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Chitosan-*g*-polyaniline: a creatine amidinohydrolase immobilization matrix for creatine biosensor

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Abstract. A novel matrix composed of chitosan-graft-polyaniline (CHIT-*g*-PANI) was electrochemically prepared to investigate the immobilization of creatine amidinohydrolase (CAH). CAH enzyme was covalently immobilized with the CHIT-*g*-PANI matrix using glutaraldehyde as a linker. The resulting CAH/CHIT-*g*-PANI biomatrix was characterized with Fourier transform infrared spectroscopy (FTIR), atomic force microscopy (AFM), contact angle measurement and cyclic voltammetry (CV) taking CHIT-*g*-PANI as a reference. The influence of various parameters on CAH enzyme activity within the matrix was investigated including pH, temperature, and time. The Michaelis-Menten constant and apparent activities for the CAH enzyme were calculated to be 0.51 mM and 83.59 mg/cm^2, respectively; indicating CHIT-*g*-PANI matrix has a high affinity to immobilize CAH enzyme.

Keywords: *polymer synthesis, molecular engineering, chitosan-graft-polyaniline, creatine amidinohydrolase, immobilization matrix*

1. Introduction

Creatinine is an important clinical analyte for the diagnosis of renal and muscular dysfunction [1]. It is a dehydrogenated form of creatine (i.e., a metabolic byproduct of amino acid) that provides energy to muscles tissue. The normal clinical range of creatinine in the human blood is ranging from 44 to 106 μM; however, it can exceed up to 1000 μM during nephrons malfunction [2]. Therefore, precise monitoring of creatinine in the blood is compulsory during routine check up.

Most of the existing creatinine biosensors utilize creatine amidinohydrolase (CAH) as a sensing element [3–5]. Typically, CAH is a homodimer enzyme, which hydrolyzes creatinine into urea and sarcosine [6]. The concentration of creatinine is quantitatively measured by monitoring the librated hydrolyzed byproducts using a range of transducers such as amperometric, potentiometric, optical, etc. [7, 8]. However, these biosensors are offered only limited biosensor stability due to low functional stability of CAH enzyme within the matrix [9]. In practice, performance of enzyme based biosensors usually depends on the physicochemical properties of the electrode materials as well as process of the enzyme immobilization and also enzyme concentration on the electrode surface [10]. Although, various matrices are reported in the literature for the immobilization of CAH enzyme to use in creatinine biosensors, the method of immobilization and electrode matrices, both are considered promising factor during the determination of the operational and

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storage stability of the biosensors [11]. The low intrinsic stability of CAH enzyme has encouraged for applying biomaterials engineering to improving stability [12]. Specifically, this study investigates the feasibility of CHIT-*g*-PANI as potential CAH immobilization matrix.

Polyaniline (PANI) is a unique conducting polymer and its reversible conductivity can be controlled by the protonation of the imine sites or the oxidation of the main polymer chain [13]. However, traditional PANI does not have such enzyme loading capability as its chemically modified forms [14]. Meanwhile, Chitosan (CHIT) has become a widespread biopolymer owing to its remarkable chemical and biological characteristics. It is a hydrophilic material due to the presence of both amino and hydroxyl groups; however, CHIT is insoluble in water and aqueous basic media [15]. It was chosen as the orientation directing matrix because there are large quantities of amino and hydroxyl groups on the CHIT units, which have a strong binding ability to enzyme and DNA [14, 16]. Moreover, in acidic condition CHIT showed a cationic nature that may provide an electrostatic core environment to zwitterion molecules such as enzyme.

In the present work, we describe the fabrication of a novel matrix based on CHIT-*g*-PANI, covalent immobilization and stabilization conditions for the CAH enzyme. Further, Michaelis-Menten kinetic parameter and apparent activities for the CAH enzyme were calculated correspondingly using electrochemical and photometric techniques.

2. Experimental

2.1. Materials

Chitosan (CHIT, >85% deacetylated, M_w $1.86 \cdot 10^5$), aniline (99%), creatinine (anhydrous) and urease (Urs, from *Canavalia ensiformis*) were purchased from Sigma-Aldrich, USA. Creatine amidinohydrolase (CAH, from *Actinobacillus* sp., CRH-211) was taken from Toyobo Co., Ltd. and used without further purification. All supplementary chemicals were of analytical grades and solutions were prepared with nanopure water. Indium-tin-oxide (ITO) coated glass sheets (Balzers) with a resistance of 15 Ω/cm^2 were used as substrates for the deposition of electrodes.

