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Ultrathin polyaniline film coated on an indium–tin oxide cell-based chip for study of anticancer effect

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ABSTRACT

Polyaniline emeraldine base (EB) coated indium–tin oxide (ITO) electrode was prepared for the construction of a cell-based chip. Ultrathin polyaniline PANI film on an ITO was electroactive at neutral pH without co-deposition of an acidic counterion. HeLa cells were cultured on a PANI/ITO substrate and utilized to assess the biological toxicity of anticancer drugs. Cell growth, cell viability and drug-related cell toxicity were evaluated by a cyclic voltammetry (CV) method under a neutral pH. We demonstrated the functionality of a PANI coated ITO electrode for use as a cell chip and found that PANI was a good surface for the HeLa cells to grow without any significant morphological changes.

1. Introduction

The factors that play an essential role in cell adhesion mechanisms are: specific surface chemistry, surface hydrophobicity, topography, surface charge, and protein interactions [1]. Conductive polymers are found to have widespread use in the development of new biosensing technologies. Enzymes, antibodies and whole living cells [2] have been incorporated as biorecognition elements into inherently conductive polymers. Amongst conductive polymers, PANI is regarded as one of the most technologically promising polymer due to its ease of synthesis, low cost and environmental stability [3,4]. Its chemical, electrical and optical properties can be used to convert chemical information or biointeractions into electrical or optical signals, which can easily be detected by modern techniques [5,6]. Unfortunately, PANI shows electrochemical activity only in acidic conditions. This restricts its applications especially in bioelectrochemistry, since most biocatalytic and immunological reactions occur optimally at neutral pH (pH 7). Many efforts have been focused on adapting PANI to be active at neutral solution pH. This has been done by the introduction of acidic groups into the PANI chains [7], or doping PANI with negatively charged polyelectrolytes [8]. However, all of these approaches cannot obtain polymers with higher degrees of homogeneity. Ultrathin films of PANI have received great interest due to their potential applications in chemical and biological sensors [9]. The Langmuir–Blodgett (LB) technique, electropolymerization, and layer-by-layer self-assembly have been used to deposit PANI thin films onto a variety of electrode materials [10]. It is difficult to prepare homogenous ultrathin (<10 nm) polymer films by electropolymerization. Relative to LB deposition self-assembly is advantageous due to both of its technical simplicity and inherent flexibility. A living cell can be properly described as an electrochemically dynamic system; many important processes in living cells have electrochemical characteristics. For example, redox reactions and changes in ionic composition derived from various cellular processes lead to electron generation and electron transfer at the interface of living cells [11,12]. Cell-based sensors are potentially useful for studying the effects of drugs and cell–external stimuli interactions [13]. In vitro immobilization of living cells is an important process in the fabrication of a cell-based chip [14], and the interaction between cells and the adhesion of cells to the chip surface can be a reliable candidate for cellular attachment without loss of viability.

In the present study, we used ultrathin PANI film deposited on ITO [15]. Unlike metal electrodes such as platinum or gold, PANI deposited on ITO electrode is electroactive at neutral pH without co-deposition of an acidic counterion. Immobilized HeLa cells were used to study the ability of this system to determine the cell viability electrochemically. This method was used to determine the effectiveness of anti-cancer drugs on cancer cell viability. The prepared ultrathin PANI film deposited on ITO surface was demonstrated to be very effective for the immobilization of cancer cells and provide a simple, low-cost and easy method for electrochemical study of cell adhesion, proliferation and the effect of anticancer drugs on cells.

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2. Experimental

2.1. Materials

Aniline and 1-methyl-2-pyrrolidinone (NMP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxyurea and cyclophosphamide were purchased from Calbiochem (Germany). All other chemicals that are used in this study were obtained commercially as reagent grade.

2.2. Cell culture

HeLa cells obtained from a human epithelial carcinoma cell were routinely cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum, and a 1% concentration of antibiotics. Cells were maintained under standard cell culture at 37 °C in an atmosphere of 5% CO_2.

2.3. Electrochemistry

The CV studies were performed using a potentiostat (CHI-660, CHI, USA). The cell/PANI/ITO was used as the working electrode, a platinum wire as the counter electrode, and an Ag/AgCl as the reference electrode. A solution of 10 mM PBS (pH 7.0) was used as the electrolyte. The scan rate was 0.1 V/s.

