

AN AMPEROMETRIC BIOSENSOR BASED ON COVALENT IMMOBILIZATION OF ASCORBATE OXIDASE ON BIOCOMPATIBLE AND LOW-TOXIC POLY(THIOPHENE-3-ACETIC ACID) MATRIX*

Dong Li^{a, b}, Yang-ping Wen^{a, b}, Jing-kun Xu^{a**}, Hao-hua He^{b**} and Ming Liu^{a, b}

^a Jiangxi Key Laboratory of Organic Chemistry, Jiangxi Science and Technology Normal University, Nanchang 330013, China

^b Key Laboratory of Crop Physiology, Ecology and Genetic Breeding, Ministry of Education, Key Laboratory of Physiology, Ecology and Cultivation of Double Cropping Rice, Ministry of Agriculture, Jiangxi Agricultural University, Nanchang 330045, China

Abstract The biocompatible and low-toxic poly(thiophene-3-acetic acid) (PTAA) matrix was successfully electrosynthesized in ionic liquid 1-butyl-3-methylimidazolium hexafluorophosphate (BmimPF₆) in comparison with the electrosynthesis of PTAA matrix in acetonitrile (ACN). Ascorbate oxidase (AO) was used as a model for the development and application of biosensor. Vitamin C (VC) biosensors were facilely fabricated by the covalent immobilization of AO molecules on PTAA matrices electrosynthesized in ACN containing tetrabutylammonium tetrafluoroborate and BmimPF₆, respectively. Electrochemical impedance spectroscopy, scanning electron microscopy and FTIR spectroscopy indicated that AO molecules were covalently immobilized on PTAA matrices. Parameters of the as-obtained biosensors such as working potential, pH and temperature have been optimized. The amperometric biosensor based on PTAA matrix electrosynthesized in BmimPF₆ exhibited wider linear range, lower detection limit, higher sensitivity and bioaffinity, and better operational and storage stability than that electrosynthesized in ACN under optimal conditions. The as-obtained biosensor based on PTAA matrix electrosynthesized in BmimPF₆ was employed for the detection of VC content in commercial juices, and the result was close to the data given by manufacturers. Excellent results indicate that the PTAA matrix electrosynthesized in ionic liquid is a promising platform for the covalent immobilization of biologically-active species and the development of biosensors.

Keywords: Poly(thiophene-3-acetic acid); Ionic liquids; Ascorbate oxidase; Vitamin C; Biosensor.

INTRODUCTION

The conducting poly(thiophene-3-acetic acid) (PTAA), as one of inherently conducting polymers (ICPs) substituted with functional groups, has attracted much attention due to its high electrical conductivity, good environmental stability and excellent electrochemical activity^[1–3]. More importantly, PTAA is a promising candidate for the covalent immobilization of biologically-active species because of its carboxylic group. A number of studies have been reported on the covalent immobilization of protein or DNA biomacromolecules on PTAA matrices^[4–6]. However, these PTAA matrices, because of the high polymerization potential of the monomer, were usually electrosynthesized in traditional organic solvents such as acetonitrile (ACN),

* This work was financially supported by the NSFC (Nos. 50963002, 51073074), Key Projects in the National Science & Technology Pillar Program in the Eleventh Five-year Plan Period (Nos. 2006BAD02A04, 2006BAD01A01), Jiangxi Provincial Department of Science and Technology (2006BAD01A01-2-5).

** Corresponding author: Jing-kun Xu (徐景坤), E-mail: xujingkun@tsinghua.org.cn
Hao-hua He (贺浩华), E-mail: hhhua64@163.com

Received November 30, 2011; Revised February 29, 2012; Accepted March 12, 2012
doi: 10.1007/s10118-012-1167-6

chlorobenzene and chloroform^[3–7] containing supporting electrolytes like tetrabutylammonium tetrafluoroborate, tetraethylammonium perchlorate and lithium perchlorate trihydrate^[4–7], which were not beneficial for application in biosensors due to high toxic and poor biocompatibility of traditional organic solvents. Consequently, the synthesis of high-biocompatible and low-toxic PTAA matrix was very interesting and significant in the development of efficient biosensors, but still a great challenge.

Ionic liquids (ILs) are viewed as a novel class of organic molten electrolytes, consist of negatively and positively charged ions at ambient temperature. They have interesting specific physical and chemical properties, which have attracted considerable attention in both industry and academic research as diverse as electrochemistry, synthesis, catalysis, materials, separations, extraction and biotechnology^[8–12]. Thus, some ACS meetings were dedicated to ILs^[13–16], and also some papers on journals including Nature and Science^[8–12], reviews^[17–20] and books^[21, 22] were published collecting the beginning of the research performed on and about ILs. Compared with traditional organic solvents, ILs have environmentally benign features derived from non-volatility and non-flammability, negligible vapor pressures, high thermal stability, broad liquid temperature ranges and high specific solvent abilities. As electrolytes with high ionic conductivity and wide potential window, ILs are expected to be particularly suitable media for electropolymerization. The electrochemistry of ICPs in ionic liquids (ILs) has attracted considerable attention in recent years^[23–25]. Moreover, the utility of ICPs in ILs is very promising for actuators, batteries, supercapacitors, light-emitting electrochemical cells, electrochromic windows and displays, photovoltaic cells, sensors and biosensors^[26–29]. However, there had no report on the electrosynthesis of PTAA in ILs for the covalent immobilization of biologically-active species.

In this paper, we report the covalent immobilization of ascorbate oxidase (AO) molecules on the biocompatible and low-toxic PTAA matrix electrosynthesized in BmimPF₆ in comparison with PTAA matrix electrosynthesized in ACN for fabricating amperometric biosensors. Working parameters and performance of biosensors, together with the practical detection of the VC content in commercial juices were investigated in detail.

EXPERIMENTAL

Materials

AO (from cucumber *Cucurbita sp.*, Enzyme Committee 1.10.3.3, 250 U mg^{−1}) was purchased from Aldrich. Thiophene-3-acetic acid (TAA) and *N*-hydroxysuccinimide (NHS) were obtained from TCI. 1-Butyl-3-methylimidazolium hexafluorophosphate (BmimPF₆) was purchased from Center for Green Chemistry and Catalysis, LICP, CAS. Tetrabutylammonium tetrafluoroborate (Bu₄NBF₄) was purchased from Acros Organics. Acetonitrile (ACN) was obtained from Tianjin Guangfu Fine Chemical Research Institute. 1-Ethyl-3-(3-dimethylamio)propyl carbodiimide hydrochloride (EDC·HCl) was obtained from Sigma. Disodium hydrogen phosphate dodecahydrate (Na₂HPO₄·12H₂O) and sodium dihydrogen phosphate dihydrate (NaH₂PO₄·2H₂O) were purchased from Sinopharm Chemical Reagent Co. Ltd. 50 mmol/L phosphate-buffered saline solution (PBS, pH 6.5) was prepared from 50 mmol/L NaH₂PO₄·2H₂O aqueous solution and 50 mmol/L Na₂HPO₄·12H₂O aqueous solution. Vitamin C (VC) was purchased from Bio Basic Inc. All reagents were analytical grade, and were used as received without further purification. All solutions were prepared using deionized water.

