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Preparation of substituted polyaniline/chitosan composites by *in situ* electropolymerization and their application to glucose sensing

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ABSTRACT

Substituted polyaniline/chitosan (sPANI/Ch-H_2SO_4) composites were electrochemically synthesized in 0.10 M H_2SO_4 acid medium. The composites were characterized by electrochemical measurements, FTIR, SEM and AFM analyses. Anodic and cathodic current responses of substituted polyaniline/chitosan composites increased linearly with increasing the scan rate. SEM images of chitosan show smooth surfaces, whereas sPANI/Ch-H_2SO_4 composites show more porous surfaces which will be suitable for the immobilization of biomolecules. Glucose oxidase (GOD) was immobilized on to the surface of these composites by physical adsorption. The PNMANI/Ch-H_2SO_4/GOD electrode was utilized for glucose sensing for bigger concentrations range than other sPANI/Ch-H_2SO_4 composites and Ch/GOD by the amperometric method. The response current of PNMANI/Ch-H_2SO_4/GOD increases linearly with glucose concentration from 0.06 to 1.83 mM. The surface topography of the electrodes was studied using atomic force microscopy. PNMANI/Ch-H_2SO_4/ITO composite film electrode exhibited a relatively rough surface topography than Ch/ITO, which may facilitate the immobilization of biomolecules.

1. Introduction

Composites of conducting polymers and chitosan (Ch) have shown good properties as conducting and biocompatible material, for various applications. However, conducting polymers have limited biocompatibility whereas Ch has limited conductivity which has been a subject of intensive research over the last decade. The biopolymer chitosan is a type of matrix for enzyme immobilization with such attractive properties as excellent film-forming ability, high permeability for water, good adhesion, nontoxicity, and biocompatibility (Luo, Xu, Du, & Chen, 2004; Du, Luo, Xu, & Chen, 2007). However, its poor electrical conductivity results in poor response time and a high operational voltage limits its applicability in devices. Composites have been prepared by incorporating a rigid conducting polymer (such as PANI) into a flexible matrix (such as chitosan) combining the good processability of the matrix and the electrical conductivity of the conducting polymer (Kim, Shin, Spinks, Kim, & Kim, 2005). Additionally, conducting polymers have the ability to efficiently transfer the electric charges produced by biochemical reactions to electronic circuits (Cha et al., 2003; Tahir, Alocilja, & Grooms, 2005). Biosensors prepared by using a conducting polymer as a support material have fast response times and high storage and operational stability (Mu & Xue, 1996). Polyaniline is extensively researched for its electrical, optical, chemical and electrochemical properties due to its simple synthesis method, stability in air, and potential range of applications (Winokur, 1998). It is the best known semi-flexible rod-like conducting polymer system with chemical and structural flexibility surrounding its amine nitrogen linkages for binding with biological materials (Genies, Boyle, Lapkowski, and Tsintavis (1990)). However, the major disadvantages of PANI are its insolubility in common organic solvents and its infusibility. One possible method for preparing soluble PANIs is substituted groups. In our previous work we presented chemically substituted polyaniline/chitosan composites (Yavuz, Uygun, & Bhethanabotla, 2009). Due to their application areas, different synthesis methods show different advantages. In terms of biological applications, electrochemical polymerization is widely used because of several advantages: (i) it is performed at ambient temperatures and microelectrodes or electrodes with a large surface area can be used; (ii) the polymer film formed is confined to the electrode and its shape can thus be controlled by electrode design, while the thickness can be controlled in the nanometer to micrometer range; (iii) the properties of the conducting polymer (CP) film can be widely modulated by varying electrochemical polymerization conditions (Wallace, Smyth, & Zhao, 1999). The main goal of this work is to electrochemically synthesize substituted polyaniline/chitosan (sPANI/Ch-H_2SO_4) composites in 0.10 M H_2SO_4 acid medium and to investigate their biosensor properties. The sPANI/Ch compos-

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ites were characterized using electrochemistry, FTIR, SEM and AFM techniques.

2. Materials and methods

2.1. Materials

Aniline (ANI), N-methylaniline (NMANI), N-ethylaniline (NEANI), 2-ethylaniline (2EANI) were obtained from Aldrich and were under nitrogen prior to use. Chitosan with medium molecular weight also purchased from Aldrich and H_2SO_4 (Aldrich) were used as received. Glucose oxidase (GOD) (EC 1.1.3.4, 179,000 units/g, type VII-S from *Aspergillus niger*, Sigma), D-(+)-Glucose anhydrous (Fluka) were utilized. The buffer solution was prepared using $NaH_2PO_4{\cdot}2H_2O$ (Riedel De Haen).

2.2. Synthesis of the substituted polyaniline/chitosan composites

The electrochemical synthesis of sPANI/Ch-H_2SO_4 composites was carried out in a compartment with a three-electrode configuration. All electrochemical experiments were carried out with the conventional three-electrode cell, using the modified Pt electrode as a working electrode, a saturated calomel electrode (SCE) as a reference electrode, and a platinum wire as an auxiliary electrode. The working electrode was a platinum electrode with 1 mm^2 of surface area. Prior to electropolymerization, it was polished to a mirror finish using alumina slurries of 0.05 μm.