2.4. Raman spectroscopy and topological analysis by atomic force microscopy (AFM)

The immobilization of PANI on ITO substrate was investigated with atomic force microscopy with semi-contact mode, and Raman spectroscopy using AFM-Raman NTEGRA spectra (NT-MDT, Russia). The maximum scan-range, *XYZ* was 100 μm × 100 μm × 6 μm, and the resolution of the spectrometer in the *XY* plane was 200 nm and along the *Z* axis it was 500 nm. Raman spectra were recorded using a He–Ne laser emitting light at a 633 nm wavelength. Five scans of 10 s from 400 to 1800 cm^{-1} were recorded and the mean of these scans was used.

2.5. Preparation of polyaniline base

Emeraldine salt (ES) was chemically synthesized according to the standard method [16]. Briefly, 2.5 ml 0.25 M ammonium persulfate ($(NH_4)_2S_2O_8$) aqueous solution was added drop by drop to 1.5 ml 0.5 M aniline dissolved in 1 M HCl solution, both solutions being precooled to 0 °C. The reaction was allowed to proceed for about 2 h under stirring in an ice bath. Then the precipitate (ES) which had formed was removed by filtration, washed repeatedly with 1 M HCl and dried under vacuum for about 48 h. The above obtained PANI-HCl salt form was converted into the polyemeraldine base (EB) form by treating it with a 0.1 M ammonium hydroxide (NH_4OH) solution for about 24 h while stirring. The obtained powder was then dried under vacuum for 48 h. The mechanism of oxidative chemical polymerization of aniline and the conversion of ES into EB are shown in Fig. 1a.

2.6. Preparation of PANI thin film and electrode preparation

EB was dissolved in NMP by sonicated for 12 h, the concentration of PANI was 1 mg/mL, then the solution was filtered, followed by depositing onto cleaned ITO substrates using the self-assembly technique at different deposition time (15 min, 30 min and 50 min) [17]. It was then rinsed with DI water for 15 s, and then soaked in DI water for 5 min followed by drying gently under N_2 stream, Fig. 1b shows Schematic of the PANI film deposition process using slides and cell immobilization. The dimension of the working electrode (ITO) was 2 cm × 1 cm, while the active area for PANI deposition was