2.2. Preparation of CHIT solution

CHIT solution was prepared by dissolving 2.0 g of CHIT flakes into 100 ml of 1.0% acetic acid and stirred for three hours at room temperature until completely dissolved. The CHIT solution was stored in a refrigerator when not in use.

2.3. Electrochemical synthesis of CHIT-*g*-PANI

Aniline (180 µl), and 2.0% CHIT solution (75 µl) was mixed with 10 ml of 0.5M HCl in an electrochemical cell and the mixture was ultrasonically agitated for about four hours. The CHIT-*g*-PANI was chronoamperometrically synthesized onto an ITO coated glass surface using a three-electrode assembly with ITO glass as working, platinum as counter, and Ag/AgCl as reference electrodes at a potential of 0.9 V and duration of 200 seconds. The resulting CHIT-*g*-PANI/ITO electrode was washed with deionized water followed by a phosphate buffer saline (PBS) solution of pH 7.0 in order to neutralize the electrode surface.

2.4. Immobilization of CAH enzyme

The CAH enzyme was covalently immobilized over the CHIT-*g*-PANI matrix using glutaraldehyde as a linker. Ten µl of 25% gluteraldehyde solution was spread over the CHIT-*g*-PANI/ITO

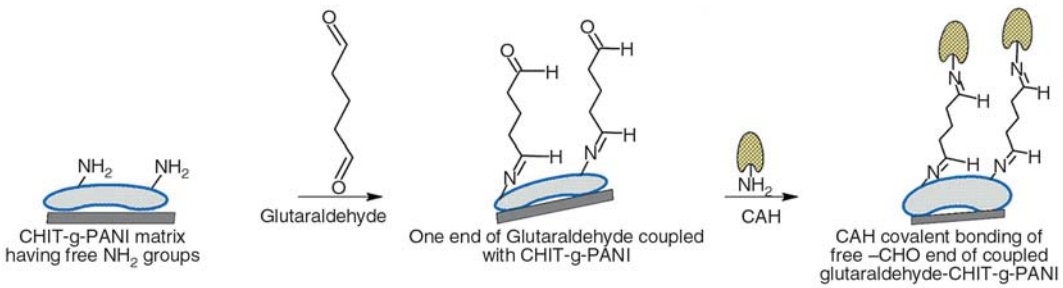

Figure 1. Covalent immobilization of the CAH on CHIT-*g*-PANI graft copolymer matrix using gluteraldehyde as a linker

electrode and kept for five hours at room temperature, then 10 µl CAH (350 units/µl) was spread on top of the electrode and dried (Figure 1). The resulting CAH/CHIT-*g*-PANI/ITO bioelectrode was thoroughly washed with PBS of pH 7.0 to rinse off any loosely bound CAH enzyme from the bioelectrode.

2.5. Characterization

CHIT-*g*-PANI and CAH/CHIT-*g*-PANI biomatrix were characterized using FTIR, AFM, contact angle, and CV measurements. FTIR spectra were recorded on a Perkin Elmer, Spectrum BX II spectrophotometer. The surface topology of the electrodes was studied using AFM (Veeco DICP2) under the tapping mode. Contact angles of the electrodes were measured with a video-based, automatic research grade contact angle measurement system, FDSC-OCA 20 using water as liquid phase.

Electrochemical measurements of the electrodes were carried out on a Potentiostat/Glavanostat (Princeton Applied Research, 273A) unit with three electrodes in a 50 mM phosphate buffer solution (pH 7.0, 0.9% NaCl) containing 5 mM $Fe(CN)_6^{3-/4-}$. The working electrode was either CHIT-*g*-PANI/ITO or CAH/CHIT-*g*-PANI/ITO. Platinum foil and Ag/AgCl were used as the counter and reference electrodes, respectively. All measurements were carried out at 25°C.