4 μL of 0.01 g chitosan/ml solution in 2 wt% acetic acid was dropped onto the Pt electrode and dried at room temperature. Then, 1.85×10^{-3} mol of substituted aniline monomer was dissolved into 10 mL of 0.10 M H_2SO_4 acid. The potential was cycled between 0 and 1.05 V vs. saturated calomel reference electrode at a scan rate of 50 mV s^{-1}. Thirty cycles were used to obtain the polyaniline/chitosan (PANI/Ch-H_2SO_4) and poly(N-methyl)aniline/chitosan (PNMANI/Ch-H_2SO_4). 100 cycles were necessary for poly(N-ethyl)aniline/chitosan (PNEANI/Ch-H_2SO_4) and poly(2-ethyl)aniline/chitosan (P2EANI/Ch-H_2SO_4). Larger amounts of the composites were synthesized for FTIR and SEM characterizations using a platinum electrode with bigger surface area. To remove non-bonded Ch the composites were washed with 2 wt% acetic acid and deionized water until they became colorless. The final composites were dried at 50 °C for 24 h. PNMANI/Ch-H_2SO_4 and Ch were electrochemically coated onto ITO electrode to confirm immobilization of enzyme using SEM and AFM analysis.

2.3. Characterization methods

Electrochemical experiments were carried out using a Gamry PCI4/300 model potentiostat. A conventional three-electrode cell, modified platinum working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a Pt electrode as the auxiliary electrode, were utilized. Fourier transform infrared (FTIR) spectra were recorded between 400 and 4000 cm^{-1} with KBr pellets on a Perkin Elmer Spectrum BX FTIR system (Beaconsfield, Buckinghamshire, HP91QA, England). SEM images were taken on a scanning electron microscope model Philips XL-30 S FEG and Tescan Vega LSU.

The surface topography of the electrodes was investigated using an atomic force microscopy (AFM) in contact mode under a constant force (NanoSurf). The roughness was obtained from the 9.8 μm × 9.8 μm scan.

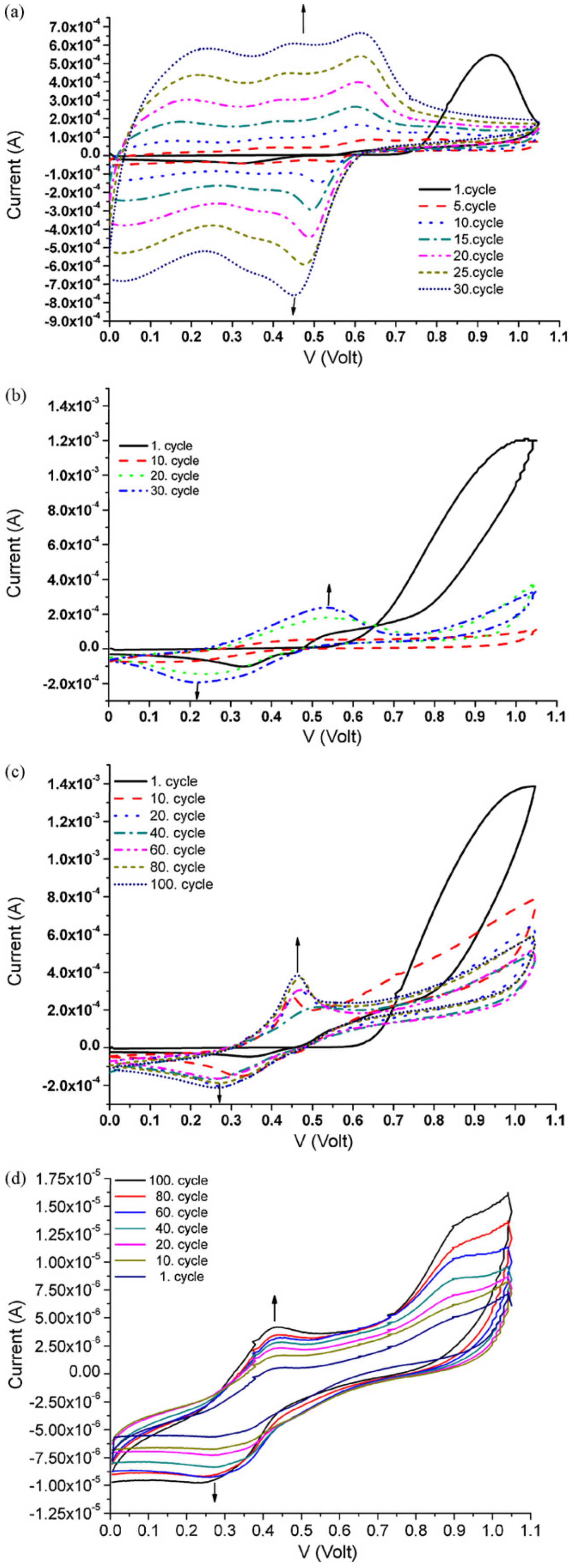

Fig. 1. Electrochemical polymerization cycles of (a) PANI/Ch-H_2SO_4, (b) PNMANI/Ch-H_2SO_4, (c) PNEANI/Ch-H_2SO_4 and (d) P2EANI/Ch-H_2SO_4 composites.

2.4. Preparation of the enzyme electrodes and amperometric measurements

The films of sPANI/Ch-H_2SO_4 composites were electrochemically synthesized using the cyclic voltammetry technique in 0.10 M H_2SO_4 for immobilizing glucose oxidase (GOD). The enzyme electrode was prepared by immersing the composite film into 5 mg/mL glucose oxidase in 0.10 M phosphate buffer (pH 7.4) solution for 20 min. Then, the enzyme electrode was washed with 0.10 M phosphate buffer after which it was ready to use. Free enzyme electrodes and chitosan enzyme electrodes (without any conducting polymer) were also prepared to compare their glucose sensing capabilities.