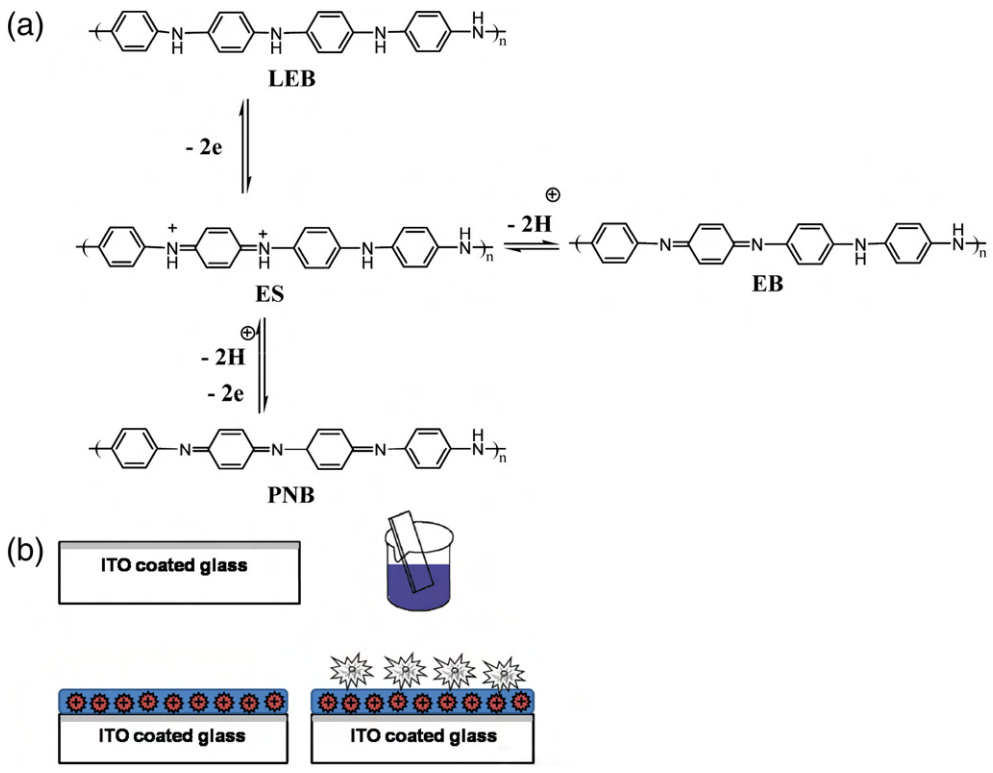

Fig. 1. (a) Schematic of PANI equilibria showing the chemical structures of the four oxidation states of PANI (fully-reduced LEB, half-oxidised EB, fully-oxidised PNB and the salt form ES) response to pH or potential changes. (b) Schematic of the PANI film deposition process using slides and cell immobilization.

1 cm × 1 cm, the substrates were attached to chamber using PDMS creating an exposure area for cell attachment had dimensions of was about 1 cm × 1 cm × 0.5 cm (width × length × height). The cells were transferred into the chip at a known cell density by infusion of new culture medium. The deposition of PANI film was conformed by using AFM, Raman spectroscopy (Fig. 2a and b) and CV (Fig. 3a).

3. Results and discussion

3.1. PANI ultrathin film

The deposition of PANI film on ITO was confirmed using Raman spectroscopy (Fig. 2a). A blank spectrum was acquired prior to film deposition, which allowed the absorbance of the film to be subsequently measured. The assignments of the Raman bands are listed in Table 1, the main absorption bands situated at 820, 1170, 1480, and 1550 cm^{-1} are attributed to the following vibrations: bending of C–H (out-of-plane) on benzene ring p-disubstituted, bending of C–H (in-plane), stretching of Caromatic-N, stretching of N–B–N ring, and stretching of C–C in benzene ring, respectively [18]. AFM experiment was carried out in order to study morphology of PANI film. Fig. 2b and c shown the topography of the PANI film on an ITO substrate observed by tapping mode AFM. The formation of uniform polymeric islands can be observed, with islands height of about 30 nm. The pH-dependent based on PANI films have been reported, in all of these studies, the PANI film was relatively thick (60–700 nm) [15]. Thinner films should respond more rapidly, these result cloud be explain the change of PANI/ITO conductivity with change of pH from 7.0 to 7.4.

3.2. Electrochemistry

Fig. 3a shows the cyclic voltammogram of PANI/ITO in the potential range from +0.4 to −0.2 V at pH 7.0. This shows a cathodic peak at −0.073 V and an anodic peak at +0.036 V. This electrochemical activity of PANI/ITO at neutral pH was related to the presence of acidic groups on the ITO surface which appeared to be function as acidic counterions [15]. The effect of deposition time on the electrochemical activity of PANI/ITO was studied (Fig. 3a). We observed that as the deposition time increased the electrochemical activity decrease. We selected 15 min as the optimum deposition time. Fig. 2b shows the effect of pH on the electrochemical properties of PANI/ITO we selected this pH range near to neutral pH (pH 7) since most of the electrochemical studies on cell had been done at pH = 7.4. As the pH increased (pH = 7.0, 7.2 and 7.4), the electrochemical activity decreased. The effect of pH may be related to the fact that, PANI is a mixed oxidation state polymer composed of reduced benzenoid units and oxidized quinoid units [19]. PANI can exist in several oxidation states ranging from the completely reduced leucoemeraldine