2.6. Photometric apparent enzyme activity measurement

Photometric study was performed using a Varian Cary 100 Bio UV-visible spectrophotometer. For photometric measurements, a CAH/CHIT-*g*-PANI/ITO bioelectrode was dipped in a 5 ml phosphate buffer solution (50 mM, pH 7.0), which contained 200 µl of Nessler's solution, 50 µl urease enzyme (50 mg/ml) and 1 ml of creatinine solution with varying concentrations. After 3 min of CAH/CHIT-*g*-PANI/ITO electrode incubation, the absorbance of the colored product (i.e., $NH_2Hg_2I_3$, a complex formed between the Nessler's reagent and ammonia produced by the enzymatic hydrolysis of creatinine) in the solution at λ_{max} 385 nm, was measured to monitor the CAH enzyme kinetics.

3. Results and discussion

3.1. Graft copolymerization, enzyme immobilization and characterization

In the electrochemical copolymerization synthesis, the aniline monomer initially became protonated with HCl and propagated to form an intermediate called PANI radical cation (Figure 2a) [17–19].

PANI radical cation simultaneously generated CHIT macro radicals (Figure 2b) by the abstraction of hydrogen from the –OH and $–NH_2$ groups of the CHIT macromolecules [15, 16].

The PANI cation radicals and CHIT macro radicals then copolymerized and yielded CHIT-*g*-PANI (Figure 2c).

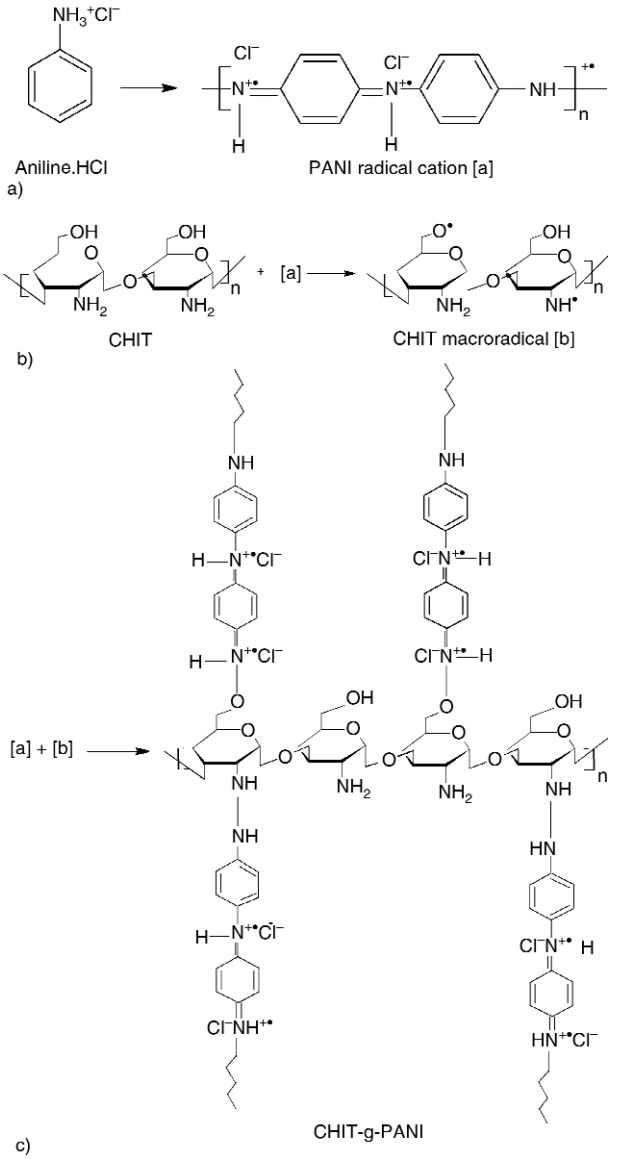

Figure 2. Electrochemical copolymerization synthesis. a) PANI radical cation, b) CHIT macro radical, c) CHIT-g-PANI