Electrochemical Measurements

Electrochemical polymerization and measurements were performed in a one-compartment cell. A platinum disc electrode with a diameter of 1 mm was acted as working electrode, a platinum wire with a diameter of 0.5 mm was used as counter electrode, and the reference electrode was a saturated calomel electrode (SCE). They were placed 0.5 cm apart during the experiments. The platinum disc electrode was polished with alumina (Al₂O₃, 0.05 μm), rinsed sequentially by ultrasonic cleaning in acetone, ethanol and deionized water for 5 min and dried in air. All solutions (except for the solutions containing VC) were deoxygenated by bubbling with dry argon for 10 min prior to each experiment. The thickness of PTAA films was controlled by the total charge passing

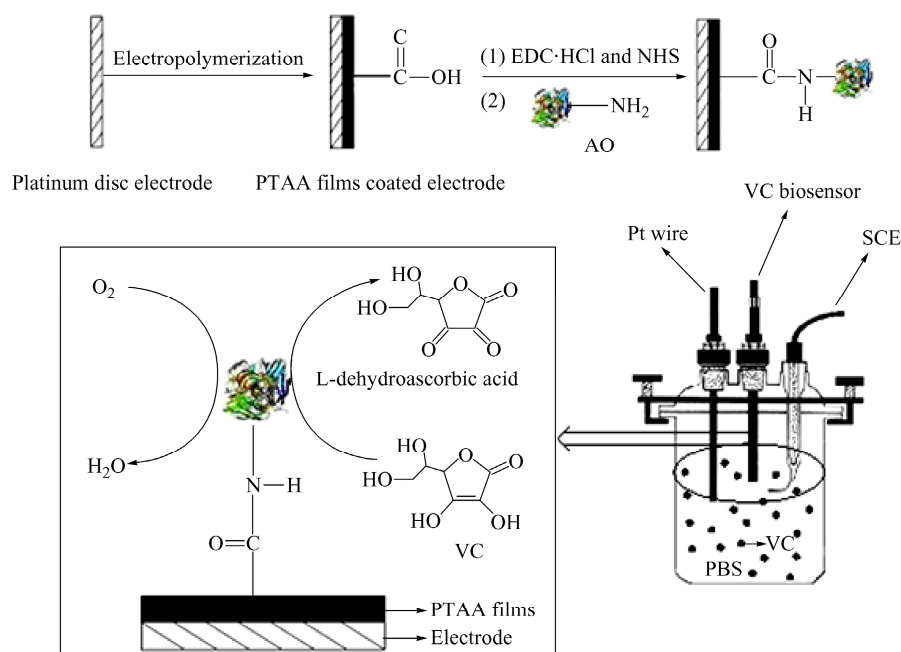
through the cell, which was read directly from the current-time curves by computer. For comparison studies of PTAA films electrosynthesized in two different solvent systems (ACN containing 0.1 mol/L Bu_4NBF_4 and BmimPF_6), the identical electropolymerization time and potential were selected for the preparation of PTAA films.

Electrochemical Preparation of PTAA Films

PTAA films were prepared by one-step potentiostatical polymerization of 0.5 mol/L TAA at 2.2 V versus SCE for 90 s in ACN containing 0.1 mol/L Bu_4NBF_4 and in BmimPF_6 , respectively. The as-obtained PTAA films were washed with deionized water to remove loosely adsorbed monomer and supporting electrolyte from the working electrode surface.

Covalent Immobilization of AO Molecules on PTAA Matrices

After PTAA matrices in two different solvent systems (ACN containing 0.1 mol/L Bu_4NBF_4 and BmimPF_6) were electrosynthesized on the platinum disc electrode surface, enzyme molecules were immobilized by the covalent attachment method. Initially, carboxylic groups of PTAA matrices were activated by immersing in a solution of 15 mmol/L EDC·HCl and 30 mmol/L NHS in 50 mmol/L PBS (pH 6.5) for 1.5 h at room temperature, and then immersed in PBS (pH 6.5) containing 0.3 mg mL^{-1} AO at 4°C for 1.5 h to covalently immobilize AO molecules. During the above process, peptide bonds formed between activated carboxylic groups and free amino groups of AO molecules leading to the formation of VC biosensor. The as-prepared biosensor was rinsed with PBS (pH 6.5) several times to remove free AO molecules and kept in PBS (pH 6.5) at 4°C when not in use. The fabricated process of VC biosensor is illustrated in Scheme 1.



Scheme 1 The fabricated process and working mechanism of VC biosensor

Working Mechanism of VC Biosensor

Amperometry technique was used to measure VC. The biocatalytical process for the oxidation of VC in the presence of AO can be summarized as follows:



AO could catalyze the oxidation of VC to L-dehydroascorbic acid and water with the oxygen present in

solution, and then the PTAA matrix acted as an electron-transfer mediator (Scheme 1).

Detection of VC Content in Commercial Juices

All commercial juices used in our tests were purchased from a local supermarket. VC sample solutions were prepared from the commercial juices containing VC and 50 mmol/L PBS (pH 6.5). Finally, the as-fabricated biosensor was used to detect all kinds of VC sample solutions. These values obtained by as-fabricated biosensors were compared to those given by the manufacturers.

Characterization

All electrochemical experiments were carried out with a three-electrode cell equipped with a potentiostat-galvanostat (Model 263A, EG&G Princeton Applied Research). Scanning electron microscopy (SEM) measurements were performed with a Hitachi S-3000N scanning electron microscope. pH values were measured with a Delta 320 pH meter (Mettler-Toledo Instrument, Shanghai, China). Infrared spectra were recorded using a Bruker Vertex 70 FTIR spectrometer with KBr pellets. Temperature was controlled with a HHS thermostat (Shanghai, China). The electrochemical impedance spectroscopy (EIS) investigations were performed with an Autolab Frequency Response Analyzer System (AUT30. FRA2-Autolab, Echemie, B.V.) in the frequency range from 100 kHz down to 10 mHz using an alternating voltage amplitude of 10 mV.

RESULTS AND DISCUSSION

Electrochemical Polymerization of PTAA Films

Cyclic voltammograms (CVs) of TAA in two different solvent systems were represented in Fig. 1. As cyclic voltammetric scans continued, PTAA films from two different solvent systems were formed on the working electrode surface. The increase in redox wave currents implied that the amount of the polymer on the electrode was increasing. In addition, the potential shift of the current wave maximum provided information about the increase of the electrical resistance of the polymer film and the over-potential needed to overcome this resistance^[30]. All these phenomena indicated that high-quality PTAA films were formed on the working electrode surface. However, from the first cycle of CVs, the oxidation onset potential of TAA was estimated to be 1.66 V versus SCE in ACN, which was relatively higher than that in BmimPF₆ (1.38 V versus SCE). This implied that the oxidation of TAA in BmimPF₆ was easier than that in ACN. Generally, the lower is the oxidation potential, the less possible are the side polymerization reactions. From this point of view, the IL is more appropriate for electrochemical syntheses of PTAA films.

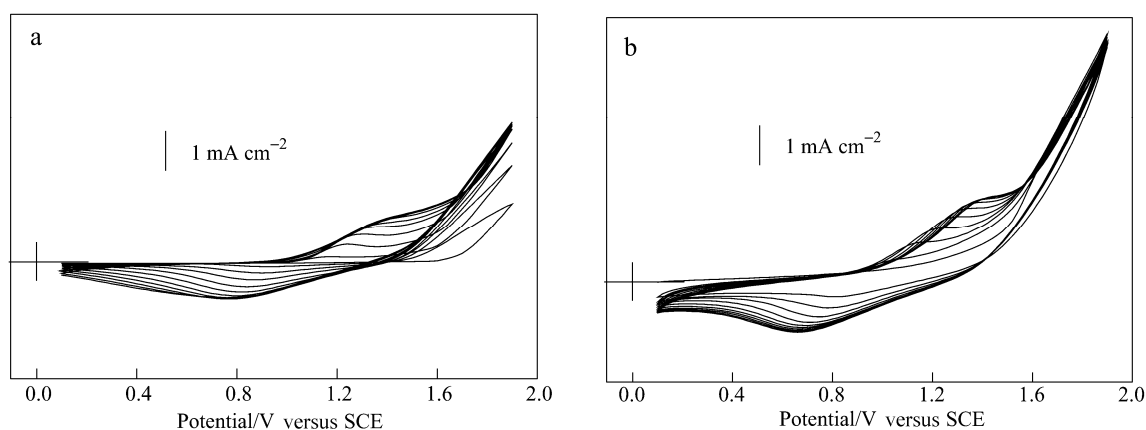


Fig. 1 CVs of 0.5 mol/L TAA in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b)
Potential scan rates 100 mV s⁻¹

