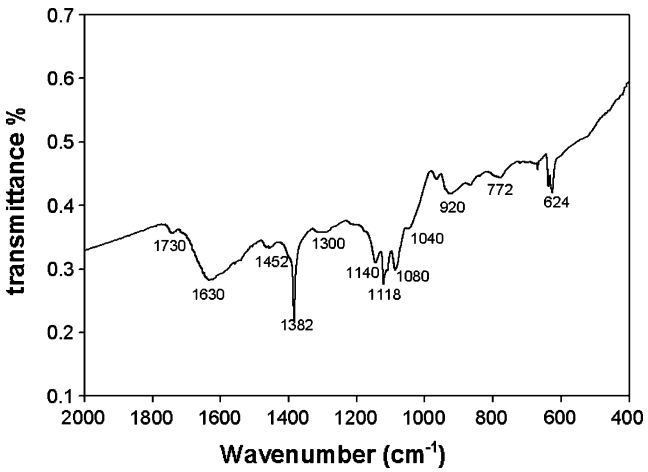

Fig. 1 FTIR spectrum of poly(pyrrole-2-aminobenzoic acid)

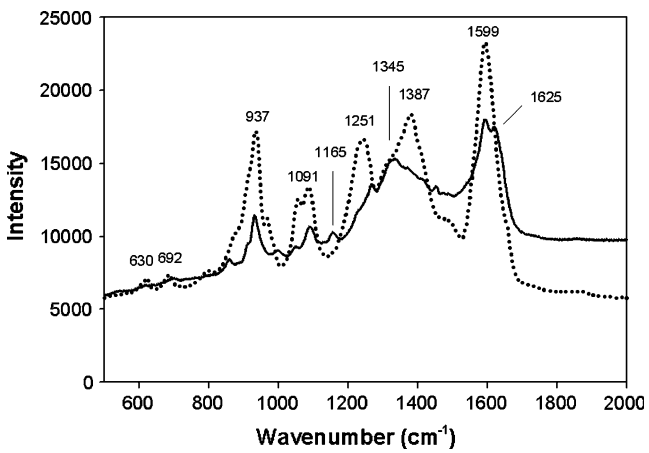

Fig. 2 SERS spectrum of poly(pyrrole-2-aminobenzoic acid)

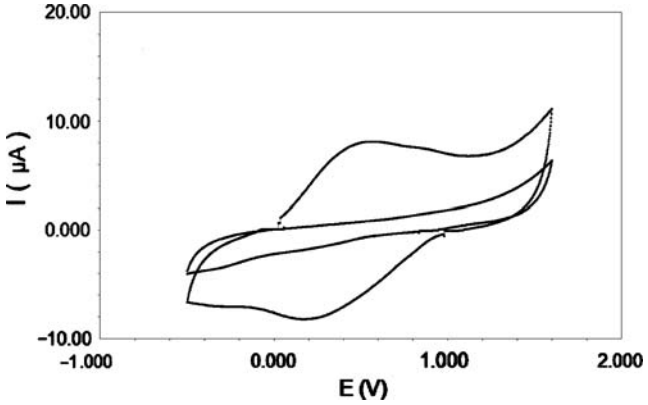

Fig. 3 The cyclic voltammetric response of PP2ABA and PP2ABA-enzyme film at 100 mV/s scan rate in aqueous solution containing 0.1 M $LiClO_4$

SERS spectra of PP2ABA and polypyrrole are given in Fig. 2. The scattering modes of polypyrrole and PP2ABA can be demonstrated with sharp peaks due to the SERS spectrum. For polypyrrole, the characteristic Raman bands appeared at about 1,599 and 1,387 cm^{-1} due to the C=C backbone stretching of polypyrrole and the ring stretching mode of polypyrole, respectively, as shown in Fig. 2 [33, 34]. The spectrum of PP2ABA film shows new band at 1,625 cm^{-1} due to carboxylate group. The spectrum also shows indication of nitrogen site of PP2ABA due to the presence of peak at 1,345 cm^{-1} [35, 36].

Immobilization of enzyme

At initial experiments, covalent coupling of enzyme was carried out using typical *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride, *N*-hydroxy-succinimide, covalent coupling reagent, but we did not observe any response from resulting polymer electrode. Covalent coupling of enzyme was performed to the resulting polymer electrode using glutaraldehyde which is known to be a bifunctional compound mainly used in modification of proteins. In the electrochemical synthesis of polyaniline, large number of free NH_2 groups available at the end of each polyaniline chain which binds with the NH_2 groups of enzyme with glutaraldehyde as a cross-linker [5]. To verify and confirm the presence of enzyme in polymer film, the electrochemical behavior of the PP2ABA-enzyme film was also investigated. A typical cyclic voltammogram recorded for PP2ABA and PP2ABA-enzyme film at 100 mV/s scan rate is shown in Fig. 3. As can be seen in Fig. 3, the conducting film of PP2ABA showed a broad oxidation peak with a peak potential of +0.5 V vs Ag|AgCl and PP2ABA-enzyme film underwent slow electron transfer during the electrochemical reaction.