The response of the GOD enzyme electrode to glucose was investigated by the amperometric technique. Oxygen was introduced into electrochemical cell at a constant flow rate to obtain an oxygen-saturated solution. Oxygen flow over the solution was continued to keep it saturated during the measurements. When the background current stabilized to a constant value, a known amount of glucose solution was injected, followed by 5 s of stirring. The steady-state current was measured in 10 mL of 0.10 M phosphate buffered solution (pH 7.4) under a polarizing potential of 0.6 V (Pan, Kan, & Yuan, 2004) vs. SCE at 25 °C. Then, an increase in time-dependent current with the oxidation of hydrogen peroxide was recorded. The reaction of glucose with glucose oxidase (GOD) enzyme on the electrode is shown in the following equations:

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$H_2O_2 \rightarrow O_2 + 2H^+ + 2e^- \quad (2)$$

The amount of hydrogen peroxide produced in Eq. (1) is determined by the amperometric method by oxidation at the working electrode, according to Eq. (2).

3. Results and discussion

3.1. Cyclic voltammetry studies

Fig. 1a–d shows the cyclic voltammograms recorded between 0.00 and 1.05 V, with a scan rate 50 mV s^{-1} during the synthesis of polyaniline/chitosan (PANI/Ch-H_2SO_4) and substituted polyaniline/chitosan (sPANI/Ch-H_2SO_4) onto Pt electrodes coated with chitosan, which are utilized as a working electrodes. As can be seen from Fig. 1a–d, the intensity of oxidation and reduction peaks increases as the PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 films grow with successive CV scans onto the working electrodes, and oxidation potentials shift to higher values which indicate the electropolymerization of PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 (Duran, Turhan, Bereket, & Sarac, 2009). This indicates building up of electroactive sPANI/Ch-H_2SO_4 films onto the platinum electrode. At the end of the cycles, PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 films can be seen visually. After repeated scans, the coated PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 electrodes were removed from the monomer solution and rinsed with 0.1 M H_2SO_4 to remove the monomer and oligomeric species. PANI/Ch-H_2SO_4 has three oxidation peaks at 0.2, 0.4 and 0.6 V, whereas PNMANI/Ch-H_2SO_4, PNEANI/Ch-H_2SO_4 and P2EANI/Ch-H_2SO_4 indicate oxidation current peaks at 0.55, (Sivakumar & Saraswathi, 2003) 0.45 and 0.42 V, respectively. Moreover, in reverse cycling PANI/Ch-H_2SO_4 shows three peaks at 0.45, 0.33 and 0.05 V. PNMANI/Ch-H_2SO_4, PNEANI/Ch-H_2SO_4, P2EANI/Ch-H_2SO_4 show one reduction peak at 0.23, 0.27 and 0.28 V, respectively. It is known that N-alkyl groups can change the π-electron distribution in the aromatic rings.

After electrodeposition of PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 films, their electrochemical behavior was investigated in monomer free solutions at various potential sweep rates (25–200 mV s^{-1}). Fig. 2a–d shows the scan rate dependance for sPANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4. If the electroactive species are electrode-confined, both the anodic (I_{ac}) and cathodic current (I_{cc}) responses will increase linearly with the scan rate. As seen from Fig. 2a–d, both anodic and cathodic peak current values increase linearly with an increasing scan rate, indicating that the electroactive polymer is well adhered to the working electrode surface (Cihaner, 2007).

The scan rate dependence of PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 films in monomer free solution is given with smaller graphs in Fig. 2a–d. Anodic and cathodic peak current densities related with the oxidation and reduction of PANI/Ch-H_2SO_4 and sPANI/Ch-H_2SO_4 are plotted vs. scan rate (up to 200 mV s^{-1} in monomer free 0.1 M H_2SO_4 solution). Peak current is proportional to the range of scan rates where diffusion control applies. This demonstrates that both the electrochemical processes are diffusion controlled.

3.2. FTIR results

Fig. 3 shows the comparison of FTIR spectra of chitosan and substituted polyaniline/chitosan composites. In the spectrum of chitosan, distinctive absorption bands at 3435 cm^{-1} (–NH_2 stretching), and at 1654, 1597 cm^{-1} (–NH_2 bending) are found. The band around 2875 cm^{-1} is ascribed to the C–H stretching mode in chitosan (Ismail et al., 2008; Yavuz et al., 2009). The absorption bands at 1155 cm^{-1} (anti-symmetric stretching of C–O–C bridge), 1070 cm^{-1} (skeletal vibration involving the C–O stretching) are characteristics of its saccharide structure (Xu et al., 2006).

The characteristic bands at 1560–1593 cm^{-1} for the nitrogen quinone (Q) structure, and 1475–1496 cm^{-1} bands related to benzene ring (B) structure. 1295–1307 and 1100–1102 cm^{-1} are the stretching peaks of C–N and C=N, respectively (Yavuz & Gok, 2007). Additionally, it can also be noted, by comparing spectra of chitosan and sPANI/Ch-H_2SO_4 composites, sPANI/Ch-H_2SO_4 composites have characteristic bands of chitosan at 1653, 1375, and 1403 cm^{-1}. And also, broadening in characteristic bands associated with substituted polyanilines and chitosan was observed in FTIR spectra. This broadening of FTIR bands between 2875 and 3435 cm^{-1} is due to H-bonding interaction between substituted polyanilines and chitosan (Khan & Dhayal, 2009). FTIR results suggest that there is an interaction between substituted anilines with the active sides of chitosan, that is –OH and –NH_2.