Electrochemical Behaviors of PTAA Films

Electrochemical behaviors of as-formed PTAA films electrosynthesized in two different solvent systems were

studied in corresponding monomer-free ACN containing 0.1 mol/L Bu_4NBF_4 (Fig. 2A) and in corresponding monomer-free BmimPF_6 (Fig. 2B), respectively. Steady-state CVs represented broad anodic and cathodic peaks. Peak current densities were proportional to scanning rates (Fig. 2, inset), indicating a redox couple fixing on the electrode^[31]. Furthermore, these ICPs films could be repeatedly cycled between the conducting (oxidized) and insulating (neutral) state without significant decomposition, indicating the high redox stability of the polymer. Additionally, from the figure, the PTAA films could be oxidized and reduced from 0.66 V (anodic peak potential, E_a) to 0.56 V (cathodic peak potential, E_c) in ACN, and from 0.36 V (E_a) to 0.33 V (E_c) in BmimPF_6 . The difference of ($E_a - E_c$) related to the kinetics of doping-dedoping reaction, was equal to 0.1 V in ACN and 0.03 V in BmimPF_6 , respectively. These values implied that the doping-dedoping processes of PTAA films electrosynthesized in BmimPF_6 were easier and faster than those in ACN.

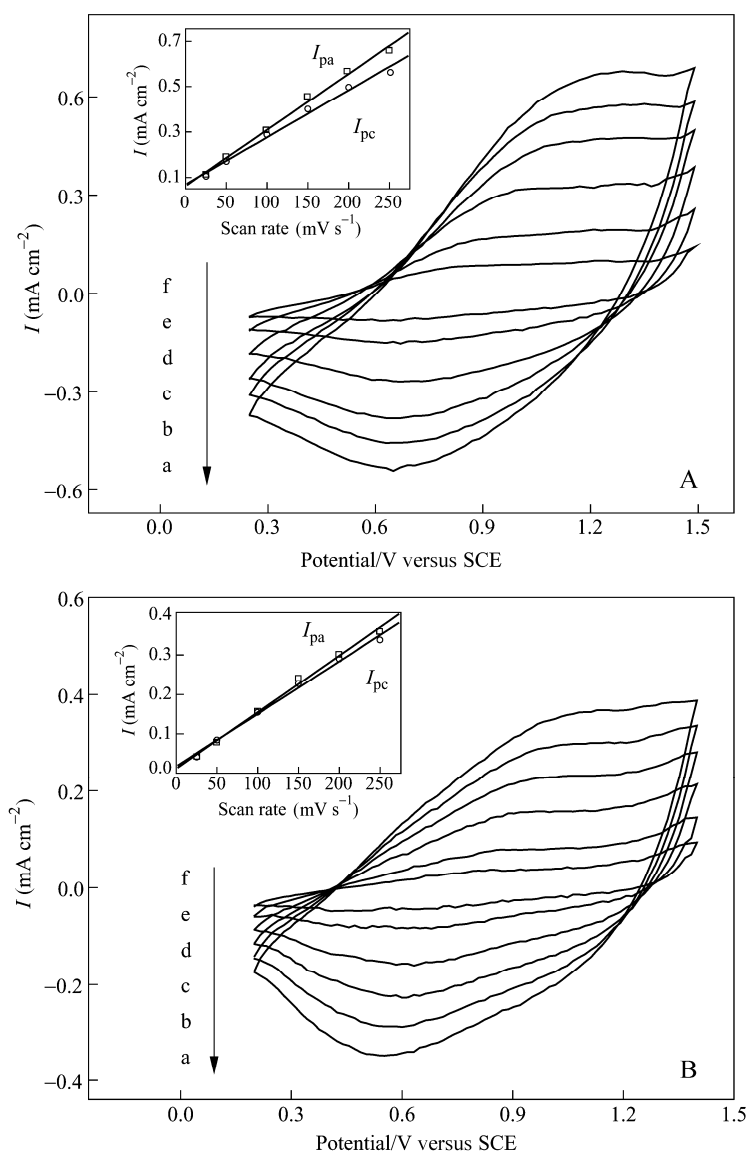


Fig. 2 CVs of PTAA films from two different solution systems (ACN solution containing 0.1 mol/L Bu_4NBF_4 and BmimPF_6) in corresponding monomer-free ACN containing 0.1 mol/L Bu_4NBF_4 (A) and in corresponding monomer-free BmimPF_6 (B) at different potential scan rates of (a) 250, (b) 200, (c) 150, (d) 100, (e) 50, and (f) 25 mV/s densities, respectively. I is the current density, and I_{pa} and I_{pc} denote the anodic and cathodic peak current density.

Impedance Plots

The electrochemical impedance spectroscopy (EIS) can offer information on impedance changes of the electrode surface during the modification process. In Nyquist plots of EIS, the semicircle part at higher frequencies corresponds to the charge transfer limited process, whereas the linear part at lower frequencies corresponds to the diffusion-controlled process^[32–34]. The semicircle diameter in the impedance spectrum equates to the charge-transfer resistance (R_{ct}) at the electrode surface. The increase or decrease in R_{ct} values exactly characterizes the modification of the electrode surface. According to Fig. 3, the lowest R_{ct} on the bare platinum disc electrode (A) indicated the fastest charge-transfer rate between the bare platinum disc electrode and the $\text{Fe}(\text{CN})_6^{3-}/\text{Fe}(\text{CN})_6^{4-}$ pair. With the PTAA matrix electrosynthesized in ACN modified electrode surface (B), the R_{ct} increases dramatically, owing to the coverage by PTAA matrix. After immobilization of AO molecules on PTAA matrix (C), R_{ct} increased accordingly in comparison with that of B. Similarly, R_{ct} values of PTAA matrix electrosynthesized in BmimPF₆ (D) were much larger than those of the bare platinum disc electrode (A), but smaller than those of the PTAA/AO films (E). Therefore, all these results implied that AO could be immobilized covalently on PTAA matrices electrosynthesized in ACN and BmimPF₆, respectively. In addition, The R_{ct} values of PTAA matrix electrosynthesized in BmimPF₆ (D) were much lower than those in ACN. The lower R_{ct} meant that PTAA matrix electrosynthesized in BmimPF₆ might mediate a faster charge transfer in the PTAA matrix and solution interface.

