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Modified Indium-Doped Tin Oxide Glass with Polyaniline-Fe_3O_4 Nanocomposites for Immunosensing Application

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Abstract :
The evolution of the recent electrochemical biosensors reflects a simplification and enhancement of the transduction pathway. Modification of the transducer with nanomaterials has become increasingly studied with many advantages. In this study,Indium Tin oxide (ITO) electrode was obtained after modification of the ITO coated glass with conducting PAni-Fe_3O_4 nanocomposites on which anti-β-Amyloid was immobilized on the new electrode for detection of Alzheimer's diseases. Thin film of PAni–Fe_3O_4 composite was deposited by the electrophoretic technique on an ITO-coated glass plate to serve as a matrix for immobilization of antibody (anti-β amyloid). Electrochemical responses were generated at −200 mV reduction potential. The calibration curve of the immunosensor showed a linear response between 0.5 μg/mL and 2 μg/mL and a detection limit of 0.03 μg/mL. The incorporation of glutaraldehyde as a mediator resulted in a well-oriented antibody immobilization of the capture antibody and consequently enhanced the sensitivity.Response studies of the Anti-β Amyloid /PAni/Fe_3O_4/ITO bioelectrode have also been carried out. The results of the biosensing studies reveal that this bioelectrode can be used to detect β Amyloid in a wide detection range of 25–500mg/dL with high sensitivity of 3.36 mAmg-1dL and a fast response time of 30 s at pH 7.4. This bioelectrode exhibits a very low value of Michaelis–Menten constant of 1.18mM indicating enhanced interactions between β Amyloid and anti-β Amyloid immobilized onto this nanostructured PAni/Fe_3O_4 matrix. In conclusion, this immunosensor can be a promising technology for the rapid and convenient detection of β-Amyloid in real serum samples

Keywords: Polyaniline, Nanocomposites, Conductivity, Fe_3O_4

1. Introduction

Polyaniline (PAni) nanostructures have been prepared by blending PAni with inorganic electrical, optical, and magnetic nanoparticles to form nanocomposites (Lu *et al.,* 2005). Among the inorganic nanoparticles, Fe_3O_4 nanoparticles have received great attention because of their interesting magnetic properties as well as extensive potential applications in colour imaging, magnetic recording media, soft magnetic materials and ferrofluids (Lu *et al.,* 2006). There are also significant researches devoted to the design of metal nanoparticle conducting polymer composite based biosensors (Feng *et al.,* 2006 & Adamu *et al.,* 2015).

The properties of conducting polymers as sensing materials can be improved by the preparation of conducting polymer-based composites. For example, the intercalation of conducting polymer in layered host inorganic materials and other structurally organized environments resulted in organic/inorganic hybrid nanocomposites (Xia *et al.,* 2010). Other example includes sensors based on conducting polymer/inorganic semiconductor composites (Mahmoud *et al.*,2022; Joshi *et al.,* 2008),

conducting polymer/piezo materials (Patil *et al.,* 2007) and conducting polymer/biomolecule composites in sensing applications (Xia *et al., 2010*). These hybrid nanomaterials are expected to display synergistic properties between the polymer and the metal nanoparticles (Yan *et al., 2008*). Their novel electrical, structural and mechanical properties make them excellent candidates as sensing materials (Xia *et al.,* 2010; Mahmoud *et al*.,2020).

Fe_3O_4/Au/polyaniline (PAni) nanocomposites were fabricated by *in situ* polymerization in the presence of mercapto carboxylic acid (Yu *et al.,* 2008). Recently, PAni/Fe_3O_4 nanocomposite has become a popular material for applications in electrical–magnetic shields, electrochemical display devices and microwave absorbing materials. There are many methods to produce PAni/Fe_3O_4 nanocomposite (Lu, *et al.,*2005). The most general method is by direct polymerization of aniline monomers in an aqueous solution in the presence of dispersed iron oxide particles (Lu, *et al.,*2005.). Other methods include (1) blending the PAni in N-methyl-2-pyrrolidone (NMP) with iron (II) sulfate aqueous solution and precipitating Fe^{2+} into maghemite, and (2) allowing the monomer to react with $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$, followed by treatment with aqueous KOH (Lu, *et al.,* 2005).

