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ORIGINAL PAPER

An amperometric urea biosensor based on covalent immobilization of urease on copolymer of glycidyl methacrylate and vinylferrocene

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Abstract A unique urea biosensor construction based on the direct covalent attachment of urease onto a polymeric electron transfer mediator, poly(glycidyl methacrylate-co-vinylferrocene)-coated electrode is described. Amperometric response was measured as a function of urea concentration, at a fixed potential of +0.35 V vs. Ag/AgCl in phosphate-buffered saline (pH 7.0). Covalent immobilization of the urease directly to the functionalized ferrocene copolymer surface produced biosensors with a short response time (about 3 s) and provided low detection limits. The stability, reusability, pH, and temperature response of the biosensor, besides its kinetic parameter, were also studied.

Keywords Mediator · Ferrocene · Covalent immobilization · Urease · Biosensor

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Introduction

Urea is widely distributed in nature, and its detection is greatly significant in biomedical and clinical analysis. The measurement of blood urea is the best way to evaluate kidney function and identify kidney disease [1]. An increase in the urea concentration (normal level in serum ranges from 15 to 40 mg/dL) causes renal failure, such as acute or chronic urinary tract obstruction with shock, burns, dehydration, and gastrointestinal bleeding. However, a decrease in urea concentration causes hepatic failure, nephritic syndrome, and cachexia [2]. Therefore, there is a pressing need to develop a device that can rapidly measure the blood urea concentration. The enzyme-based biosensor systems are generally performed to determine concentration of urea. A substrate active enzyme layer is embedded on a suitable surface of a classical sensor, which measures the concentration of products formed during the course of enzymatic reaction.

Immobilization of urease onto a suitable matrix is crucial in developing an electrochemical urea biosensor [3–7]. In this context, many electrode materials, polymers, sol–gels, conducting polymers, Langmuir-Blodgett films, nanomaterials, and self-assembled monolayers have been used to obtain enzyme biosensors. Recently, composite materials based on conducting polymers, redox mediators, metal nanoparticles, nanocomposites, and nanoclusters have been used to combine the properties of the individual components with a synergistic performance in biosensor fabrication [3]. Amperometric biosensors appear promising in determining urea concentration due to their effectiveness and simplicity. Today, pH-sensitive amperometric biosensors are most commonly utilized, as they can be used in turbid media and have instrumental sensitivity and amenability to miniaturization [8–10].

The development of a polymeric mediator for applications in sensor/biosensor is essential because polymers allow the incorporation of reagents to achieve reagentless devices. Ferrocene derivatives are widely used as mediators in mediated-amperometric biosensor construction. Direct attachment of the ferrocene-based mediators onto polymeric films prevents leaching of the mediator. Some examples of redox copolymers where covalent attachment of ferrocene has been attempted are poly(vinylferrocene-co-hydroxyethyl methacrylate) [11], poly(*N*-acryloylpyrrolidine-co-vinylferrocene) [12], acrylamide copolymers [13], ferrocene-polythiophene derivative [14], and multiwall carbon nanotubes [15].

The aim of this paper was to present our work on the preparation of a series of redox copolymers, poly(glycidyl methacrylate-co-vinylferrocene) by free-radical copolymerization of a vinylferrocene monomer with an epoxy group carrying a comonomer (GMA) and a direct covalent attachment of urease. This is an effective method, as the epoxy group is easily introduced into the copolymer, at a desired density, by adjusting the concentration of epoxy group carrying the comonomer in the polymerization mixture. This work illustrates the versatility of an epoxy-based redox copolymer poly(glycidyl methacrylate-co-vinylferrocene) as an immobilization platform for the fabrication of the enzyme electrode. The application of the resulting enzyme electrode in the amperometric detection of urea in an aqueous medium is described in detail.