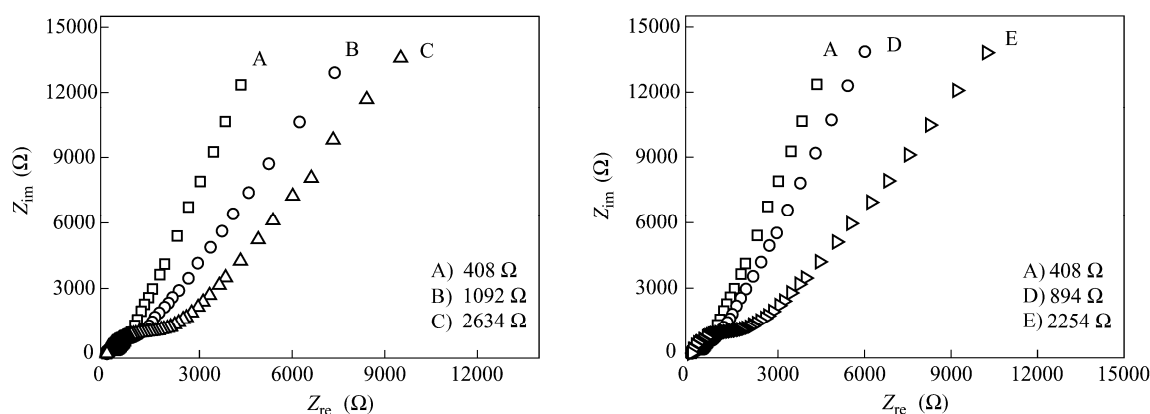


Fig. 3 EIS profiles of the bare platinum disc electrode (A), PTAA matrix electrosynthesized in ACN containing 0.1 mol/L Bu_4NBF_4 without (B) and with AO (C), and PTAA matrix electrosynthesized in BmimPF₆ without (D) and with AO (E)

Sabatani and coworkers have suggested that the change of R_{ct} is related to the apparent electrode coverage (θ) as follows: $\theta = 1 - R_{ct}^b / R_{ct}^{\text{TP}}$, where R_{ct}^b is the R_{ct} measured at a bare electrode, and R_{ct}^{TP} is the R_{ct} measured under same conditions at the thiophenol (TP)-covered electrode, respectively^[35]. From this equation one may obtained the apparent electrode coverage of TP on electrode. Therefore, the apparent electrode coverage of AO immobilized on PTAA films electrosynthesized in ACN (θ^1) was given by $\theta^1 = 1 - R_{ct}^{\text{PTAA}} / R_{ct}^{\text{PTAA-AO}}$, where R_{ct}^{PTAA} and $R_{ct}^{\text{PTAA-AO}}$ are R_{ct} of PTAA films electrosynthesized in ACN and PTAA-AO electrode based on PTAA matrices electrosynthesized in ACN, respectively. Based on the experimental data from the inset of Fig. 3, the value of θ^1 is about 59%. Similarly, the apparent electrode coverage of AO immobilized on PTAA films electrosynthesized in BmimPF₆ can be obtained ($\theta^2 = 61\%$). θ^2 was bigger than θ^1 , which indicated that AO immobilized on PTAA films electrosynthesized in BmimPF₆ possessed higher electrode coverage in comparison with that in ACN. In addition, this result further confirmed that AO molecules were covalently immobilized on PTAA films.

SEM

Surface morphologies of PTAA matrix electrosynthesized in ACN in the absence of (a) and in the presence of AO (b) and PTAA matrix electrosynthesized in BmimPF₆ in the absence of (c) and in the presence of AO (d) were observed by SEM, as shown in Fig. 4. Macroscopically, PTAA matrices electrosynthesized in two different solvent systems were homogeneous, compact and smooth. In contrast, microscopically, PTAA matrix electrosynthesized in ACN resembled globular, cauliflower-like units in agreement with previous study^[7]. However, PTAA matrix electrosynthesized in BmimPF₆ was still rather smooth, homogeneous and continuous. One of the reasons for the attaining of homogeneous PTAA matrix electrosynthesized in BmimPF₆ can be due to the fact that the high viscosity of BmimPF₆ prevents the formation of the first polymer nucleus. On the other hand, near an electrified interface structures of BmimPF₆ molecules may be much more ordered if the electric field is strong enough, which can drive a better organization of the monomer or oligomers on the electrode surface^[36]. Therefore, the use of the BmimPF₆ as both the growth medium and an electrolyte resulted in the formation of compact PTAA matrix, which may be potentially used in ion-sieving films, ionselective electrodes and matrices for hosting catalyst particles^[37]. Additionally, the surface of the two PTAA/AO films (Fig. 4b and Fig. 4d) exhibited both big, white and irregular shaped AO regions on the corresponding PTAA matrices (Fig. 4a and Fig. 4c), confirming that AO could be immobilized covalently on the PTAA matrices electrosynthesized in two different solvent systems.

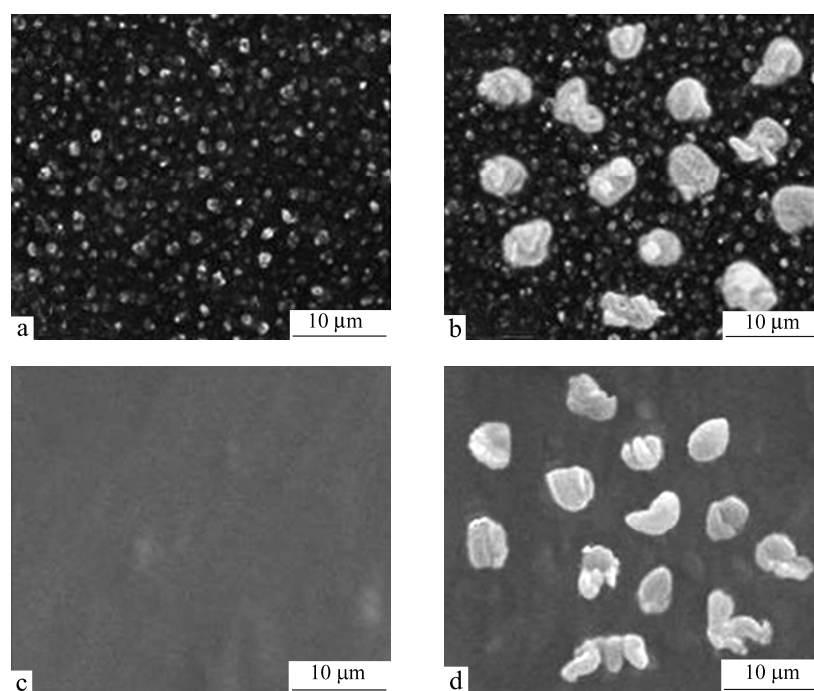


Fig. 4 The SEM images of PTAA matrix electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ in the absence of (a) and in the presence of AO (b) and PTAA matrix electrosynthesized in BmimPF₆ in the absence of (c) and in the presence of AO (d)

FTIR Spectra Analyses

Figure 5 shows FTIR spectra of PTAA matrix electrosynthesized in ACN containing Bu₄NBF₄ without (spectrum a) and with AO (spectrum b) and PTAA matrix electrosynthesized in BmimPF₆ without (spectrum c) and with AO (spectrum d). FTIR spectra (a and c) exhibit the absorption band at 3200–3500 cm⁻¹ assignable to the stretching vibration of the O–H bond and band at 1600–1900 cm⁻¹ of the C=O stretching vibration. The absorption peaks at 2800–3000 resulted from aliphatic C–H stretching vibration. All these features are attributed to the carboxylic groups at the “3” position of the thiophene ring^[38, 39]. In contrast, the additional peak

between 1520 cm^{-1} and 1550 cm^{-1} in two PTAA/AO films is assigned to the N—H bend of the AO, which confirms the formation of covalent bonds between enzyme and PTAA matrices. Besides, the O—H of carboxylic groups ($3200\text{--}3500\text{ cm}^{-1}$) vanished in spectra b and d because the carboxylic groups were linked with the enzyme. Therefore, all these described above indicated that AO molecules were successfully immobilized on PTAA matrices electrosynthesized in the two different solvent systems.