Deng et al. reported the preparation of PAni–Fe_3O_4 nanoparticles with core-shell structure via an in situ polymerization of aniline monomer in an aqueous solution, which contains Fe_3O_4 nanoparticles and surfactant NaDS (Deng *et al.,* 2003).

Most of these methods require vigorous stirring of the solution to suspend the magnetic particles and prevent the aggregation of the nanosize magnetic particles during the reaction (Lu *et al.,* 2005). The functionalization of inorganic components by PAni was found to improve the colloidal stability and the nature of the association between the components (Ahmad, 2013). The synthesis techniques, characteristics and loading amount of inorganic materials influenced the ultimate properties of the composite (Ahmad, 2013).

Antibody/antigen electron signal interaction is transduced by this conductive layer of the PAni/Fe_3O_4 nanocomposites which penetrates the insulating shell of the biomolecule and provides a means for direct electrical communication between the redox centre and the electrode surface. The signal is recorded by the electronic system (Reader) for amplification, analysis and display.

Recently, several immunosensor devices have been developed on screen-printed carbon electrodes (SPCEs) (Serafín *et al.,*2018, Viet *et al.,* 2019,). The main advantages of the screen-printed electrode include simplicity, versatility, modest cost, portability, ease of operation, reliability, small size, and mass production capabilities (Ambaye et al. 2021,Munteanu et al., 2018).

Thus this study aimed to develop an electrochemical immunosensor using PAni/Fe_3O_4 nanocomposite modified ITO for antigen detection.

2. Experimental

Reagents and materials

Aniline monomer (99.9%) was supplied by R&M chemicals and were distilled under reduced pressure before storage at a temperature below 0 ºC. All other chemicals and reagents were of analytical grade, and were used without additional purification. Ammonium peroxydisulfate (APS) was used to initiate the polymerisation, Phosphoric acid (H_3PO_4) was used as doping agent, others include N-phenyl-1,4-phenylenediamine, Acetonitrile, CH_2Cl_2, Formic acid, $FeCl_2 .4H_2O$, $FeCl_3 .6H_2O$, Diethyl ether and $NH_3.H_2O$ (25%). Succinic anhydride, Methanol, Ammonium hydroxide and glutaraldehyde was supplied by QReC. β-Amyloid, anti-β-Amyloid, and 0.1% bovine serum albumin (BSA) were supplied by Calbiochem and were prepared in a phosphate buffer (50 mM, pH 7.0). Phosphate buffer solution (PBS) pH 7.0 was used as redox mediator as well as solvent for making various antigen concentrations and 10 mM $K_3[Fe(CN)_6]+K_4[Fe(CN)_6]$ was used as redox probe during electrochemical measurements and were also supplied by QReC.

Synthesis of Aniline dimer-COOH

The reaction was carried out in a 100-mL three-necked round bottom flask equipped with a mechanical stirrer, nitrogen inlet and outlet. N-phenyl-1,4-phenylenediamine 0.921 g (5.0mmol) and

succinic anhydride 0.500 g (5.0mmol) were dissolved in 30mL CH_2Cl_2 with stirring at room temperature for 5 h. As the reaction proceeded, a white-grey precipitate was formed. At the end of the reaction, the precipitate was collected by filtration and washed with diethyl ether until the filtrate became colourless. The product was dried under a vacuum for 12 h at room temperature.

Synthesis of Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were prepared as described by (Lu *et al.,* 2005): 0.86 g of $FeCl_2.4H_2O$ and 2.35 g of $FeCl_3.6H_2O$ were dissolved under N_2 in distilled deionized water (20mL) under vigorous stirring. As the solution is been heated to 60 ^oC, $NH_3.H_2O$ (28% (w/w), 5mL) was added, quickly followed by the addition of a solution of aniline dimer-COOH (200 mg) in 2mL of acetone. The reaction was allowed to proceed for 1 h at 80 ^oC with constant and vigorous stirring to produce a stable, water-based suspension. The reaction mixture was then cooled slowly to room temperature. The suspension was washed sequentially with acetone and ethanol.