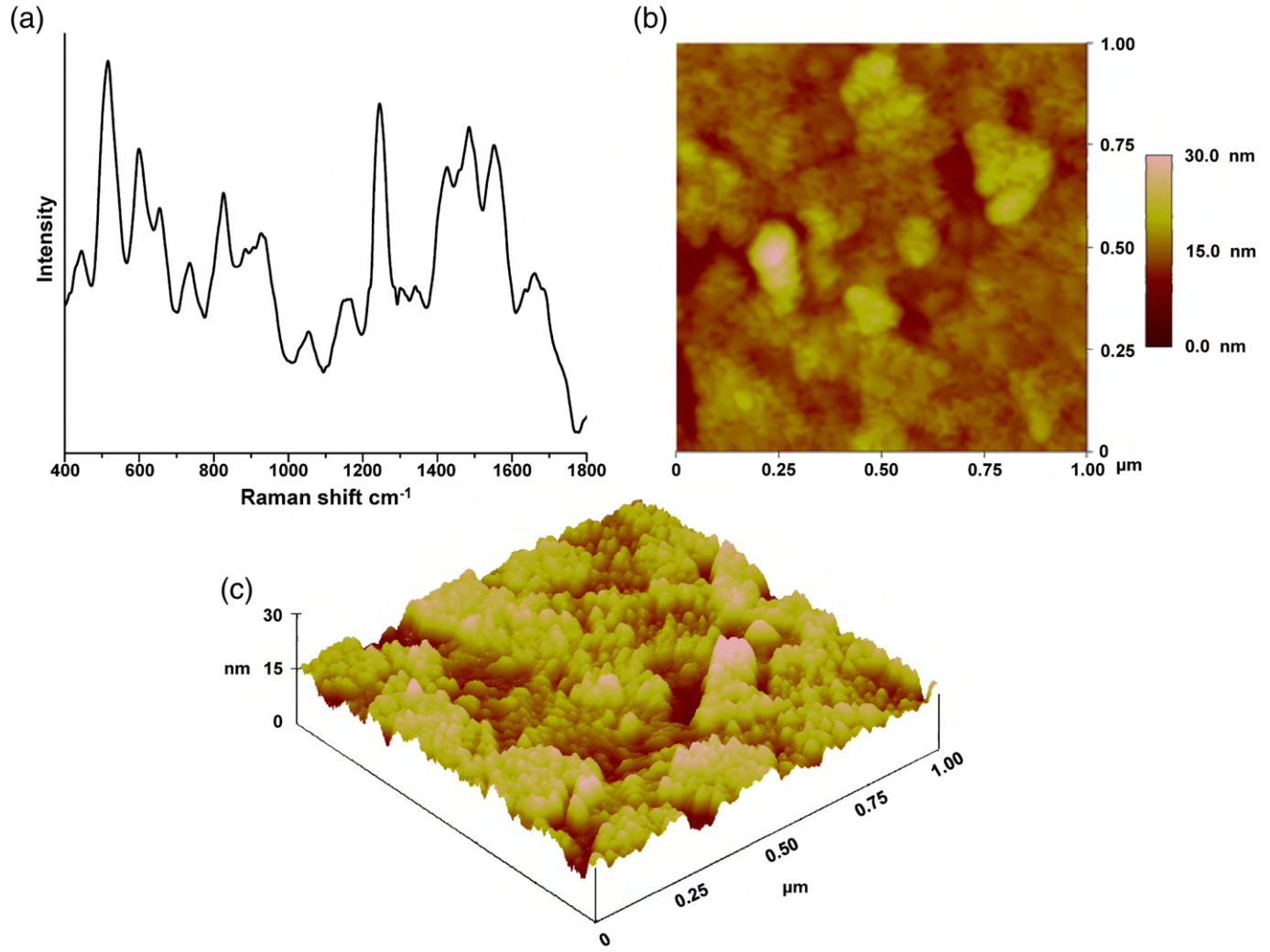

Fig. 2. (a) Raman spectra of a PANI film deposited on ITO electrode using He–Ne laser at 633 nm. (b) AFM of PANI film deposited on ITO electrode. (c) Height AFM of PANI film deposited on ITO electrode.