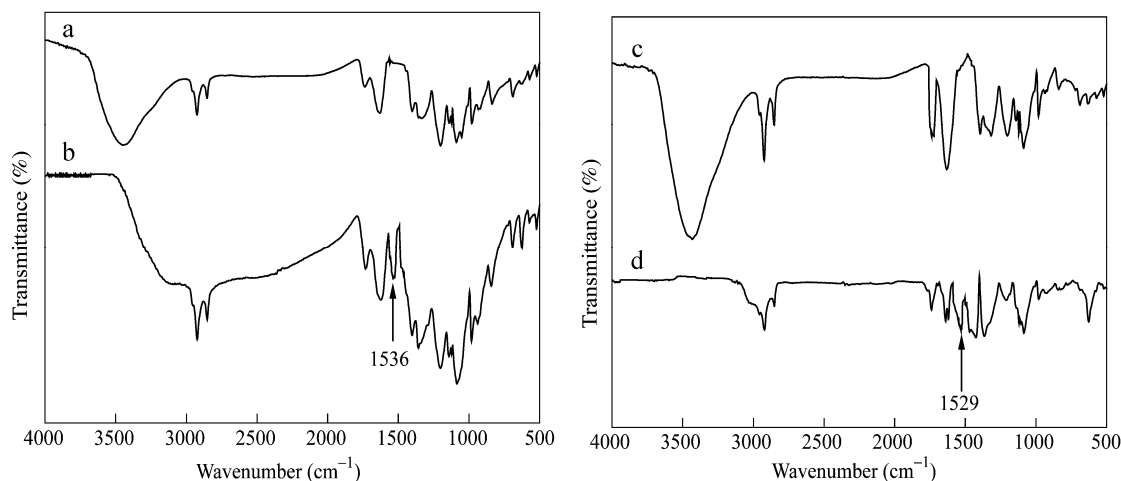


Fig. 5 FTIR spectra of PTAA matrix electrosynthesized in ACN containing 0.1 mol/L Bu_4NBF_4 without (a) and with AO (b) and PTAA matrix electrosynthesized in BmimPF_6 without (c) and with AO (d)

Optimization of Working Parameters

Optimization of working parameters for two VC biosensors based on PTAA matrices electrosynthesized in two different solvent systems, such as working potential, pH and temperature, was investigated in details.

Changes in the response current of two biosensors at different working potentials are shown in Fig. 6(A). Similar results can be observed for the two biosensors. The response current of the biosensors increased quickly for the detection of VC between 0 V and 0.4 V. When the working potential was higher than 0.4 V, the response current tended to a plateau, which could be explained by the rate-limiting process of enzyme kinetics and substrate diffusion. Considering that the higher working potential is, the easier the polymer film is to be damaged, the working potential of 0.4 V was selected in subsequent experiments.

The pH^[40, 41] is also an important factor for the bioactivity of enzyme, the effect of pH on the relative enzyme activity of two biosensors is shown in Fig. 6(B). The pH value in the range from 5 to 8 was adopted in this study. The relative enzyme activity increased from pH 5 to 6.5, while decreased above pH 6.5. The maximum relative enzyme activity of the biosensor was obtained at pH 6.5 for both biosensors. Thus, the optimal pH values of the two biosensors were adjusted to 6.5 and used in all of the subsequent experiments.

The effect of temperature on relative enzyme activity of two VC biosensors is shown in Fig. 6(C). The reaction temperature was increased from 10°C to 50°C in 5 K increments. The response current enhanced progressively with increasing temperature (10°C to 35°C), reaching a maximum response current at about 35°C and then decreased gradually. Although the two biosensors showed maximum response current at 35°C and 30°C, respectively, room temperature (25°C) was still chosen in this work for the ease of operation in the practical application and also to prevent denaturation of AO molecules at higher temperatures.

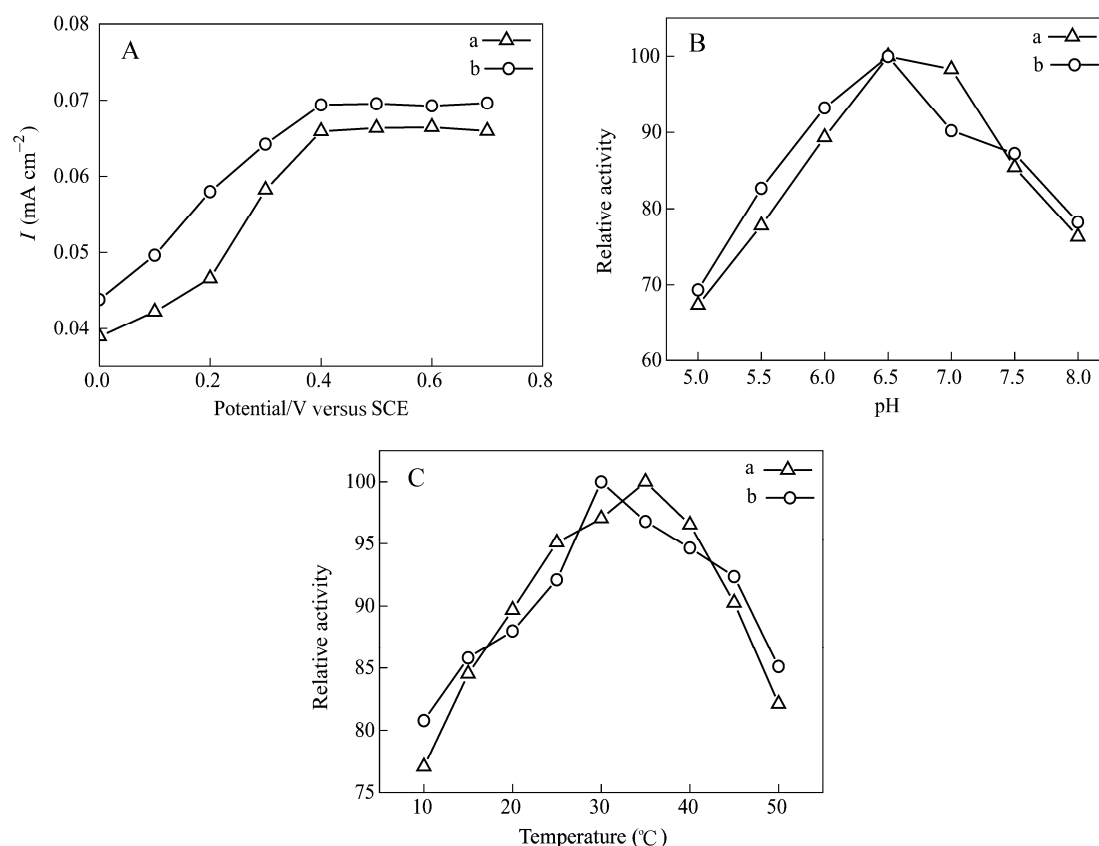


Fig. 6 (A) Effect of work potential on response current of VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively; (Response current was examined in PBS containing 1 mmol/L VC.); (B) Effect of pH on VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively; (C) Effect of temperature on VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively

Amperometric Detection of VC

The relationship between the response current of two VC biosensors based on PTAA matrices electrosynthesized in two different solvent systems and the concentration of VC is exhibited in Fig. 7. Similarly, the response current increased with increasing the concentration of VC, revealing that this method is suitable for detecting the unknown content of VC. The response current of the two biosensors was fast (within 20 s) for the detection of VC, revealing that the two biosensors both had rapid current response. The calibration plot (Fig. 7, inset) showed that the response current of the two biosensors increased linearly with increasing the concentration of VC in the range from 6.0×10^{-7} mol/L to 2.5×10^{-2} mol/L ($R^2 = 0.999$) (Fig. 7a, inset) and from 3.0×10^{-7} mol/L to 2.5×10^{-2} mol/L ($R^2 = 0.999$) (Fig. 7b, inset). The two biosensors have the detection limit of 0.114 $\mu\text{mol/L}$ ($S/N = 3$) (Fig. 7a, inset) and 0.075 $\mu\text{mol/L}$ ($S/N = 3$) (Fig. 7b, inset), and the sensitivity of 38.4 mA (mol/L)⁻¹ cm⁻² (Fig. 7a, inset) and 53 mA (mol/L)⁻¹ cm⁻² (Fig. 7b, inset), respectively. Obviously, it could be observed that VC biosensor based on PTAA matrix electrosynthesized in BmimPF₆ showed better linear range, higher sensitivity and lower detection limit in comparison with that in ACN. All results indicated that the PTAA matrix electrosynthesized in BmimPF₆ could improve biosensing performance, which was in accordance with the literature^[29, 42].