Preparation of PAni-Fe_3O_4 composites

The PAni-Fe_3O_4 nanocomposites were synthesized by ultrasonic irradiation method. In a typical procedure, 0.2mL aniline monomer was mixed with H_3PO_4 (0.07mL) and 10 or 20 wt. % Fe_3O_4 dissolved in 15mL of deionized water under ultrasonic stirring for 10min to dispersed to Fe_3O_4 nanoparticle and to form an emulsion of aniline/H_3PO_4 complex containing Fe_3O_4 nanoparticles at room temperature. Then 5mL aqueous solution of APS (0.46g) was added to the above mixture. The reaction was kept by ultrasonic stirring for 4h at a temperature between 25 and 30^oC. When APS was added the suspension turned green immediately indicating the polymerization of Aniline. The resulting precipitate (Blackish green ppt) was obtained by filtering and washing the reaction mixture with distilled water and ethanol, respectively. Finally, the product was dried in a vacuum at 70^oC for 24h.

Fabrication of composite film

Thin film of PAni–Fe_3O_4 composite was deposited by the electrophoretic technique (Dhand *et al., 2008*) on an ITO-coated glass plate with a dimension of $1.27 \times 2.54 \times 0.1 cm^2$ and a resistance value ranging from 15 to 25 ohms to serve as a matrix for immobilization of antibody (anti-β amyloid). The ITO coated glass was ultrasonically cleaned with dilute ammonia, deionized water, ethanol and deionized water for about 5 min, respectively and air-dried. For film fabrication, a colloidal suspension of PAni-Fe_3O_4 was prepared by forming a solution of 120 µl of PAni-Fe_3O_4/formic acid (1 mg ml^{-1}) in 9.9 ml of acetonitrile followed by ultrasonication for about 1 min. The set-up for the EPD consists of two electrodes with platinum foil as an anode and a pre-cleaned ITO coated glass substrate as a cathode. The two electrodes are placed parallel to each other with 1 cm separation and dipped in PAni/Fe_3O_4 colloidal suspension.

Variation of DC voltage (60, 80 and 100 V) and time (20, 30 and 40 s) was also investigated for optimum desired uniform film formation. Washing of film prepared in this way was accomplished using deionized water followed by drying. The substrate was left to air-dry and referred to as ITO-PAni/Fe_3O_4 substrate.

Characterization

The ultrasonic irradiation experiments were performed with UP100H Hielscher ultrasonic GmbH Germany, X-ray diffraction (XRD) patterns was obtained using PANalytical X'Pert^3 Powder X-ray diffraction system. Atomic force microscopy (AFM) measurements were carried out with XE-100 AFM Park system USA, in the tapping mode. IR measurements were performed with a Fourier transform infra-Red (FTIR) spectrophotometer on a PerkinElmer spectrometer (Model spectrum 100 using universal Attenuated total reflectance (ATR) sampling accessory). The pH measurements were made with a pH meter (MP 230, Mettler-Toledo Switzerland). FESEM image were conducted using (FESEM, JEOL, JSM-7600F, Japan) by Field Emission scanning electron microscopy. UV-Vis spectrophotometer (Shimadzu UV-1800) Japan. LUCAS LABS Pro4, China.

ITO coated glass used in this study were purchased from a local company (ScrintTechnology, Penang, Malaysia).

Multi Autolab/M101 Potentiostat/Galvanostat. (Metrohm autolab B.V. Netherland) was used for cyclic voltammetry and impedance measurements. The CV experiments were performed in a conventional electrochemical cell containing a three-electrode system where bare and modified ITO was used as a working electrode, platinum wire as an auxiliary electrode and Ag/AgCl as a reference electrode. Impedance measurements of the electrodes were carried out with a (Model S1 1260 impedance/gain-phase analyser Solartron analytical UK) and were performed in a background solution of 5.0×10^{-3} mol/L $K_3[Fe(CN)_6]$ + $K_4[Fe(CN)_6]$ at 25 ◦C. The frequency range is at 100 mHz–10 kHz at 220 mV versus Ag/AgCl.

FESEM and XRD studies indicated that the obtained Fe_3O_4 nanoparticles are crystalline and are embedded in the PAni matrix. The synthesized PAni/Fe_3O_4 nano-composites show different thermal, electrical and magnetic properties with an average particle size of 12 nm.