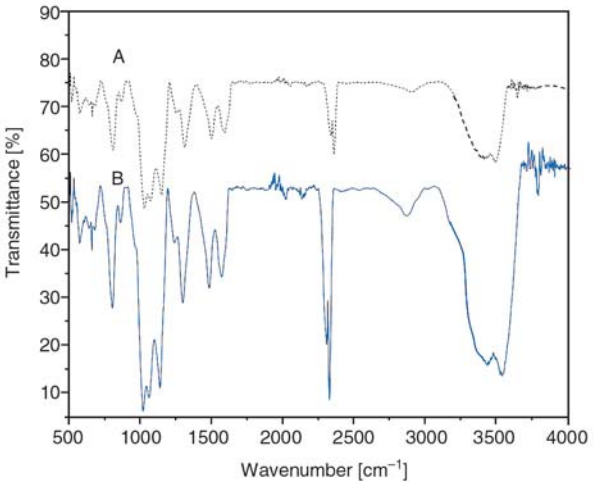

Figure 3. FTIR spectra of the (spectrum A) CHIT-*g*-PANI and (spectrum B) CAH/CHIT-*g*-PANI

The FTIR spectrum of the CHIT-*g*-PANI/ITO electrode (Figure 3, spectrum A) illustrated the characteristic peaks of PANI, as well as CHIT [16]. The following key characteristic bands were observed: 1) 3150 to 3542 cm^{-1} (free O–H stretching and N–H stretching with hydrogen bonded secondary amino groups); 2) 3021 cm^{-1} (aromatic C–H stretching); 2926 and 2862 cm^{-1} (aliphatic C–H stretching); 3) 1632 cm^{-1} (C=O stretching of carbonyl group, typical saccharide absorption); 4) 1584 cm^{-1} (C=C stretching of quinoid rings); 5) 1483 cm^{-1} (C=C stretching vibration of benzenoid rings); and 6) 1245 cm^{-1} (C–N stretching). The absorption band of the N=Q=N bending vibration of protonated pure PANI was observed at 1236 cm^{-1}, but shifted to 1123 cm^{-1} in the CHIT-*g*-PANI copolymer due to the steric effect of CHIT [16].

Stable enzyme-substrate coupling was achieved with gluteraldehyde as a cross-linking agent [20]. One end of the gluteraldehyde is attached to the –NH_2 group of the CHIT-*g*-PANI/ITO electrode through a reaction between the –CHO end group of glutaraldehyde and the –NH_2 groups of terminal PANI and CHIT. The other end of the gluteraldehyde is attached to CAH through a reaction between the –CHO group of gluteraldehyde and –NH_2 group of CAH, which resulted in a CAH/CHIT-*g*-PANI/ITO bioelectrode (Figure 1). It may be possible that some of the glutaraldehyde attached within the free –NH_2 groups of the CHIT-*g*-PANI matrix but probability of cross linking between –NH_2 functionalized electrode to enzyme is quite common [20]. The FTIR spectrum of the CAH/CHIT-*g*-PANI/ITO electrode (Figure 3, spectrum B) showed phosphate vibration at 1224 cm^{-1} (accredited from phosphate backbone of immobilized cDNA) with the peaks broadening at 1) 3123 to 3564 cm^{-1} (addition of N–H stretching vibration); 2) 3022 to 2856 cm^{-1}; and 3) 1631 cm^{-1} to 1664 due to the attachment of CAH with the CHIT-*g*-PANI matrix. Hence, FTIR spectra confirmed the immobilization of CAH onto the CHIT-*g*-PANI/ITO electrode.

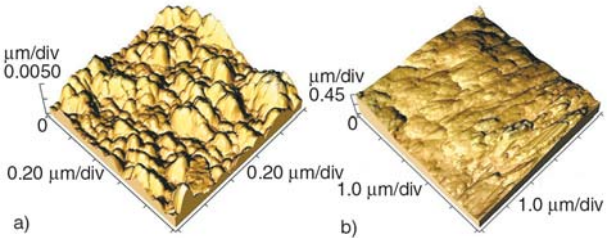

Figure 4. AFM images of the (a) CHIT-*g*-PANI and (b) CAH/CHIT-*g*-PANI

The surface morphology of the electrodes was observed with AFM and is shown in Figure 4. The CHIT-*g*-PANI/ITO electrodes exhibited a relatively rough surface topology in comparison to CAH/CHIT-*g*-PANI/ITO electrode, which may facilitate the immobilization of CAH onto the CHIT-*g*-PANI/ITO electrode. To study the relative surface hydrophilicity, the contact angles of CHIT-*g*-PANI/ITO and CAH/CHIT-*g*-PANI/ITO electrodes were measured [21] and were about 29±2° and 38±2°, respectively. The work of adhesion between the surface of the electrodes and the water droplet (W_a) can be calculated from the Young-Dupre Equation (1):