Experimental

Materials

Glycdyl methacrylate (GMA) and 2,2′-azobis(isobutyronitrile) (AIBN) were purchased from Fluka and Across Chemical Co. and used without further purification. Reagent-grade vinylferrocene (VFc) was purchased from Aldrich Co. and used without purification. Urease (EC 3.5.1.5, from jack beans) was obtained from sigma. All other chemicals were of analytical grade and were used without further purification.

Preparation of poly(GMA-co-VFc)

The redox polymer, having different compositions, was prepared according to literature [16]. A mixture of VFc and GMA at a known molar ratio was injected into a Pyrex tube, AIBN (1 mol%). The mixture was degassed with argon and sealed under vacuum. After degassing, the tubes were placed in a constant-temperature bath set to 70 °C. After 2 days, the reaction mixture was added drop-wise to rapidly stirred diethyl ether to precipitate the copolymer. Precipitated copolymer was washed with diethyl ether and reprecipitated two more times in this manner. The precipitated product was then dried under vacuum.

Preparation of enzyme electrodes

To evaluate the electrochemical property, 10 μL of 1 wt.% poly(glycidyl methacrylate-co-vinylferrocene) (poly(GMA-co-VFc)) in DMF was dropped onto the glassy carbon electrode. After drying at room temperature, the cyclic voltammetry (CV) of the copolymer was measured in 100 mM phosphate-buffered 0.8% saline (PBS, pH 7.0).

Urease was immobilized by covalent attachment on poly (GMA-co-VFc)-coated glassy carbon electrode (GCE). A 1 wt. % solution of poly(GMA-co-VFc) in DMF was dropped onto the electrode and dried in air. Functional epoxy group carrying copolymer film electrode was immersed in 100 mM phosphate buffer (pH 7.0) for 2 h and transferred to the same fresh medium containing Urs (2.0 mg/ml). Immobilization of Urs on the poly(GMA-co-VFc) film was carried out by continuously stirring the reaction medium at 24 °C for 24 h. After this period, electrode was removed from medium and washed with phosphate buffer (100 mM, pH 7.0). Electrochemical properties (CVs) of poly(GMA-co-VFc) and enzyme electrodes were measured in pH 7.0 100 mM PBS at a scan rate 5 mV/s.

Instrumentation

The morphology of the modified gold surfaces was imaged by atomic force microscopy (AFM, Park systems XE-100E). Imaging was carried out at ambient temperature in noncontact mode.

Electrochemical measurements were performed using a CHI Model 842B electrochemical analyzer. A small glassy carbon working electrode (2 mm diameter), a platinum wire counter electrode (0.2 mm diameter), Ag/AgCl-saturated KCl reference electrode, and a conventional three-electrode electrochemical cell were purchased from CH Instruments.

All amperometric measurements were carried out at room temperature. They were performed in stirred solutions by applying the desired potential and allowing the steady-state current to be reached. Once prepared, the Urs electrode were immersed in 10 ml of a pH 7.0 100 mM PBS solution, and the amperometric responses to the addition of known amount of urea solution were recorded, respectively. The data are the averages of three measurements.

Results and discussion

Characterization of poly(GMA-co-VFc) and Urs-immobilized electrodes

The most frequently used supports in biotechnology for covalent enzyme immobilization have been obtained after activation of natural and synthetic polymers such as chitin, alginic acid, cellulose, acrylic polymer, and polyvinyl alcohol