Measurements

Room temperature conductivities of the pressed pellets were measured by the standard Van Der Pauw DC four-probe method which was used to measure the electron transport behavior of PAni nanostructure containing Fe_3O_4 nanoparticles. The samples of PAni/Fe_3O_4 were pressed into pellet. Then the pellet was cut into a square. The square was placed on the four-probe apparatus. Providing a voltage, a corresponding electrical current was obtained. The electrical conductivity of samples was calculated by the following formula:

$$\sigma\ (S/cm) = (2.44 \times 10/S) \times (I/E) \quad (1)$$

Where σ is the conductivity; S is the sample side area; I is the current passed through outer probes; E is the voltage drop across inner probes (Lu *et al*., 2006).

Measurement Procedure for Electrochemical Responses

All the electrochemical responses were performed at room temperature (27±1°C). Electrochemical measurements were carried out by placing a 50 µL TMB solution onto the electrode, covering the three electrodes' areas. Fixed reduction potential of −200 mV was applied to the reference electrode to measure the electrochemical responses. Each measurement was carried out in triplicate. Cyclic voltammetric measurements were carried out by scanning at 100 $mV \cdot s^{-1}$ and potential ranging from −600 mV to 1000 mV.

3. Result and Discussion

Morphology and Structural Properties

The morphologies of the resulting PAni/Fe_3O_4 composites containing 5, 10, 15 and 20 wt. % Fe_3O_4 are shown in Figure 1. FESEM technique was used to visualize the electrode surface modification following antibody immobilization of the carbon electrode.

Figure 1 & 2 shows the FESEM image of a bare working electrode surface. However, the surface was then covered with cloudy clusters (Figure 2b) following incubation with the anti-Amyloid antibody.

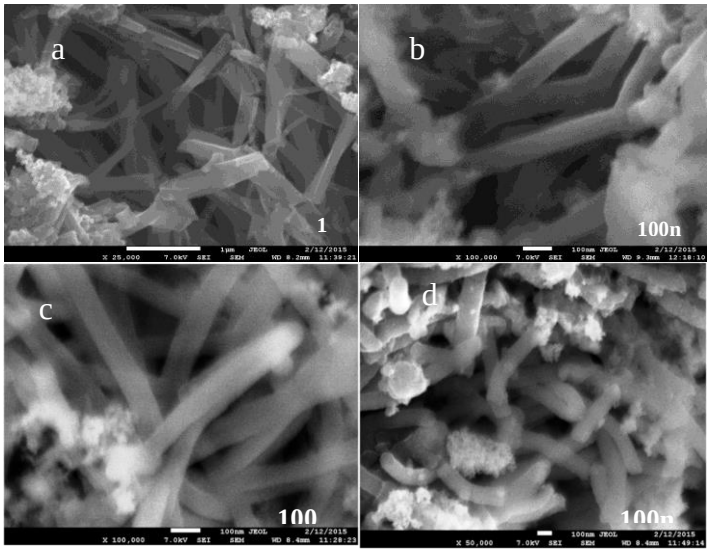

Figure 1. FESEM images of PAni nanotube containing (a)5wt% (b) 10wt% (c) 15wt% and (d) 20wt% Fe_3O_4 nanoparticle

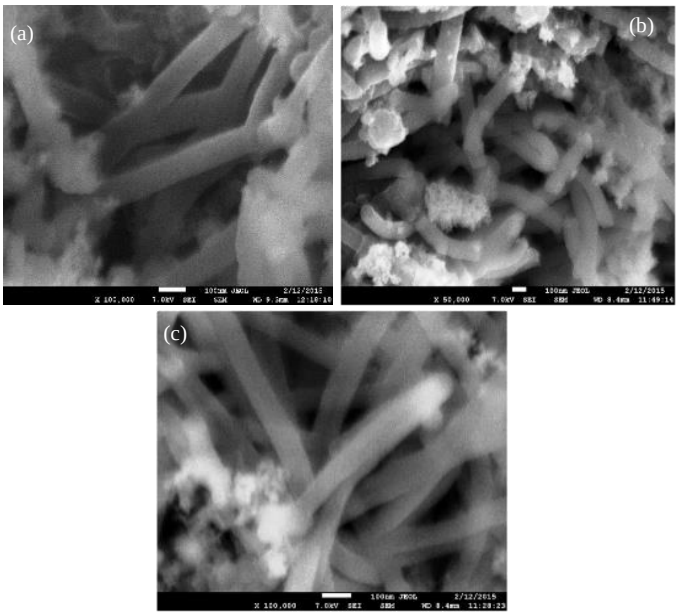

Figure 2. FESEM images of PAni nanotube containing (a) 5wt% (b) 15wt% (c) 20wt% Fe_3O_4 nanoparticles (before deposition)