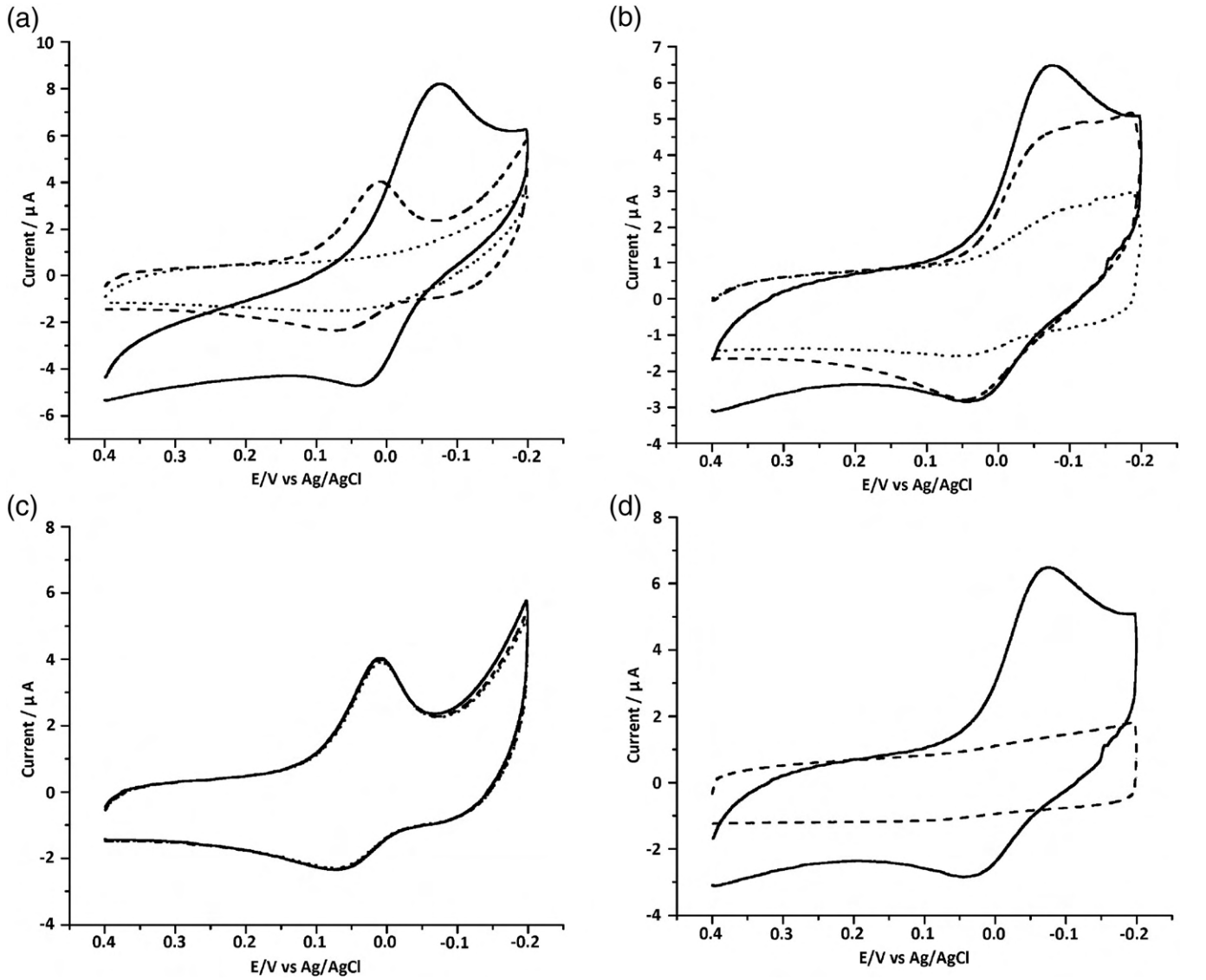

Fig. 3. CV of PANI/ITO at (a) different deposition time, (—) 15 min, — —) 30 min, (•••) 50 min. pH was 7.0 and 0.1 M phosphate buffer. (b) different pH, (—) 7.0 pH, (— —) 7.2 pH, (•••) 7.4 pH. (c) different cycles numbers (—) 2 cycles, (— —) 10 cycles, (•••) 25 cycles. (d) effect of physiological conditions on the CV of PANI/ITO (—) before immersing in media, (— —) after immersing in media for 48 h. The scan rate was 0.1 V/s.

base (LEB) state, to the completely oxidized pernigraniline base (PNB) state. The half-oxidised emeraldine base (EB) state is composed of an alternating sequence of two benzenoid units and one quinoid unit. Each

Table 1
Band assignments of the Raman spectra of PANI Film.