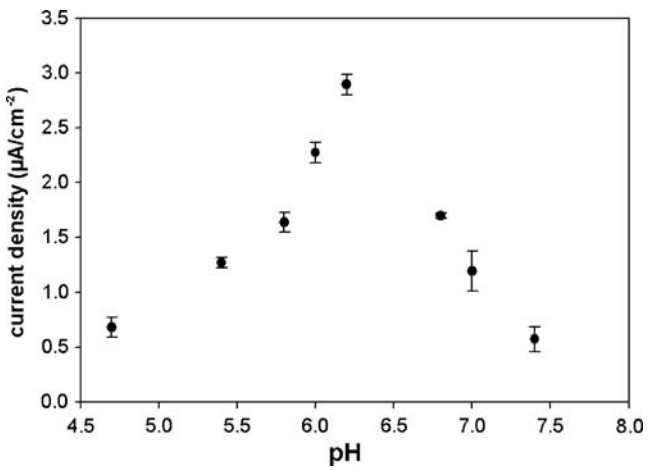

Fig. 4 The effect of pH on the measured response of PP2ABA-enzyme film

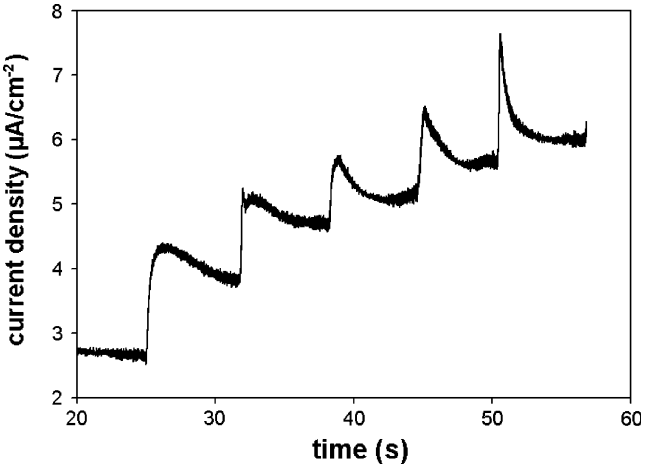

Fig. 5 A typical amperometric response of the PP2ABA-enzyme biosensor after addition of 10 µg/mL concentrations of glucose at +0.7 V vs Ag/AgCl in stirred PBS of pH 6.2

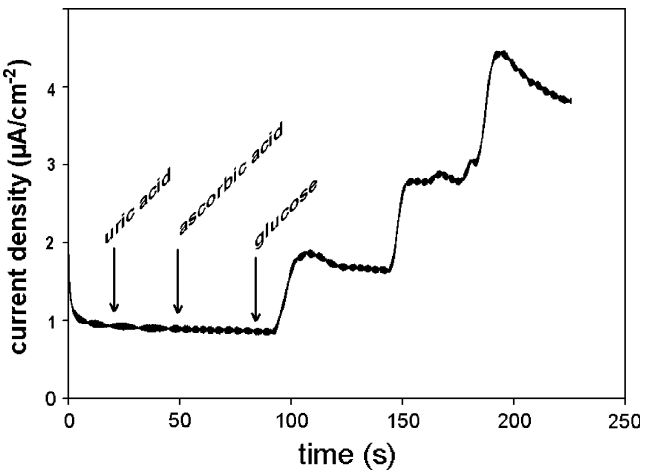

Fig. 6 A typical amperometric response of the PP2ABA-enzyme biosensor after addition of 0.05 mM ascorbic acid, 0.2 mM uric acid, and 10 µg/mL concentrations of glucose at +0.7 V vs Ag/AgCl in stirred PBS of pH 6.2

The thickness of the film is related to the number of cycles in the potential domain selected in cyclic voltammetry. The biosensor performance for different film thickness was evaluated by amperometric detection of H_2O_2 at 0.7 V vs Ag|AgCl. By increasing the number of cycles, the biosensor response became higher with a maximum for three cycles then the response decreased again.

The influence of the pH was also investigated by amperometric detection of 10 mM H_2O_2 in phosphate buffer 0.1 M at different pH the range 4.0–8.0. As shown in Fig. 4, the highest current intensity was obtained at pH 6.2. Each assay was repeated three times per each pH value. The pH 6.2 was selected for the biosensor response.

Performance of biosensor

The performance of the PP2ABA-enzyme biosensor was measured at room temperature. Figure 5 displays a typical amperometric response of the PP2ABA-enzyme biosensor after addition of various concentrations of glucose at +0.7 V vs Ag/AgCl in stirred PBS of pH 6.2. With successively increasing concentration of glucose, the biosensor shows rapid and sensitive response to the change of glucose concentration. The response time for the electrode is less than 5 s and under optimum conditions, the linear range of calibration curve is from 3 to 40 mM with a sensitivity of 0.058 μA mM^{-1} cm^{-2} and a limit of detection of 0.5 mM. Reproducibility of the biosensor was evaluated from the response for 10 mM glucose at six different biosensors and RSD value of 4.5% was observed. The enzyme electrode was found to be stable for 4 days when electrode was stored at 5 °C as dry. The apperent Mishaelis–Menten constant K_M is calculated to be 1×10^{-2} mM which indicates maximal catalytic activity of the enzyme at low substrate concentrations.

In real matrix, some electroactive species may involve the electrochemical reaction and affect the biosensor response such as ascorbic acid, uric acid, etc. The main contribution of presence of carboxylate group in the polymer structure is the exclusion of the matrix effects. The effect of these interfering substances was tested at their normal physiological concentrations. The addition of 0.05 mM ascorbic acid and 0.2 mM uric acid exhibits no effect on the response of PP2ABA-enzyme biosensor as shown in Fig. 6.

Conclusion

A functionalized stable film of PP2ABA was used as suitable matrix for enzyme immobilization and electrocatalysis. Combination with glucose oxidase, obtained conducting polymer electrode was used to fabricate a glucose biosensor. The advantage of this biosensor over previously developed sensors includes ease of preparation and rapid response time. Also, the contribution of potential interferences such as ascorbic acid and uric acid on the measured current value was not observed. The PP2ABA may offer a broad potential range for electroanalytical applications. Obtained conducting film electrode can also be easily derivatized by covalent coupling with carboxylate functional group for desired applications.

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