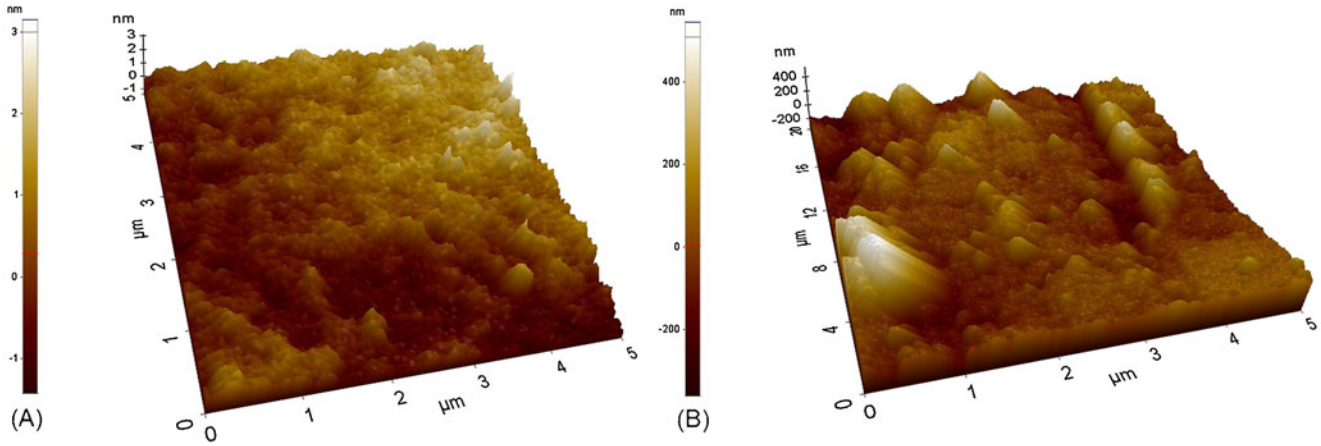

Fig. 1 AFM images of **a** poly(GMA-co-VFc) and **b** poly(GMA-co-VFc)-Urs

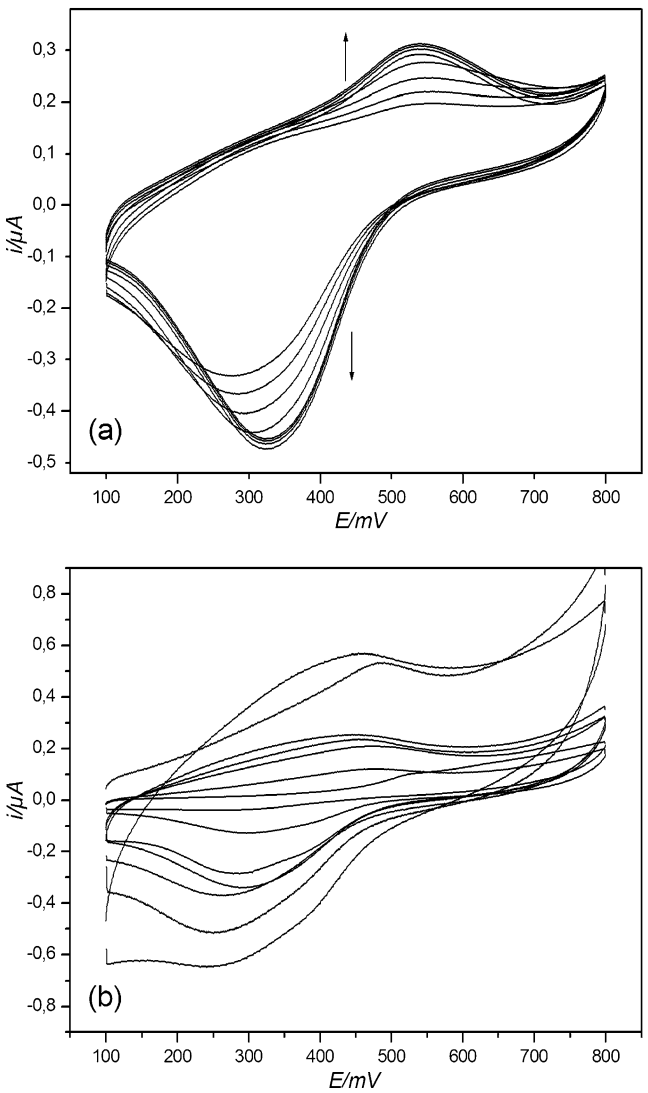

Fig. 2 Cyclic voltammogram of poly(GMA-co-VFc) in 0.1 M PBS (pH 7.4) **a** at scan rate 5 mV/s, **b** at scan rates of 1, 2.5, 5, 7.5, 10, 15, and 20 mV/s (from internal to external)