3.3. SEM results

Fig. 4a–e presents SEM images of chitosan and sPANI/Ch-H_2SO_4 composites. As seen from Fig. 4a, chitosan has a smooth surface (Demetgül & Serin, 2008). After coating substituted polyanilines onto chitosan coated electrode, there is a remarkable change of the surface morphology of chitosan. P2EANI/Ch-H_2SO_4 indicated both layered and granular structure, whereas, the other composites have smoother surfaces. Among the composites, the best morphological structure was observed in PNMANI/Ch-H_2SO_4 (Fig. 4c) composite. It also has porous structure as well, which can be more suitable for the immobilization of biomolecules. Glucose sensing experiments have also supported this result. PNMANI/Ch-H_2SO_4 showed a wider linear range than free enzyme, chitosan and other substituted polyaniline/chitosan composites.

3.4. Amperometric determination of glucose and morphological results of biosensor electrodes

The catalytic reaction of the glucose biosensor is as follows: glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase) catalyses the oxidation of β-D-glucose to D-glucono-1,5-lactone and hydrogen peroxide, using molecular oxygen as the electron acceptor. D-Glucono-1,5-lactone then hydrolyses to gluconic acid and simultaneously hydrogen peroxide is released.

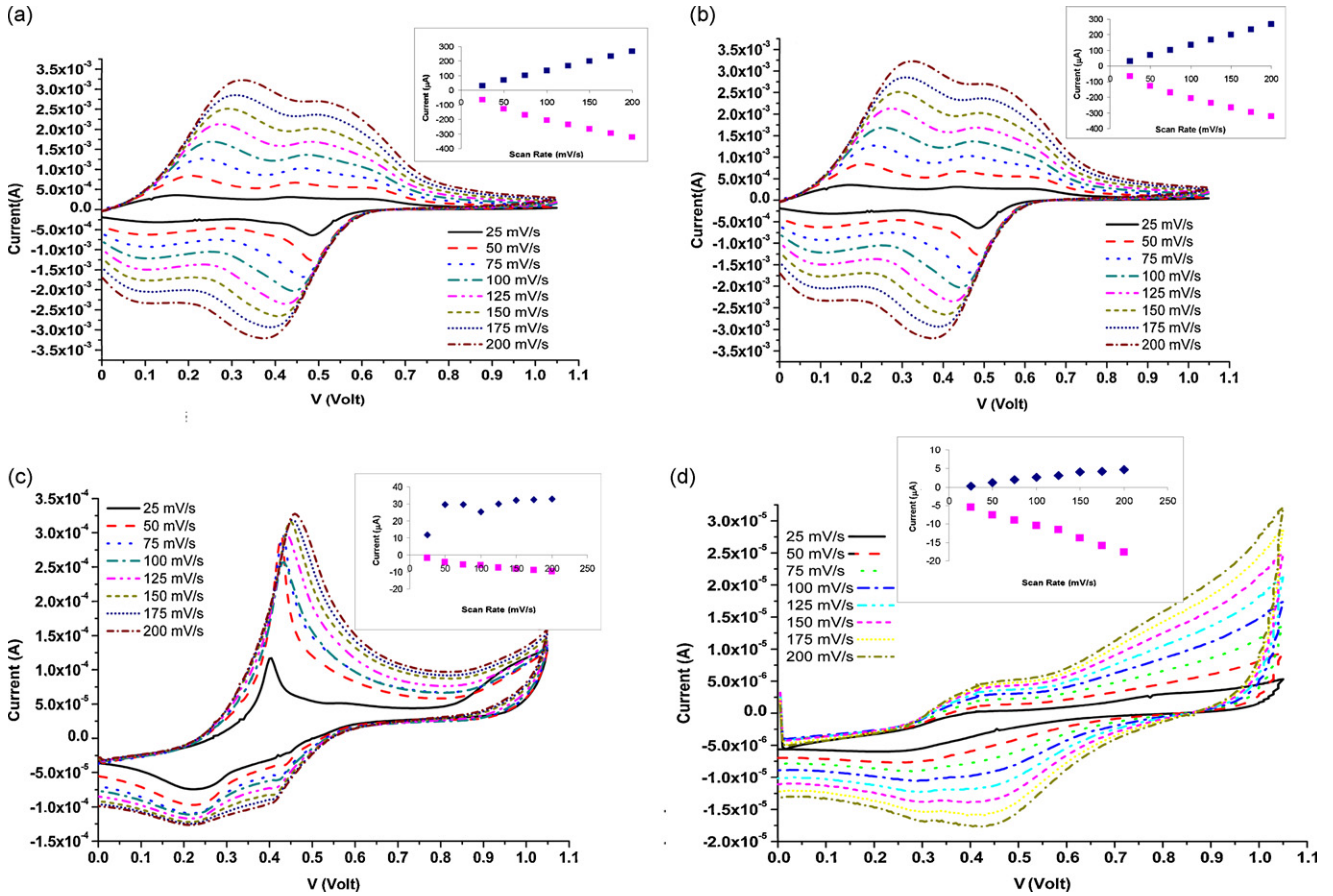

Fig. 2. (a) Cyclic voltammogram of PANI/Ch-H_2SO_4, and plot of anodic and corresponding cathodic peak current densities vs. the scan rate of the PANI/Ch-H_2SO_4 composite film up to 200 mV s^{-1} in monomer free 0.1 M H_2SO_4 solution. (b) Cyclic voltammogram of