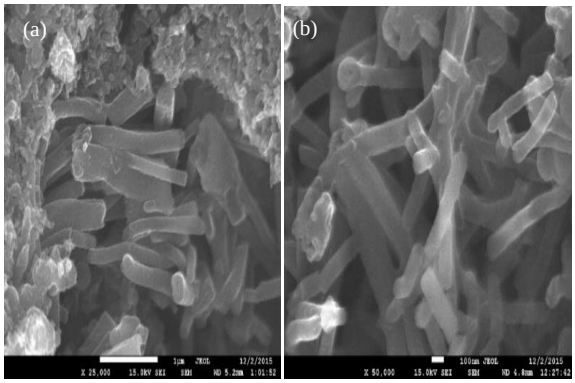

Figure 3**.** FESEM Morphology of PAni/Fe_3O_4/ITO Modified electrode with (a) 5%wt (b) (15%wt) Fe_3O_4 nanoparticles. (After deposition)

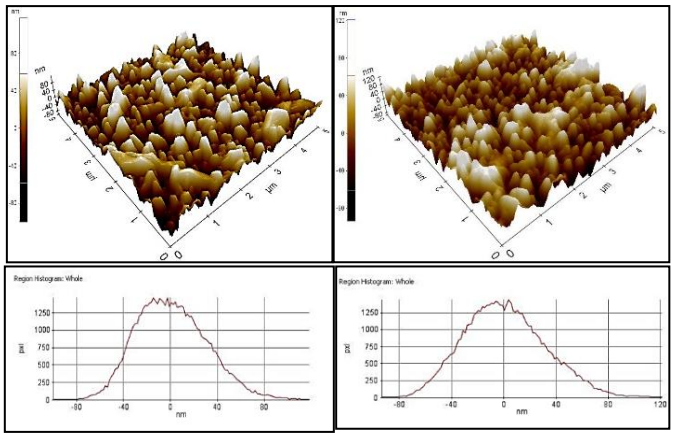

Figure 4. AFM images of PAni/Fe_3O_4 after deposition on ITO coated glass synthesize with (a) 5 % wt. Fe_3O_4 (b) 20 % wt. Fe_3O_4

Reproducibility and Stability of ITOs

The reproducibility of Indium Tin Oxide electrodes was characterized by performing cyclic voltammetry (CV) using a potassium ferricyanide redox system. The cyclic voltammogram data obtained from triplicate assays showed a relative standard deviation (RD) value of 3.22 %, suggesting a good reproducibility of the proposed immunosensor.

Another critical parameter that was investigated was the stability of the ITOs. In this study, the ITOs were subjected to 10 cycles of CV at 100 mV/s scan rate in 5 mM ferricyanide solution prepared in 0.1 M KCl. As shown in Figure 6, a pair of well-defined redox peaks were observed after each cycle. An RSD value of 4 % showed that the electrode was stable enough for electrochemical analysis.

Electrochemical characterization of the modified electrode

Electrophoretic deposition (EPD) was used to deposit PAni/Fe_3O_4 onto the surface of ITO. The FESEM image of PAni/Fe_3O_4/ITO (Figure 3) clearly shows the presence of the nano-composites distribution on the ITO. From the cyclic voltammogram Figure 6 and 7, we can find the peak currents decrease after PAni/Fe_3O_4 was adsorbed on the modified electrode indicating that the membrane is becoming less conductive due to the slow movement of the electrons.

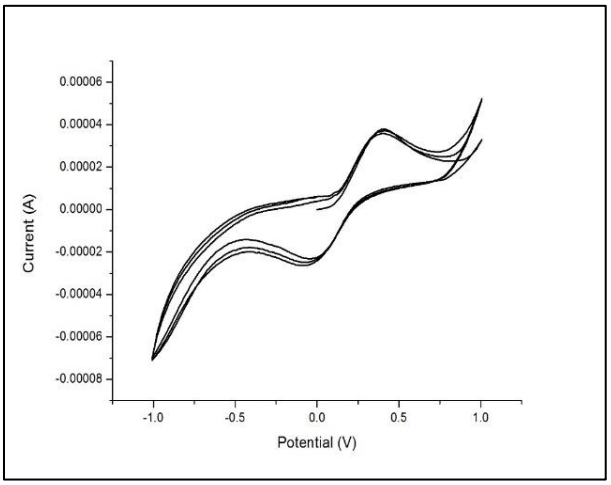

Figure 6. Cyclic voltammogram profile of the ITO with 10 cycles. Scanning was performed in 5 mM ferricyanide prepared in 0.1 M KCl at the scan rate of 100 mV/s