[17–19]. Poly(GMA-co-VFc) possessing a different monomer ratio was prepared from glycidyl methacrylate and vinylferrocene monomers, as described in section "Experimental" [16]. The present method proved effective as the reactive epoxy group was readily introduced into the polymer structure without any modification. The epoxy group can bind the protein molecules via their amine, thiol, hydroxy, and carboxyl groups in the pH range, where the enzyme is stable and does not lose its activity. The C–N or O–C bonds formed between the epoxy groups and biomolecules are stable, so that epoxy group containing supports enable enzyme immobilization.

AFM was used to determine the surface roughness of polymer-coated and enzyme-immobilized electrodes. A smooth surface with roughness of 3 nm was observed when the electrode surface was coated with poly(GMA-co-VFc) film alone (Fig. 1a). As shown from Fig. 1b, the surface morphology of the enzyme-immobilized electrode is obviously different from that of the polymer-coated electrode. After immobilization of enzyme onto the surface of the coated electrode, the roughness of the film surface significantly increased to ~300 nm. This indicated that the urease was successfully immobilized onto the polymer-coated electrode surface via direct covalent attachment.

The cyclic voltammograms of poly(GMA-co-VFc) were obtained between 0.1 and 0.8 V in a PBS after the copolymer was casted onto a GCE. Figure 2a shows that the anodic and cathodic currents of the copolymer film rise continuously with potential scans, until a distinct redox couple of Fc occurred and reached steady-state after 12–16 cycles (Figs. 3 and 4). After equilibrium was established, the peak potential Epa and Epc values remained constants. The steady-state values for Epa and Epc of the cyclic voltammograms shown in Fig. 5a are 0.33 and 0.54 V, respectively.

Typical cyclic voltammograms of the electrode loaded with a copolymer in PBS, when the scan rate was altered from 1 to 20 mV/s are as shown in Fig. 2b. A linear

correlation between anodic peak current Ipa and square root of scan rate $V^{1/2}$ is obtained. This result indicates that the charge propagation in the polymer occurs by a diffusion-like process, such as electron hopping among neighboring redox sites and counter-ion motion. Initially, the ferric ion in the VFc units exists in both the reduced (Fe (II)) and oxidized forms (Fe (III)). During the forward scan, Fe (II) is oxidized to Fe (III), and subsequently, an oxidation current peak is observed. During the reverse scan, Fe (III) is reduced. The difference of the redox peaks is increased with the increasing scan rate. The voltammetric behavior also indicates that ferrocene has been immobilized on the surface of the glassy carbon electrode.

Catalytic current for enzyme electrode

Figure 3 exhibits a comparison of typical amperometric responses between the enzyme electrodes fabricated separately with different compositions of poly(GMA-co-VFc). As evident in Fig. 3, the current response of the developed enzyme electrode with GMA-co-$VFc_{0.6}$ (Fig. 3c) is much higher than that of the others. The results given above demonstrate again that the synergistic use of GMA-co-$VFc_{0.6}$ can endow the as-prepared enzyme sensor with increased electrochemical performances towards direct and sensitive urea detections, including a larger loading amount and higher bio-catalysis enzyme activity.