PNMANI/Ch-H_2SO_4, and plot of anodic and corresponding cathodic peak current densities vs. the scan rate of the PNMANI/Ch-H_2SO_4 composite film up to 200 mV s^{-1} in monomer free 0.1 M H_2SO_4 solution. (c) Cyclic voltammogram of PNEANI/Ch-H_2SO_4 and plot of anodic and corresponding cathodic peak current densities vs. the scan rate of the PNEANI/Ch-H_2SO_4 composite film up to 200 mV s^{-1} in monomer free 0.1 M H_2SO_4 solution. (d) Cyclic voltammogram of P2EANI/Ch-H_2SO_4 films with different scan rates between 25 and 200 mV s^{-1} and plot of anodic and corresponding cathodic peak current densities vs. the scan rate of the P2EANI/Ch-H_2SO_4 composite film up to 200 mV s^{-1} in monomer free 0.1 M H_2SO_4 solution.

The effect of glucose concentration was investigated on the response of free enzyme, Ch/GOD and sPANI/Ch-H_2SO_4/GOD electrodes. The linear dynamic ranges and equations of the response time and coefficient values of these electrodes are given in Table 1. The response current increases somewhat linearly with glucose concentration. For Ch/GOD system, a linear calibration graph (Fig. 5) was obtained for current density vs. substrate concentration between 0.06 and 0.64 mM glucose. A linear relation was defined by equation: $y = 1.657x + 5.109$ ($R^2 = 0.972$) (y is the sensor response in current density (A cm^{-2}) and x is the substrate concentration in mM). The PNMANI/Ch-H_2SO_4/GOD composite biosensor has a bigger linear range and higher sensitivity (larger slope)

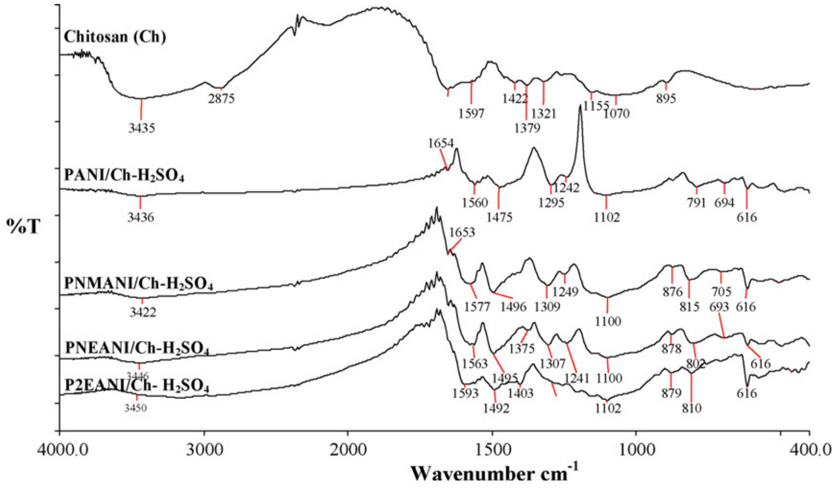

Fig. 3. FTIR spectra of the Ch (Chitosan) and composites.