$$W_a = \gamma (1 + \cos \theta) \quad (1)$$

where γ is the surface tension and θ is the contact angle. If the liquid is attracted to the solid surface (e.g., water on a strongly hydrophilic solid), the droplet will completely spread out on the solid surface and the contact angle will be close to 0°. According to this equation, the work of adhesion is higher at a lower contact angle. Since the contact angle increased after the CAH was immobilized on the surface of the CHIT-*g*-PANI/ITO electrode, this indicates that the CAH/CAH/CHIT-*g*-PANI/ITO electrode surface has a weaker affinity to the hydrophilic moiety than CHIT-*g*-PANI/ITO electrode surface.

3.2. Electrochemical measurement

Figure 5 shows the CVs of the electrochemical cells using either CHIT-*g*-PANI/ITO or CAH/CHIT-*g*-PANI/ITO electrode at a constant 50 mVs^{-1} scan rate in 50 mM phosphate buffer solution (pH 7.0, 0.9% NaCl) containing 5 mM $Fe(CN)_6^{3-/4-}$. The current of the electrochemical cell using the electrode CHIT-*g*-PANI/ITO ($7.69 \cdot 10^{-6}$ A) was about five times of that using the CAH/CHIT-*g*-PANI/ITO bioelectrode ($1.72 \cdot 10^{-6}$ A).

Thus, immobilizing CAH onto the bare electrode reduced the current. A decrease in current after the immobilization of CAH may be attributed to a slower redox behavior when compared with the bare CHIT-*g*-PANI/ITO electrode. The covalent binding of CAH on the CHIT-*g*-PANI/ITO electrode controls the moment of the supporting electrolytes [11]. Also, the non-conducting nature of the CAH molecules might have contributed to the decrease in current when using the CAH/CHIT-*g*-PANI/ITO electrode.

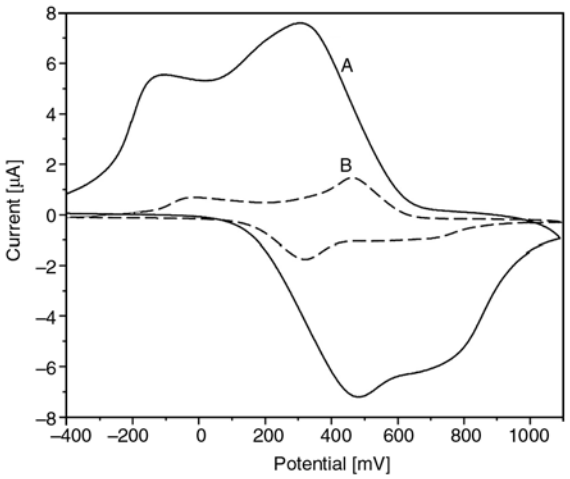

Figure 5. Cyclic voltammograms of the (A) CHIT-*g*-PANI/ITO and (B) CAH/CHIT-*g*-PANI electrodes in PBS (50 mM, pH 7.0, 0.9% NaCl, 5 mM $Fe(CN)_6^{3-/4-}$) at 50 mVs^{-1} scan rate

The affinity of CAH to the CHIT-*g*-PANI/ITO graft copolymer matrix was estimated using the Hanes plot [22]. The Michaelis-Menten kinetic parameter (K_m^{app}) was calculated to be 0.51 mM for the CAH/CHIT-*g*-PANI/ITO electrode. K_m^{app} usually depends on the electrode material as well as the enzyme immobilization process [23]. The K_m^{app} of the CAH/CHIT-*g*-PANI/ITO bioelectrode is much less than that of these previously reported biosensors (typically 2 to 7 mM). The small K_m^{app} value indicates a high affinity of CAH to the CHIT-*g*-PANI matrix over the electrode surface, which may be attributed to 1) the advantageous $-NH_2$ functionalized surface of the CHIT-*g*-PANI matrix for the enzyme immobilization that can favor conformational changes of the enzyme, and 2) the high positive electrostatic interaction, which can help to effectively immobilize CAH onto the CHIT-*g*-PANI/ITO electrode.