Response of enzyme electrode to urea at constant potential

Under optimized conditions, the typical current–time response reveals a good analytical performance (Fig. 4). The catalytic current change was monitored as aliquots of urea were added in PBS solution. A well-defined reduction current proportional to the urea concentration was observed. As shown in Fig. 4, during the successive addition of 0.1 mM urea, a

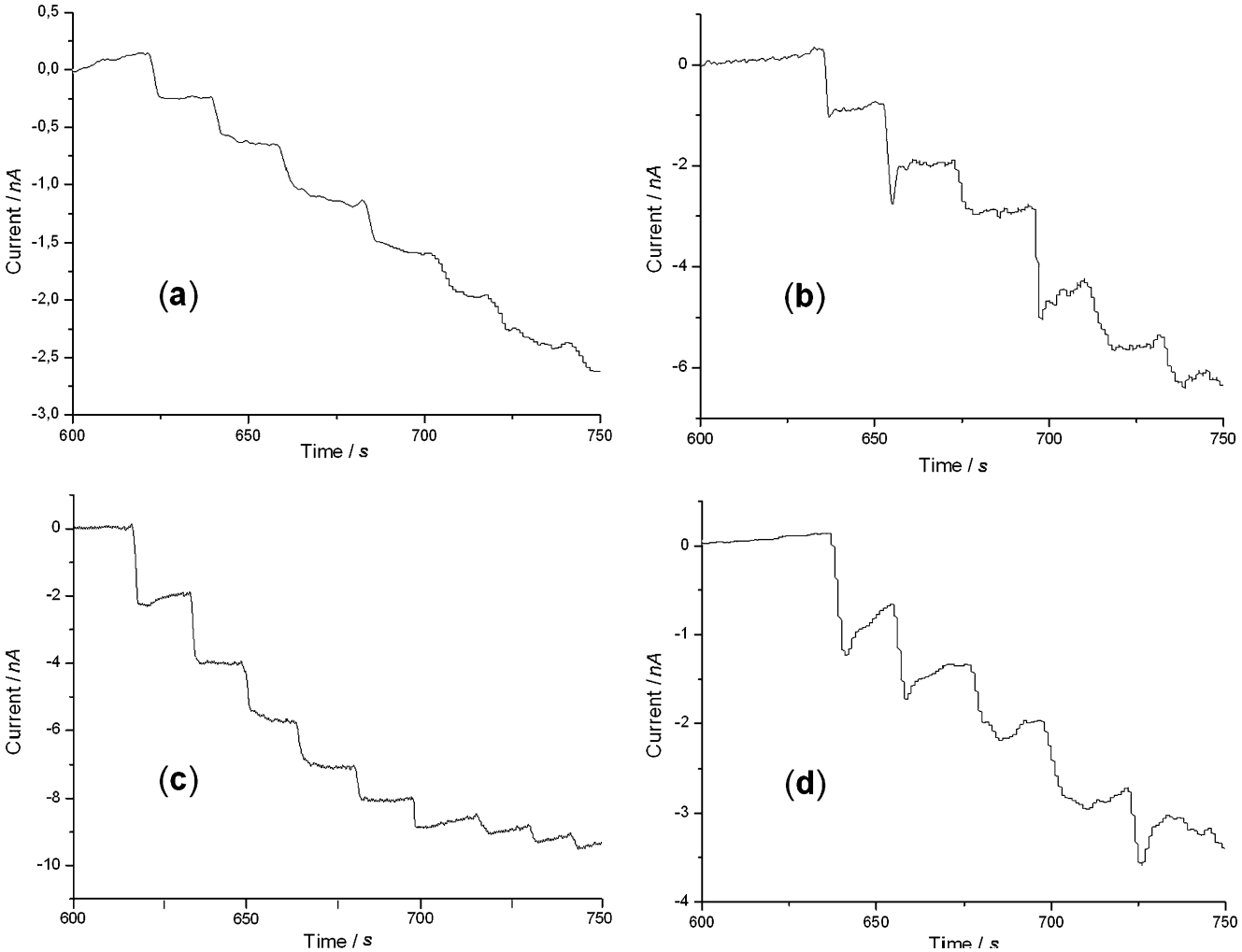

Fig. 3 Comparison of amperometric responses of the enzyme electrode fabricated with **a** GMA-co-$VFc_{0.2}$, **b** GMA-co-$VFc_{0.4}$, **c** GMA-co-$VFc_{0.6}$, and **d** GMA-co-$VFc_{0.8}$ to the successive addition of urea, at constant potential pH 7.0 10 mM PBS