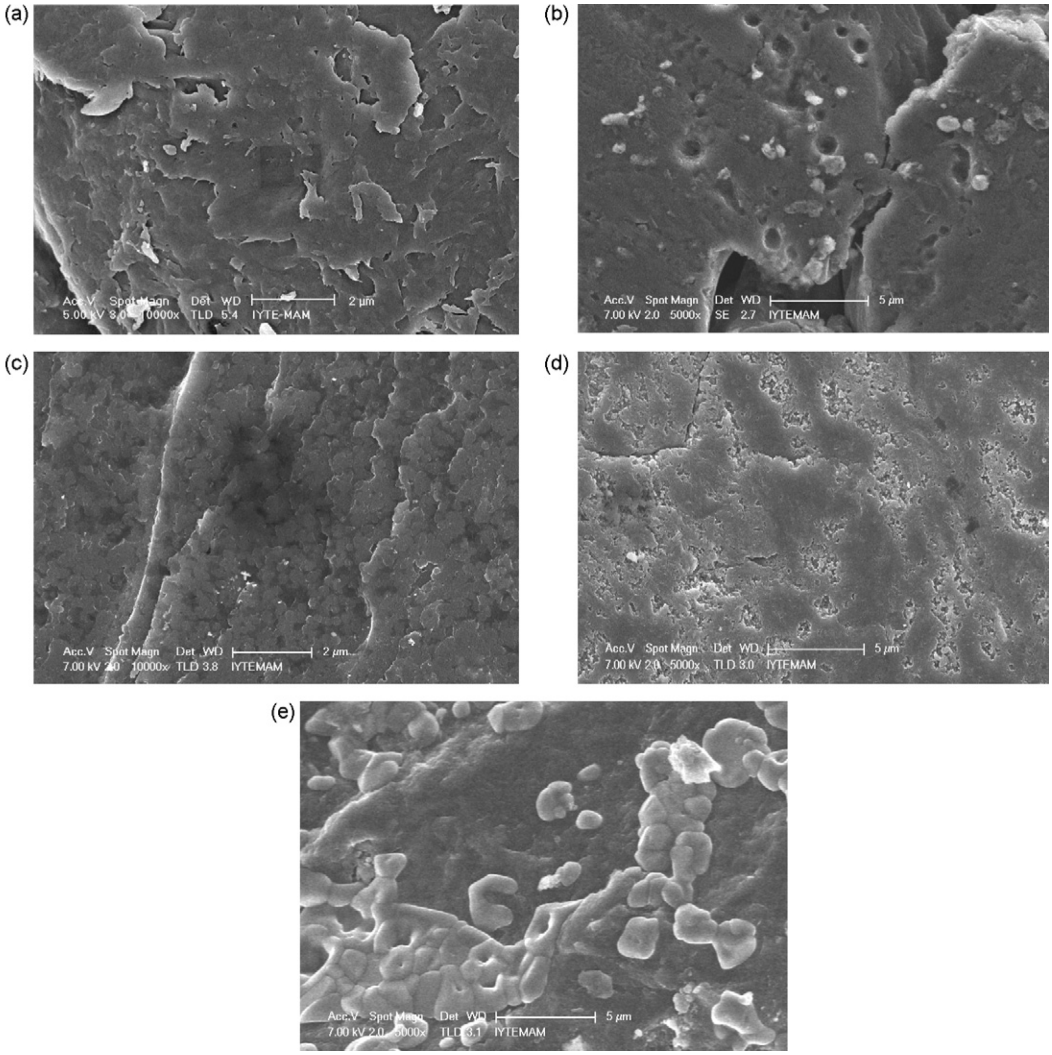

Fig. 4. SEM images of (a) Ch, (b) PANI/Ch-H_2SO_4, (c) PNMANI/Ch-H_2SO_4, (d)PNEANI/Ch-H_2SO_4 and (e) P2EANI/Ch-H_2SO_4.

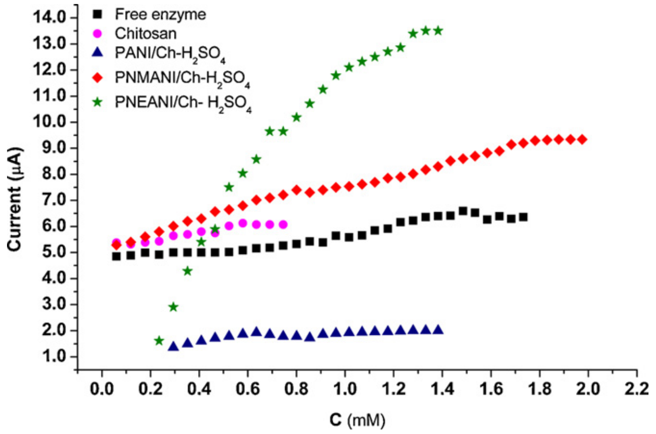

Fig. 5. Changes in the response of free enzyme, chitosan/GOD, and sPANI/Ch-H_2SO_4/GOD enzyme electrodes with glucose concentration (at 0.6 V in 0.10 M phosphate buffer solution (pH 7.4), 5 mg/mL GOD, 25 °C).

than free enzyme, other substituted polyaniline/chitosan/GOD, chitosan/GOD biosensors, respectively (Table 1). It is evident that higher current responses and shorter response times are observed for sPANI/Ch-H_2SO_4 electrodes. They could contribute by facilitating electron transfer between oxidative enzymes and the electrode surfaces.

By comparing the response times of these bioelectrodes, the PANI/Ch-H_2SO_4/GOD electrode has the shortest time at 40 s, PNMANI/Ch-H_2SO_4/GOD and PNEANI/Ch-H_2SO_4/GOD follow with 70 and 80 s, respectively, which are better than the 100 s response time for the Ch/GOD electrode. According to the Lineweaver–Burk form of the Michaelis–Menten equation, the relation between the reciprocal of the response current and the reciprocal of glucose concentration is linear. Generally, K_m has been used to evaluate enzyme activity. As the enzyme loading increases, a bigger fraction of the GOD locates near to the surface of the electrode, so that the average enzyme–substrate binding possibility increases, with K_m decreasing accordingly (Zang et al., 2007; Li & Lin, 2007; Sahmetlioglu, Yürük, Toppare, Cianga, & Yagci, 2006).

The apparent Michaelis–Menten constant K_m (slope of this line) of the biosensor based on the sPANI/Ch-H_2SO_4 electrodes is fitted to between 0.36 and 5.32 mM glucose (Table 1). These values can be compared with literature results for the polyaniline composite PANI/Ch/GOD of Garjonyte & Malinauskas (2000) and Gaikwad et al. (2007) who report 22.7 and 7.14 mM for the Michaelis–Menten constant. Our values for sPANI/Ch-H_2SO_4/GOD electrodes are much smaller.

SEM and AFM techniques were used to confirm the GOD enzyme immobilization onto the electrodes. As sample, surface morpho-

Table 1
Comparison of the linear range, response time, R^2, K_m for free enzyme, Ch/GOD, sPANI/Ch-H_2SO_4/GOD electrodes.