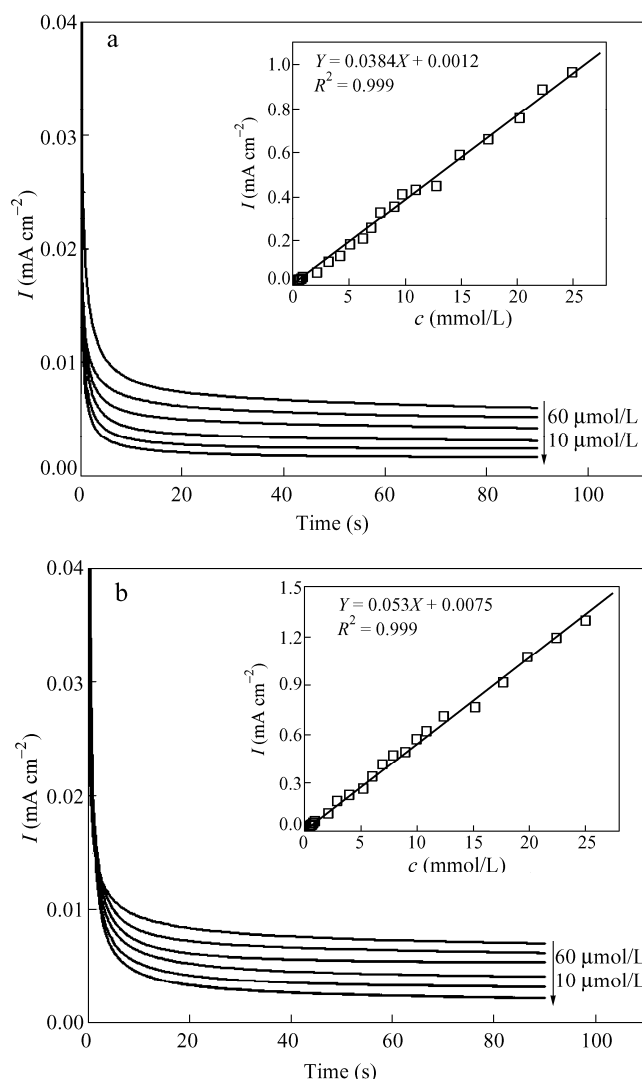


Fig. 7 The evolution of response current with the concentration of VC on the fabricated biosensors based on PTAA matrices electro synthesized in ACN containing 0.1 mol/L Bu_4NBF_4 (a) and BmimPF_6 (b), respectively. Inset: the calibration curves for the response current of the biosensors based on PTAA matrices electro synthesized respectively in ACN containing 0.1 mol/L Bu_4NBF_4 (a) and BmimPF_6 (b) versus different concentrations of VC.

Bioaffinity of VC Biosensors

The response current of two fabricated biosensors increased with increasing the concentration of VC and finally tended to a platform at VC concentrations higher than 2.5×10^{-2} mol/L (Fig. 8). The response current platform implied that AO molecules were gradually saturated with excessive substrates resulting in gradual cessation of enzymatic reaction, showing a characteristic of the Michaelis-Menten kinetic mechanism. The apparent Michaelis-Menten constant (K_m), which is an indication of both the enzymatic affinity and enzyme-substrate kinetic constants, can be calculated from the electrochemical version of the Lineweaver-Burk equation, as follows:

$$1/I = 1/I_{\max} + K_m/I_{\max}c$$

where I is the steady-state current after the addition of substrate, c is the bulk concentration of the substrate, and $I_{\max}$ is the maximum current measured under saturated substrate conditions^[43, 44]. From the curve of I^{-1} versus c^{-1} , based on experimental data from the inset of Fig. 8(b), the K_m value was estimated to be 11.02 mmol/L. The

K_m value of VC biosensor based on PTAA matrix electrosynthesized in BmimPF₆ was smaller than that in ACN (22.61 mmol/L), from the inset of Fig. 8(a), which indicated that AO molecules immobilized covalently on PTAA matrix electrosynthesized in BmimPF₆ exhibited higher bioaffinity in comparison with that in ACN.

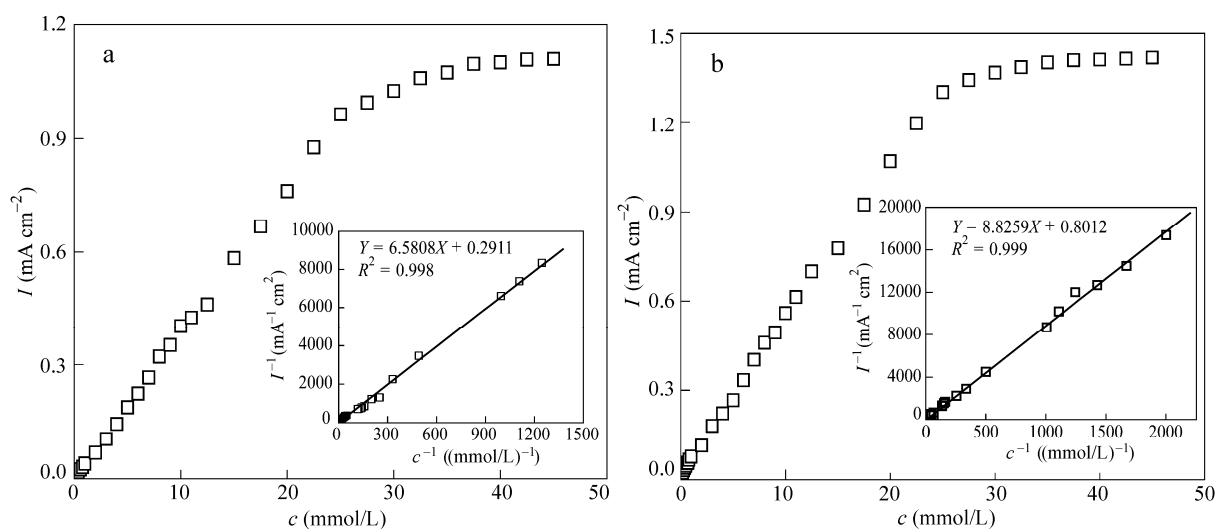


Fig. 8 The relationship between the response current of VC biosensors based on PTAA matrices electrosynthesized respectively in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and in BmimPF₆ (b) and the concentration of VC
Inset: Lineweaver-Burk plots of VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively

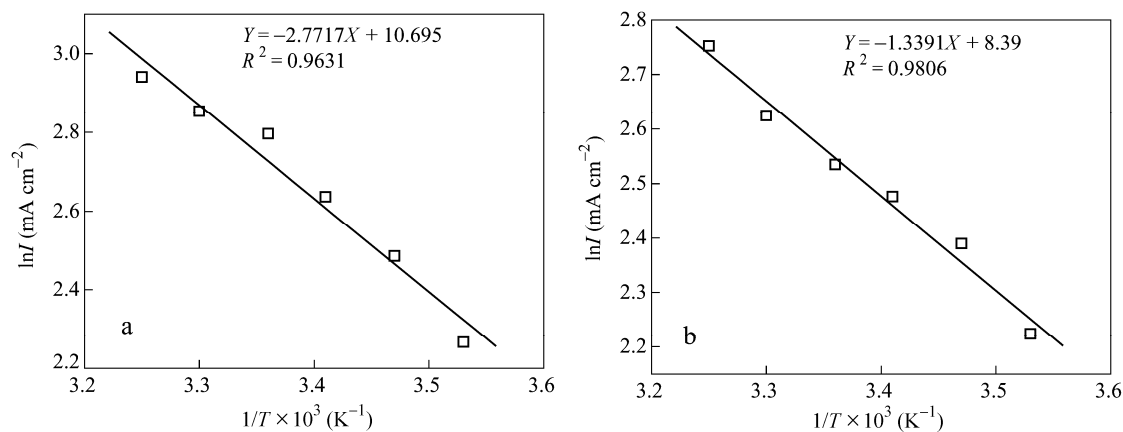


Fig. 9 The plots of the $\ln I$ versus T^{-1} for VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively

The apparent activation energy (E_a) of two fabricated biosensors was obtained by $\ln I$ versus $1/T$ graphs (Fig. 9). The dependence of response current on temperature can be expressed as an electrochemical version of the Arrhenius equation^[45, 46]:

$$\ln I = \ln I_0 + E_a/RT$$

where I_0 represents a collection of currents, R is the molar gas constant, T is the absolute temperature in Kelvin and E_a is the activation energy. E_a values of the two biosensors were calculated as 23.03 kJ (mol/L)⁻¹ (Fig. 9a)

and $11.13 \text{ kJ (mol/L)}^{-1}$ (Fig. 9b), respectively. Generally, the lower value of E_a meant higher bioactivity and bioaffinity. Therefore, the AO molecules immobilized covalently on PTAA matrix electrosynthesized in BmimPF₆ showed higher bioactivity and bioaffinity to substrates than those in ACN, which is well consistent with the K_m result. In conclusion, all these results indicated that the electrosynthesis of PTAA matrix in ACN due to its higher toxicity and lower biocompatibility was not suitable for maintaining the long-term bioactivity of AO molecules.

Stability of VC Biosensors

One of the most important performance parameters of biosensors is their stability^[47]. The operational stability of two VC biosensors based on PTAA matrices electrosynthesized in two different solvent systems is given in Fig. 10(A) and the inset of Fig. 10(A). The relative standard deviation (RSD) of the two biosensors was 2.93% (Fig. 10A) and 1.22% (Fig. 10A inset) for 20 successive assays under the same condition, respectively. The higher value of RSD indicated that the PTAA matrix electrosynthesized in ACN had a negative influence on enzyme bioactivity due to higher toxicity of traditional organic solvent. Moreover, Hence, VC biosensor based on PTAA matrix electrosynthesized in BmimPF₆ exhibited better operational stability than that in ACN.

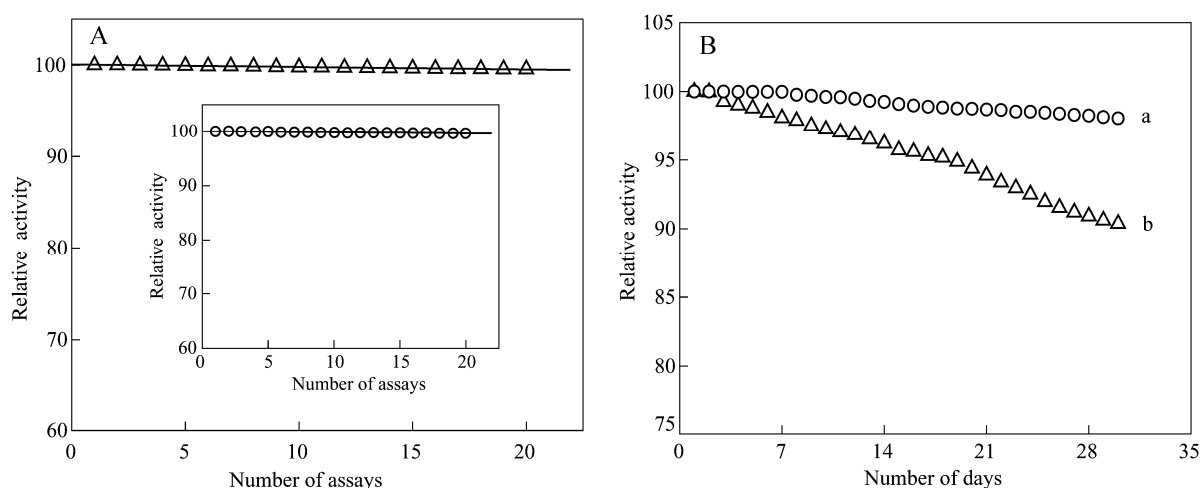


Fig. 10 (A) The operational stability of VC biosensor based on PTAA matrix electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (Inset: The operational stability of the biosensor based on PTAA matrix electrosynthesized in BmimPF₆); (B) The storage stability of VC biosensors based on PTAA matrices electrosynthesized in ACN containing 0.1 mol/L Bu₄NBF₄ (a) and BmimPF₆ (b), respectively

Figure 10(B) illustrates the storage stability of two VC biosensors based on PTAA matrices electrosynthesized in two different solvent systems. The biosensor based on PTAA matrix electrosynthesized in ACN lost 10% of its original response after 30 days of storage. For comparison, the amperometric response of the biosensor based on PTAA matrix electrosynthesized in BmimPF₆ retained almost 100% of its original activity when it was stored for 7 days, and 97% of its original activity was retained on the 30th day. This result indicated the VC biosensor based on PTAA matrix electrosynthesized in BmimPF₆ had better storage stability than that in ACN. The main reason is that PTAA matrix electrosynthesized in BmimPF₆ could possess lower toxicity and better biocompatibility in comparison with that in ACN, which is a very good choice for the development and application of biosensors.

Detection of VC in Commercial Juices

Table 1 gives analytical results for the detection of VC in commercial juices. Values obtained by using two as-fabricated biosensors were in agreement with those given by the manufacturers. This also showed the reliability and feasibility of the present method. In comparison, the biosensor based on PTAA matrix electrosynthesized in BmimPF₆ had a result closer to the data given by manufacturers than that in ACN. Therefore, these good results

also indicated the PTAA matrix electrosynthesized in BmimPF₆ could be employed for the covalent immobilization of biologically-active species and the development and application of biosensors.

Table 1. The detection of VC in commercial juices using the as-fabricated biosensors

Commercial juices	[VC] (mg/mL)	VC content A ^b		VC content B ^c	
		Mean $\pm$ SD (mg/mL) ^a	RSD (%)	Mean $\pm$ SD (mg/mL) ^a	RSD (%)
Vitamin water	0.2	0.1913 $\pm$ 0.00096	0.500	0.1979 $\pm$ 0.00073	0.037
Mizone	0.2	0.1937 $\pm$ 0.000267	0.138	0.1985 $\pm$ 0.000112	0.056
Lemon juice	0.225	0.2199 $\pm$ 0.00092	0.418	0.2208 $\pm$ 0.00046	0.210
Grape juice	0.1	0.09924 $\pm$ 0.00066	0.670	0.09938 $\pm$ 0.00058	0.580

^a Average value of five replications; ^b VC content measured by biosensor with PTAA matrix electrosynthesized in CAN; ^c VC content measured by biosensor with PTAA matrix electrosynthesized in BmimPF₆; SD: standard deviation; RSD: relative error

CONCLUSIONS

In conclusion, two amperometric biosensors were fabricated by immobilizing covalently AO molecules on the PTAA matrices electrosynthesized in two different solvent systems (ACN containing 0.1 mol/L Bu₄NBF₄ and BmimPF₆). The fabricated biosensor based on PTAA matrix electrosynthesized in BmimPF₆ exhibited better linear range, faster current response, lower detection limit, higher enzyme activity and bioaffinity, and better operational and storage stability in comparison with that in ACN containing 0.1 mol/L Bu₄NBF₄. The good performance of the as-fabricated biosensors based on PTAA matrix electrosynthesized in BmimPF₆ and good results for the detection of VC in commercial juices will be more beneficial to agricultural applications in the near future, and the high-performance PTAA matrix electrosynthesized in BmimPF₆ also provides an interesting candidate for the design and application of biosensors.