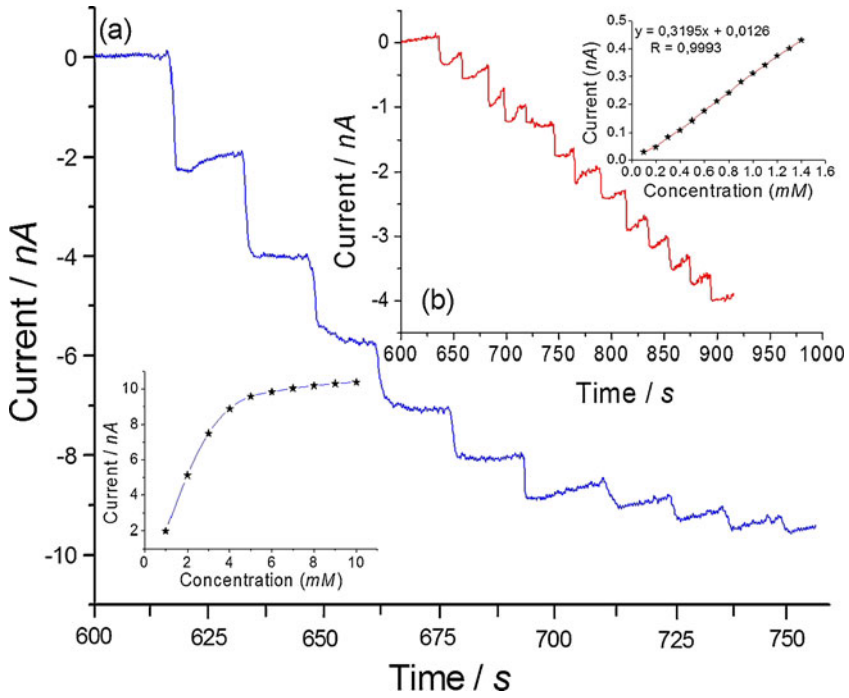

Fig. 4 Amperometric response of the enzyme electrode at constant potential pH 7.0 10 mM PBS, **a** successive addition of 1 mM and **b** 0.1 mM: *inset* plot of amperometric current vs. urea concentration, and linear calibration curve for determination of *K*m

well-defined response was noted. The plot of response current vs. urea concentration was linear over the concentration range 0.1 to 4 mM. The calibration plot over the concentration range 0.1–1.5 mM had a slope of 0.32 nA/mM (sensitivity), a correlation coefficient of 0.9993, and a detection limit of 0.06 mM. An extremely attractive feature of the enzyme electrode is its fast response time (3 s). When the concentration of urea was higher than 3 mM, a response plateau was seen, displaying the characteristics of the Michaelis–Menten kinetic mechanism. The apparent Michaelis–Menten constant (K_m^{app}), which indicates enzyme–substrate kinetics, can be calculated from the electrochemical version of the Lineweaver–Burk equation,

$$\frac{1}{I_{ss}} = \frac{I}{I_{max}} + \frac{K_m^{app}}{I_{max}} C$$

where I_{ss} 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 condition. The K_m^{app} is determined by analyzing the slope and intercept for the plot of the reciprocals of the cathodic current versus urea concentration. The K_m^{app} value of the urea biosensor is determined by the steady-state amperometric response and noted to be 3.25 mM for Urs-immobilized on the electrode surface. The smaller K_m^{app} value implies that the immobilized Urs possesses higher enzymatic activity, and the present electrodes exhibit a higher affinity to urea. The immobilization of Urs mentioned above appears to enhance and improve the biosensor's performance.