Sample	Linear range (mM)	Response time (s)	R^2	Equation	K_m
Free enzyme (GOD)	0.06–1.73	90	0.963	$y = 1.525x + 4.1917$	0.50
Chitosan (Ch)/GOD	0.06–0.64	100	0.971	$y = 1.657x + 5.1087$	1.00
PANI/Ch-H_2SO_4/GOD	0.29–1.28	40	0.988	$y = 1.749x + 0.8726$	0.36
PNMANI/Ch-H_2SO_4/GOD	0.06–1.83	70	0.984	$y = 2.167x + 5.3915$	0.72
PNEANI/Ch-H_2SO_4/GOD	0.24–1.28	80	0.965	$y = 12.199x + 0.3687$	5.32
P2EANI/Ch-H_2SO_4/GOD	–	–	–	–	–

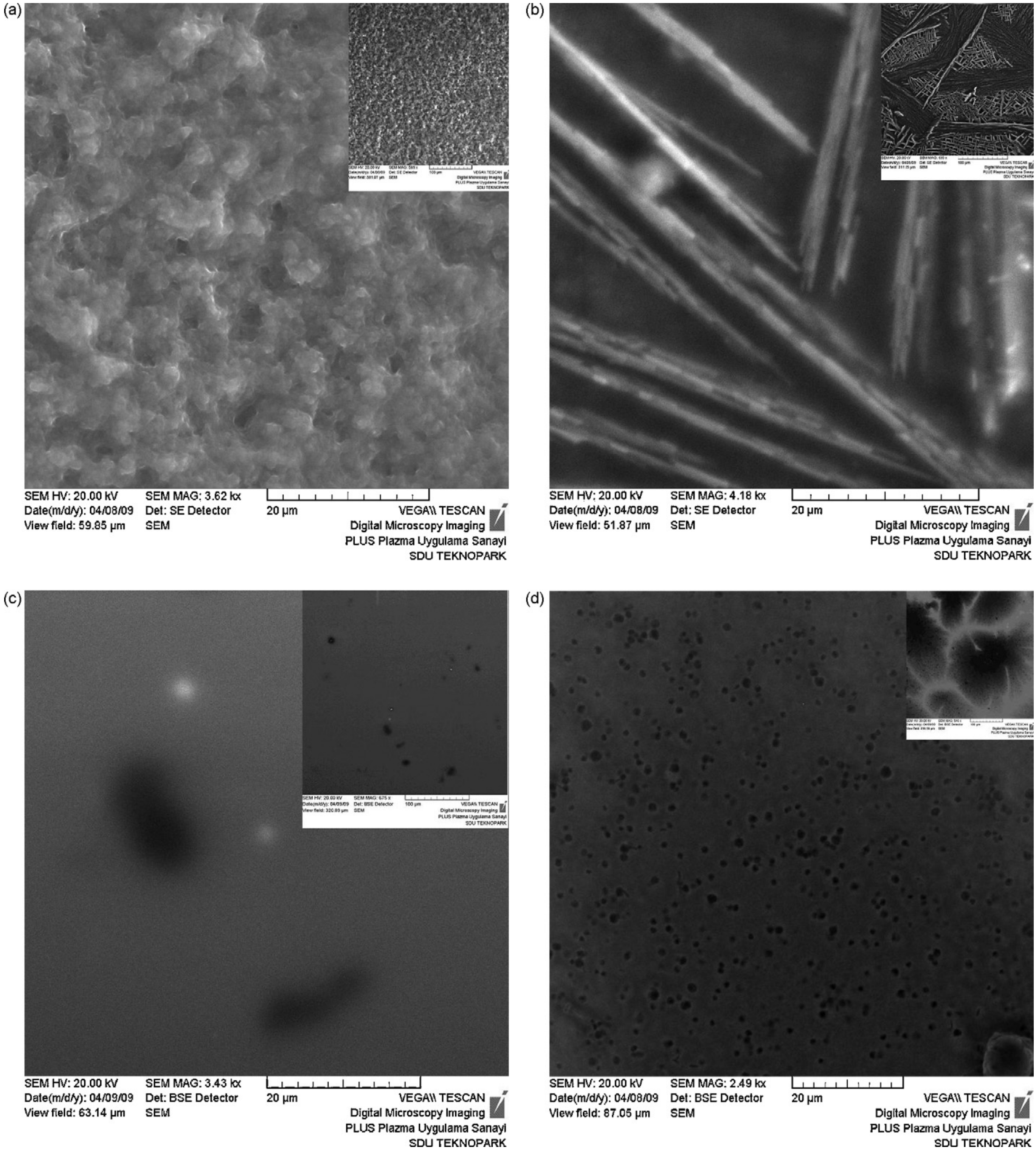

Fig. 6. SEM images of the (a) PNMANI/Ch-H_2SO_4, (b) PNMANI/Ch-H_2SO_4/GOD, (c) Ch (chitosan) and (d) Ch/GOD, electrodes onto ITO.