3.3. Photometric study

A photometric study was performed to calculate the apparent enzyme activity of the CAH enzyme. CAH catalyzes the hydrolysis of creatinine (II) to produce sarcosine (III) and urea (IV). In the presence of urease, urea further hydrolyzed into ammonia, which in turn reacts with the Nessler's reagent (K_2 Hg_2 I_4) to form a colored product, $NH_2Hg_2I_3$ (Figure 6). Through taking the absorbance of $NH_2Hg_2I_3$ at 385 nm, apparent enzyme activity of the CAH enzyme can be determined [24]. The apparent enzyme activity ($^{enz}a_{app}$) was calculated using Equation (2):

$$^{enz}a_{app} = \frac{AV}{\varepsilon ts} \tag{2}$$

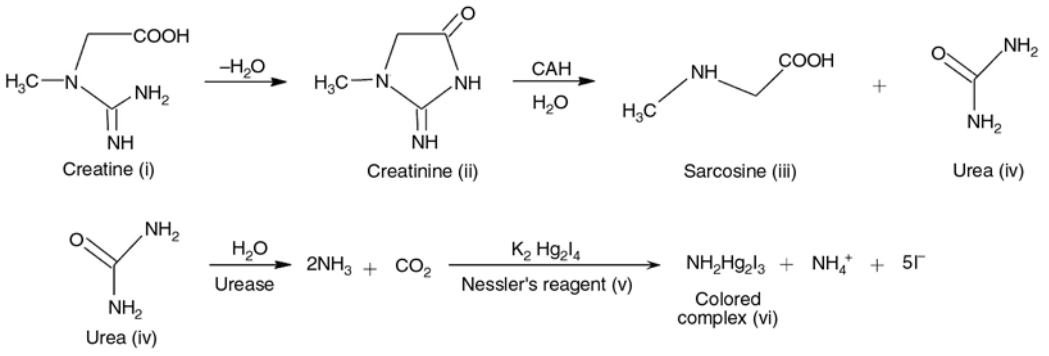

Figure 6. Production of photomerically active $NH_2Hg_2I_3$ as a sensing element resulting from the reaction of Nessler's reagent and librated NH_3 (i.e., obtained from enzymatic hydrolysis of creatinine)

where A is the difference in absorbance before and after incubation, V is the total volume of the solution, ε is the millimolar extinction coefficient, t is the reaction time and s is the surface area of the electrode. The apparent enzyme activity was calculated to be 83.59 mg/cm^2; indicating 83.59 mg of CAH was actively immobilized per unit area of CHIT-*g*-PANI matrix.

3.4. Effect of pH, temperature and time

The peak current varied with the value of pH in the range 6.0–8.0; the optimum current is obtained at pH 7.0. It suggests that optimum CAH enzyme activity is found at pH 7.0. The thermal stability of the CAH/CHIT-*g*-PANI/ITO bioelectrode was studied by measuring the current at different temperatures ranging from 25 to 45°C in the presence of creatinine 150 µM and a phosphate buffer (50 mM, pH 7.0). It was observed that the reaction rate increased with the temperature up to 32°C and the optimum temperature range was between 30–32°C due to the increased kinetic energy of the reacting molecules. The storage stability of the CAH/CHIT-*g*-PANI/ITO electrode was amperometrically measured and a similar current response was found after it was stored for ~300 days at 4°C. Hence, the CAH/CHIT-*g*-PANI/ITO bioelectrode exhibited an excellent operational and storage stability.

4. Conclusions

Creatine amidinohydrolase was covalently immobilized onto the CHIT-*g*-PANI graft copolymer matrix. The relatively low Michaelis-Menten constant of 0.51 mM indicates that the CHIT-*g*-PANI matrix had a high affinity for the CAH enzyme. The enzyme holding capacity of graft copolymer matrix was determined to be 83.59 mg/cm^2 and was affected by pH, temperature, and time. Present efforts aim to use CAH/CHIT-*g*-PANI/ITO bioelectrodes with a good shelf life to fabricate an efficient creatinine biosensor that can detect creatinine from the blood and urine.

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