The effect of the pH

The bioactivity of an enzyme depends greatly on the pH of solution, and extreme pH changes can cause enzyme denaturation. The pH dependence of the response of the enzyme electrode was determined using 100 mM PBS, and the pH varied between 6.0 and 8.0. Figure 5 shows that the maximum activity was obtained at pH 7.0. For each point in Fig. 5, a new enzyme electrode was prepared to eliminate the errors that could arise from reusing the enzyme-loaded copolymer.

The effect of the temperature

The effect of the temperature of the buffer solution on the response of the poly(GMA-co-VFc)-mediated urease biosensor system was studied in the range of 25–45 °C (Fig. 6). The amperometric response was observed to initially increase with temperature and decrease later. The response reached a maximum, at about 40 °C. The decrease of the response after 45 °C could be attributed to the thermal inactivation of the enzyme. The values of ln$I$ versus 1/T (K) were plotted, and the activation energy (Ea) for the reaction of the immobilized enzyme was found to be 4.18 kcal/mol from the slope of this Arrhenius plot (R^2=0.9732).

Operational and storage stabilities

The operational stability of Urs electrodes was obtained by running measurements on the same day. Between each

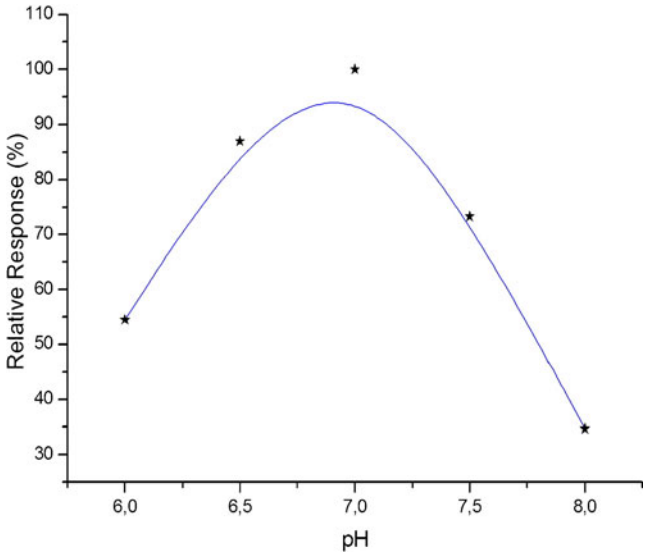

Fig. 5 Effect of pH on Urs-immobilized electrodes. Data collected with freshly prepared enzyme electrodes with reference to the average of three experiments (~25 °C)

subsequent measurement, the electrodes were stored at 4 °C in the buffer solution for 30 min. The first six measurements revealed the same response; a 50% activity loss was observed with subsequent use (Fig. 7a).

The response of the Urs electrode was measured by its response to 10 mM urea for a 44-day-period. As in Fig. 7b, the amperometric response of the enzyme electrode remained almost constant for 14 days, followed by 40% activity loss. Covalent attachment of the urease on the electrode could be responsible for protecting the enzyme from environmental effects and leaking.