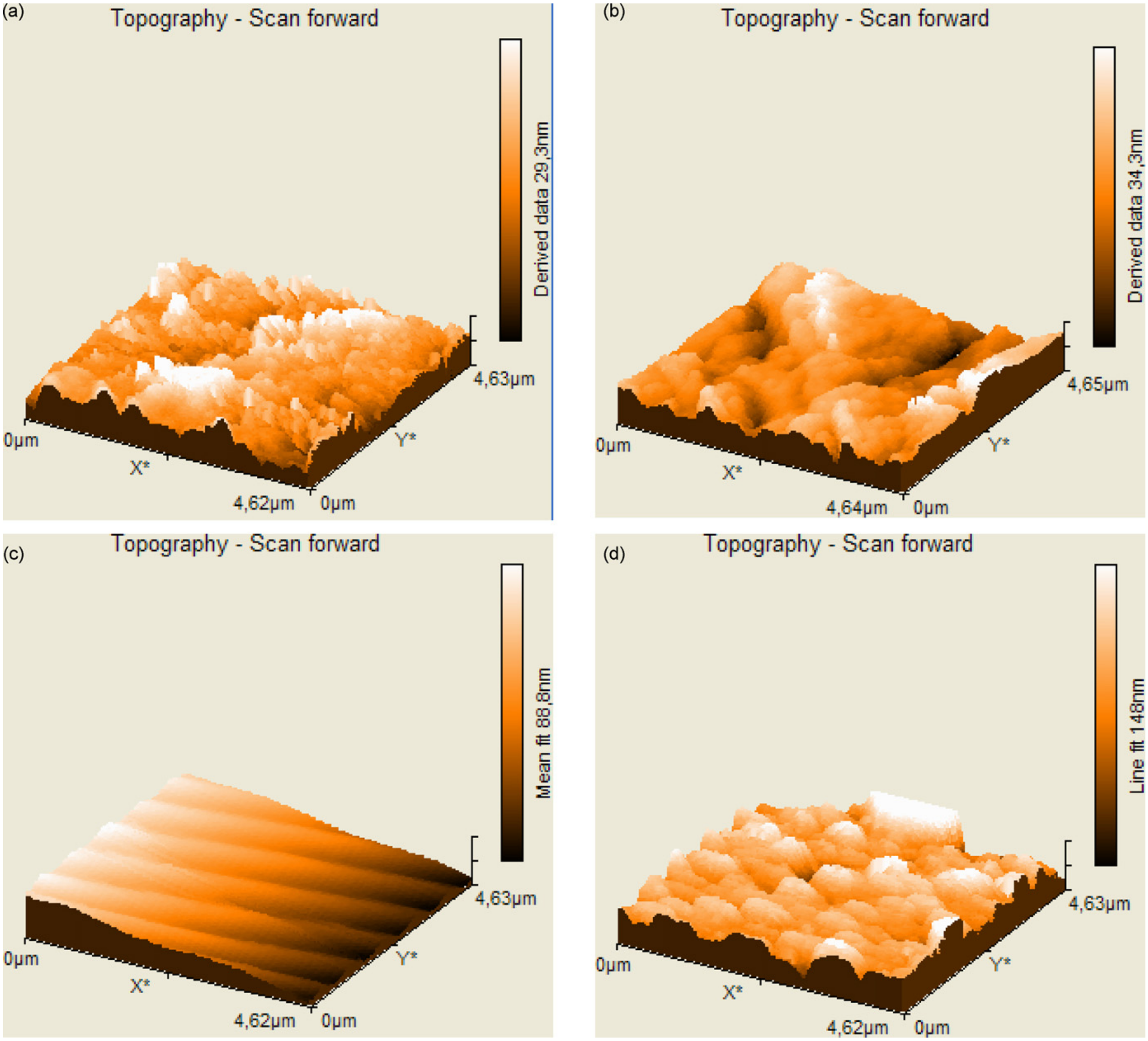

Fig. 7. AFM micrographs of the (a) PNMANI/Ch-H_2SO_4, (b) PNMANI/Ch-H_2SO_4/GOD, (c) Ch (chitosan) and (d) Ch/GOD electrodes onto ITO.

logical studies of PNMANI/Ch-H_2SO_4, PNMANI/Ch-H_2SO_4/GOD, Ch and Ch/GOD bioelectrode film onto ITO electrode have been investigated using scanning electron microscopy (Fig. 6a–d).

The granular porous surface of the PNMANI/Ch-H_2SO_4 composite film (Fig. 6a) is suitable for the immobilization of biomolecules. As GOD is immobilized onto the PNMANI/Ch-H_2SO_4 composite film matrix (Fig. 6b), the morphology of the surface changes to the well-regulated form. The film of chitosan contains smooth surface as shown in Fig. 6c, whereas the surface of GOD immobilized onto Ch has more porous morphology as shown in the SEM micrograph (Fig. 6d).

The surface topography of the enzyme electrodes was also studied using atomic force microscopy. AFM images of Ch/GOD/ITO (Fig. 7d) and PNMANI/Ch-H_2SO_4/GOD/ITO (Fig. 7b) biosensor electrodes have a relatively rough surface topology than that of Ch/ITO (Fig. 7c) and PNMANI/Ch-H_2SO_4/ITO (Fig. 7a), which may facilitate the immobilization of GOD. SEM and AFM results have confirmed the immobilization of biomaterials onto the bioelectrode surfaces (Uygun, 2009).

3.5. *Conclusions*

We introduce a new series of conductive polymer and Ch composites, which have been prepared by *in situ* electropolymerization, and characterized by electrochemical measurements, FTIR, SEM and AFM analysis. FTIR results have confirmed that there is an interaction between polyanilines and chitosan. The granular porous surface of the PNMANI/Ch-H_2SO_4 composite film is more suitable for the immobilization of biomolecules. The surface topology of the electrodes is also studied using atomic force microscopy and PNMANI/Ch-H_2SO_4/ITO composite film electrode exhibited a relatively rough surface topology than that of Ch/ITO, which may facilitate the immobilization of biomolecules. These results show that PNMANI/Ch-H_2SO_4 conducting polymer is expected to have superior biocompatibility, which can be revealed by further studies.

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