Moreover, the electron diffraction patterns indicate that the obtained Fe_3O_4 were crystalline. The formation of the nanotube is not regular when 5 and 20 wt. % Fe_3O_4 was added to PAni as shown in Figure 2(a) & (c) respectively, there is also a little aggregation of Fe_3O_4 nanoparticles in the composite nanotubes sample containing 20 wt. % Fe_3O_4 nanoparticles Figure 2(b).

Table 1 show the effects of both Fe_3O_4 and H_3PO_4 respectively on the conductivity of the composites, when the amount of (H_3PO_4) is increased the pH is reduced, the resulting conductivity at room temperature increases from 7.40×10^{-5} to about 6.06×10^{-3} S/cm as the dopant is increased from 0.05-0.07ml, further increasing of H_3PO_4 results in a reduction of the composite's conductivity at room temperature. The increase in conductivity may be attributed to an increase in the dopant concentration which is believed to be the major factor possibly playing a more important role (Haldorai *et al.,* 2011). The conductivity values obtained are similar to those informed by (Deng, *et al.,* 2003, Mahmoud et al., 2022) for PAni-Fe_3O_4 composites with core-shell structure from a magnetic fluid and dodecylbenzensulfonic acid sodium salt as a surfactant and dopant for a sample containing 43.86 % magnetic oxide.

Table 1. Conductivity of PAni with different amounts of Fe_3O_4 and H_3PO_4.

Sample	Fe_3O_4 content (wt.%)	Conductivity (S/cm)	H_3PO_4 (ml)	Conductivity (S/cm)
PAni		1.04×10^{-3}		1.04×10^{-3}
Pani/Fe_3O_4	5 %	6.49×10^{-4}	0.05	5.26×10^{-5}
Pani/Fe_3O_4	10 %	7.46×10^{-4}	0.06	3.04×10^{-4}
Pani/Fe_3O_4	15 %	6.06×10^{-3}	0.07	3.52×10^{-3}
Pani/Fe_3O_4	20 %	1.07×10^{-4}	0.08	2.07×10^{-4}

Immobilization of the anti-β amyloid

An indirect method was also employed to examine the immobilization of the anti-β amyloid antibody in the immunosensor. The stepwise immobilization of the anti-β amyloid antibody on ITO was accomplished by CV using 5 mM $[Fe(CN)_6]^{3-}$ prepared in 0.1 M KCl solution as the redox probe. A decrease in the anodic and cathodic peaks was observed when the ITO were modified with the anti-β amyloid antibody.

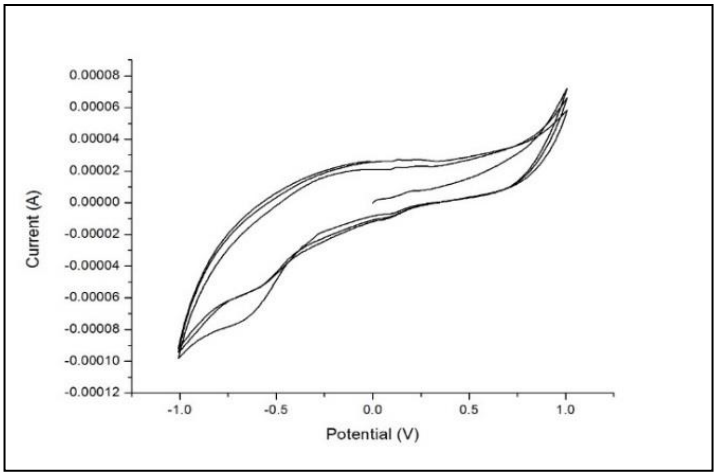

Figure 7. Cyclic voltammogram of the ITO electrode coated with Pani/Fe_3O_4

After polymerization the solution of aniline, APS, phosphate acid and some quantity of Fe_3O_4 nanoparticles under magnetic stirring, could only yield PAni/Fe_3O_4 powder, no PAni/Fe_3O_4 nanotubes could be obtained. However, under ultrasonic irradiation, PAni/Fe_3O_4 composite nanotubes could be observed. This was thought to be attributed to ultrasonic irradiation.