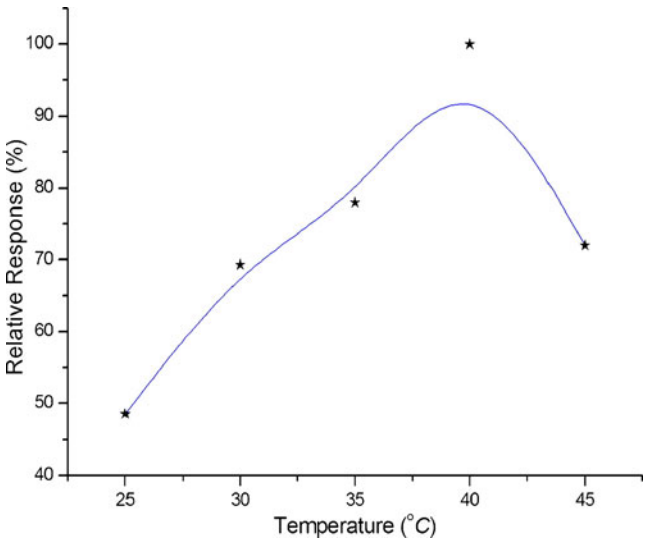

Fig. 6 Effect of temperature on Urs-immobilized electrodes. Data collected with freshly prepared enzyme electrodes with reference to the average of three experiments (pH 7.0)

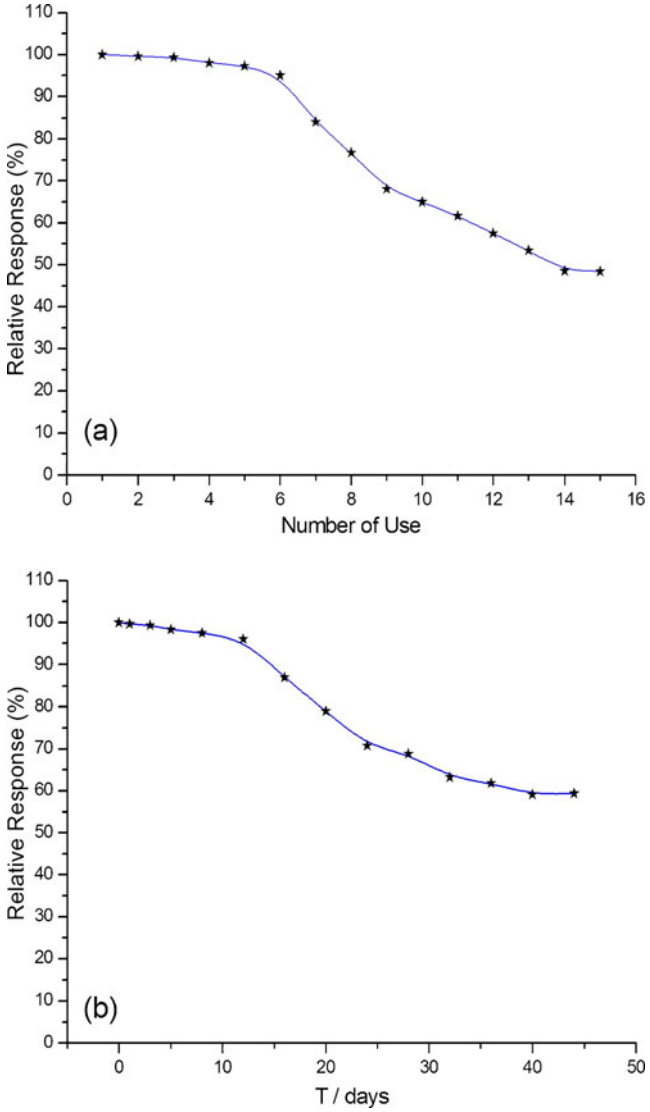

Fig. 7 **a** Operational stability of the Urs-immobilized electrode. Each data point represents the average of data collected by the three electrodes. **b** Storage stability of Urs-immobilized electrode. The amperometric responses of these enzyme electrodes are regularly checked during 45 days (pH 7.0; ~25 °C)

Conclusions

In this work, a new amperometric biosensor for the determination of urea was developed and characterized. The catalytic current responses are highly sensitive, increasing almost linearly with an increase in urea concentrations up to 8 mM. The catalytic response of the enzyme electrode achieves maximum with increasing the VFc composition up to a 0.6 ratio. The amperometric enzyme electrode developed in this study provided linearity to the urea in the 0.1–4.0 mM urea concentration range. This new urea enzyme electrode, whose characteristics are described above, appears to be simple to prepare, fast to respond, inexpensive, and reasonably sensitive.

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