REFERENCES

- Huang, H. and Yang, X., *Colloids Surf. A*, 2005, 255: 11
- Giglioti, M., Trivinho-Strixino, F., Matsushima, J.T., Bulhoes, L.O.S. and Pereira, E.C., *Sol. Energ. Mat. Sol. C*, 2004, 82: 413
- Malitesta, C., Guascito, M.R., Mazzotta, E. and Picca, R.A., *Thin Solid Films*, 2010, 518: 3705
- Liu, C., Kuwahara, T., Yamazaki, R. and Shimomura, M., *Eur. Polym. J.*, 2007, 43: 3264
- Nien, P.C., Chen, P.Y. and Ho, K.C., *Sensors*, 2009, 9: 1801
- Lin, M., Cho, M.S., Choe, W.S. and Lee, Y., *Biosens. Bioelectron.*, 2009, 25: 29
- Toth, P.S., Janaky, C., Hiezl, Z. and Visy, C., *Electrochim. Acta*, 2011, 56: 3448
- Seddon, K.R., *Nat. Mater.*, 2003, 2: 363
- Earle, M.J., Esperança, J.M.S.S., Gilea, M.A., Lopes, J.N.C., Rebelo, L.P.N., Magee, J.W., Seddon, K.R. and Widegren, J.A., *Nature*, 2006, 439: 831
- Rogers, R.D., *Nature*, 2007, 447: 917
- Zhao, H., Holladay, J.E., Brown, H. and Zhang, Z.C., *Science*, 2007, 316: 1597
- Harris, K.R. and Kanakubo, M., *Faraday Discuss.*, 2012, 154: 425
- Rogers, R.D. and Seddon, K.R., "Ionic liquids: industrial applications for green chemistry, ACS Symposium Series 818", American Chemical Society, Washington DC, 2002
- Rogers, R.D. and Seddon, K.R., "Ionic liquids as green solvents: progress and prospects, ACS Symposium Series 856", American Chemical Society, Washington DC, 2003
- Rogers, R.D. and Seddon, K.R., "Ionic liquids IIIA: fundamentals, progress, challenges, and opportunities: properties and structure, ACS Symposium Series 901", American Chemical Society, Washington DC, 2005

- 16 Rogers, R.D. and Seddon, K.R., "Ionic liquids IIIB: fundamentals, progress, challenges, and opportunities: transformations and processes, ACS Symposium Series 902", American Chemical Society, Washington DC, 2005
- 17 Welton, T., *Chem. Rev.*, 1999, 99: 2071
- 18 Buzzeo, M.C., Evans, R.G. and Compton, R.G., *Chem. Phys. Chem.*, 2004, 5: 1106
- 19 Pandey, S., *Anal. Chim. Acta*, 2006, 556: 38
- 20 Petkovic, M., Seddon, K.R., Rebelo, L.P.N. and Pereira, C.S., *Chem. Soc. Rev.*, 2011, 40: 1383
- 21 Seddon, K. and Rogers, R., "Ionic liquids: coiled for action", John Wiley & Sons, Inc., Hoboken, New Jersey, 2008
- 22 Shiddiky, M. and Torriero, A., "Electrochemical biosensor based on ionic liquids", Nova Science, USA, 2010
- 23 Pringle, J.M., Efthimiadis, J., Howlett, P.C., Efthimiadis, J., MacFarlane, D.R., Chaplin, A.B., Hall, S.B., Officer, D.L., Wallace, G.G. and Forsyth, M., *Polymer*, 2004, 45: 1447
- 24 Kubisa, P., *J. Polym. Sci., Part A: Polym. Chem.*, 2005, 43: 4675
- 25 Zhang, J., Lei, J., Liu, Y., Zhao, J. and Ju, H., *Biosens. Bioelectron.*, 2009, 24: 1858
- 26 Stenger-Smith, J.D., Webber, C.K., Anderson, N., Chafin, A.P., Zong, K. and Reynolds, J.R., *J. Electrochem. Soc.*, 2002, 149: 973
- 27 Ding, J., Zhou, D., Spinks, G., Wallace, G., Forsyth, S., Forsyth, M. and MacFarlane, D., *Chem. Mater.*, 2003, 15: 2392
- 28 Yang, C., Sun, Q., Qiao, J. and Li, Y., *J. Phys. Chem. B*, 2003, 107: 12981
- 29 Jiang, Y., Wang, A. and Kan, J., *Sens. Actuators, B*, 2007, 124: 529
- 30 Otero, T.F. and Larreta-Azelain, E.D., *Polymer*, 1988, 29: 1524
- 31 Fritz, B. and Paul, R., *Electrochim. Acta*, 2000, 45: 2467
- 32 Lu, L., Zhang, L., Zhang, X., Wu, Z., Huan, S., Shen, G. and Yu, R., *Electroanalysis*, 2010, 22: 473
- 33 Lu, Q. and Li, C.M., *Biosens. Bioelectron.*, 2008, 24: 769
- 34 Zhang, Z.H., Feng, X.Z., Yan, F.F., Wang, L.Z., Zhang, Z.H. and Ma, Z., *Acta Polymerica Sinica (in Chinese)*, 2011, (4): 381
- 35 Sabatani, E., Cohen-Boulakia, J., Bruening, M. and Rubinstein, I., *Langmuir*, 1993, 9: 2978
- 36 Dong, B., Song, D., Zheng, L.Q., Xu, J.K. and Li, N., *J. Electroanal. Chem.*, 2009, 633: 63
- 37 Ohno, H., "Electrochemical aspects of ionic liquids", John Wiley & Sons, Inc., Hoboken, New Jersey, 2005
- 38 Kim, B.S., Chen, L., Gong, J.P. and Osada, Y., *Macromolecules*, 1999, 32: 3966
- 39 Zhang, F.X. and Srinivasan, M.P., *Mater. Chem. Phys.*, 2008, 112: 224
- 40 Liu, J.J., Yan, Y.F. and Yao, P., *Chinese J. Polym. Sci.*, 2011, 29(4): 397
- 41 Xue, Y.N., Zhang, M., Liu, L., Li, W.X. and Huang, S.W., *Acta Polymerica Sinica (in Chinese)*, 2011, (8): 874
- 42 Santhosh, P., Manesh, K.M., Uthayakumar, S., Komathi, S., Gopalan, A.I. and Lee, K.P., *Bioelectrochemistry*, 2009, 75: 61
- 43 Wang, G., Xu, J.J., Chen, H.Y. and Lu, Z.H., *Biosens. Bioelectron.*, 2003, 18: 340
- 44 Tan, X.C., Li, M.J., Cai, P.X., Luo, L.J. and Zou, X.Y., *Anal. Biochem.*, 2005, 337: 117
- 45 Su, C.M. and Puls, R.W., *Environ. Sci. Technol.*, 1999, 33: 165
- 46 Barnett, S.J., Soutsos, M.N., Millard, S.G. and Bungey, J.H., *Cem. Concr. Res.*, 2006, 36: 439
- 47 Zhu, H., Hou, C. and Li, Y.F., *Acta Polymerica Sinica (in Chinese)*, 2011, (8): 864