Raman shift cm^{-1}	Ring	Assignments
~380	Benzene	In-plane ring deformation
~440	Quiniod structure	Out-plan C–H wag
~520		Out-plane C–N–C torsion
~600	Benzene	In-plane amine deformation
~650	Quiniod structure	In-plane ring deformation
~740	Quiniod structure	Imine deformation
~820	Quiniod structure	Out-plan C–H deformation
~880	Benzene	In-plane ring deformation
1170–1180	Quiniod structure	In-plane ring binding
~1250	Benzene	C–N stretching
1330–1340	Semiquiniod	$C–N^+$
~1480	Quiniod structure	C=N stretching
~1550	Benzene	C–C stretching
~1650		Cyclized structure containing nitrogen formed by crosslinking

of the above mentioned three forms of PANI acts as an insulator. However, the insulating EB form can be non-redox doped with protonic acids (HA) to yield the emeraldine salt (ES) form [20]. Both the redox doping process and the non-redox doping process are reversible, the ES form can be converted back to its corresponding EB forms if the conditions change, either physically (for non-redox doping) or (electro-) chemically (for redox-doping). The equilibrium potential of the emeraldine/leucoemeraldine (LE) redox couple is also dependent on pH; consequently, the conductivity of an electrode supported PANI film is sensitive to pH [15]. We selected pH 7.0 as an optimum pH at which PANI/ITO electrode shown high electrochemical activity. The CV of PANI/ITO at different cycle number until 25 cycles (Fig. 3c) showed a slight decrease in peak current. This result provides the stability for PANI/ITO electroactivity. Also the conductivity of PANI/ITO under physiological conditions for 48 h was studied. The electrical conductivity decreased over time (Fig. 3d). The effect of media may be related to the effect of media which posses weak alkaline effect (pH 7.4) for long time, which may be change PANI from one oxidation state to another especially in this study we have used non-conductive emeraldine base (EB) and the electrochemical activity of PANI/ITO at neutral pH that are related to the presence of acidic groups on the ITO surface [15].

3.3. Cyclic voltammetry of HeLa cells at PANI/ITO electrode

HeLa cells on ITO and PANI/ITO were allowed to grow for two days before analysis. Fig. 4a shows the CV of HeLa cells in the potential range from +0.4 to −0.2 V. Cells adhered onto bare ITO showed no redox peaks due to ITO toxicity. HeLa cells on PANI/ITO show a quasi-reversible process, with a cathodic peak at −0.092 V and an anodic peak at +0.078 V. Since bare PANI/ITO electrode has no redox peaks after immersing to the media (Fig. 3d), these two redox peaks at −0.092 and +0.078 are related to the presence of HeLa cells. This result has provided the evidence that PANI film has protected the cells from ITO toxicity. The cell viability was examined using optical microscopy (Fig. 4b). Fig. 4c shows cyclic voltammograms at different cell numbers, inset figure shows the linear plot of reduction current peak as a function of cell number, we have chosen the reduction current peak to make simple study to aviation the confusing of negative values in case of oxidation peak. The peak current was found to be increased with increasing cell number. We extracted some redox enzymes from HeLa cells using 2-D electrophoresis techniques including NADH dehydrogenase (ubiquinine) flavoprotein 2, quinone oxidoreductase-like (QOH-1) (data not shown). From these results we could conclude that the redox enzyme in HeLa cells may have a relation with the voltammetric behavior, hence as the cell number increased or as the cell viability the contents of these enzymes were increased which might be responsible to the linear relationship between the cell number and CV behavior. Cyclic voltammograms at different scan rates of HeLa cells attached to PANI/ITO are shown in Fig. 4d. Inset figure shows linear plot of reduction current peak as a function of scan rate. With an increasing scan rate from 20 to 200 mV/s, the current peaks were increased, while the separation between the potential peaks at different scan rates |Epc–Epa| exceeded 59 mV. The peak current ratio at different scan rates was ipa/ipc ≠ 1, which was indicative of a distinct quasi-reversible character of the cell electrode process. These results demonstrated that, under the same conditions we can use the cyclic voltammetry to determine the cell number by measuring the peak current.

3.4. Voltammetric study of the effect of anticancer drugs on HeLa cells

Hydroxyurea is an effective inhibitor for DNA synthesis in HeLa cells and leads to a state of unbalanced growth [21]. The clinical activity of cyclophosphamide is associated with a decrease in aldehyde dehydrogenase 1 (ALDH1) activity [22]. HeLa cells were allowed to attach and grow for 24 h, then fresh culture medium containing different concentrations of anticancer drugs was supplied and the CV signals were detected after 24 h. Fig. 5a shows the effect of different concentrations of