Lu et al. studied the mechanism of PAni nanofibers produced by interfacial polymerization and they thought that PAni preferentially forms as nanofibers in aqueous solution during chemical oxidative polymerization (Lu, *et al.,* 2006). The nanotubes produced in the early stage of polymerization during slow-feeding reactions were subject to secondary growth, which led to the large agglomerates containing irregularly shaped particles. Pure nanotubes could be obtained by preventing secondary growth. In our method to synthesize PAni, the secondary growth was prevented effectively after using ultrasonic irradiation, and a nanotube is obtained.

FTIR spectra are used to characterize the molecular structures of PAni/Fe_3O_4 composite nanotubes. Figure 8 shows the characteristic peaks of PAni nanotubes with four different quantities of Fe_3O_4 nanoparticles. The spectrum of pristine Fe_3O_4 had a band at 689 cm^{-1} ascribed to the Fe–O stretching vibration. It is found that PAni/Fe_3O_4 composite nanotubes have characteristic peaks at around 3124cm^{-1} (N–H stretching), 1541 cm^{-1}, 1385 cm^{-1} (C=C stretch deformation of the quinoid and benzenoid ring, respectively), 1303 cm^{-1} (C–N stretch of secondary aromatic amine), 1140cm^{-1}, and 889cm^{-1} (out-of-plane deformation of C–H in the 1,4-disubstituted benzene ring) which indicate the availability of PAni containing Fe_3O_4. In Figure 8b of these peaks are similar to those of PAni synthesized by a common method (Haldorai, *et al.,* 2011).

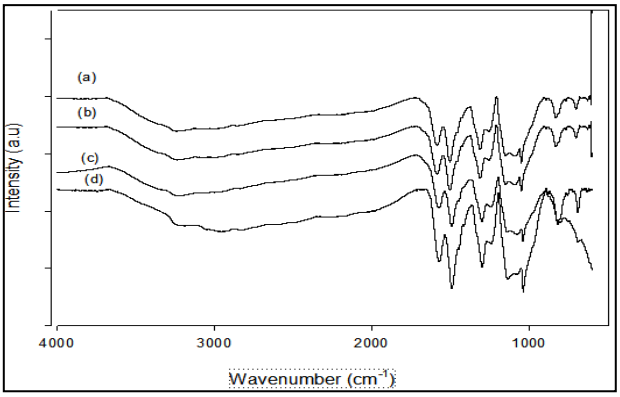

Figure 8. FTIR spectra of PAni nanotubes containing (a)5 wt.% (b) 10wt% (c) 15wt% and (d) 20wt% Fe_3O_4 nanoparticles.

However, the incorporation of Fe_3O_4 nanoparticles leads to the shift of some FT-IR bands of the PAni. This may be ascribed to the fact that the interaction of Fe_3O_4 and PAni was followed by the formation of H-bonding between the proton on N–H and the oxygen atom on the Fe_3O_4 surface (Qiu, *et al.,* 2006).

Table 1 also shows that when 5% Fe_3O_4 was incorporated into PAni, the conductivity of PAni composite was greatly reduced from 1.04×10^{-3} of pure PAni to 6.49×10^{-4} S/cm. Further increasing of Fe_3O_4 content from 5 to 10 wt.%, results in a slight reduction of the conductivity at room temperature. Although at 15wt%, the conductivity is higher than the other values but still slightly lower than the pure PAni. Both the insulating behaviour of iron oxide in the core of the nanoparticles and the decrease in the doping degree is thought to play a major role in the decrease of the room temperature conductivity of the PAni/ Fe_3O_4 nano-composites.

4. Conclusion

In this study, a simple method to synthesize surface-modified PAni/Fe_3O_4 nano-composites *via* ultrasonically-assisted chemical oxidative polymerization was demonstrated. Under ultrasonic irradiation, the aggregates of Fe_3O_4 nanoparticles were broken down and the particles were redispersed in an aqueous solution, the nano-composites are found to be stable and electrically conductive. It is therefore possible to prepare a nano-composite with moderate conductivity that can be used as a conductive matrix for the development of sensors.

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