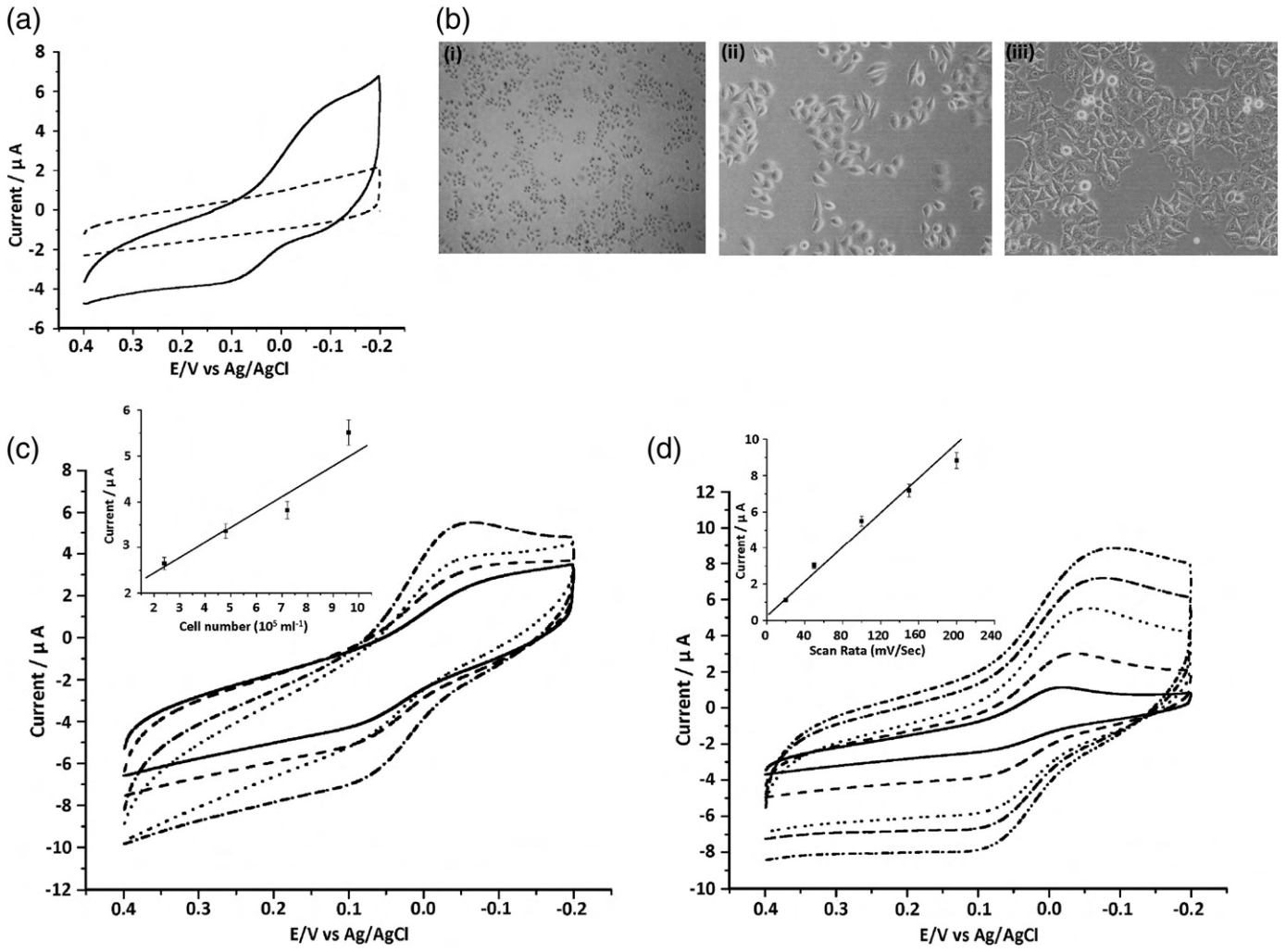

Fig. 4. (a) CV for HeLa cell on, (—) ITO and (— —) thin film of PANI/ITO, cell number was 5.8×10^5 per ml. (b) Microscopy image of HeLa on (i) ITO incubated for 48 h, (ii) thin film of PANI/ITO incubated for 24 h and (iii) thin film of PANI/ITO incubated for 48 h. (c) CV for HeLa cells on PANI/ITO at different cell numbers, (—) 2.4×10^5, (— —) 4.8×10^5, (•••) 7.2×10^5, (—•—•) 9.6×10^5. Inset linear plot of reduction current peak as a function of cell number. The scan rate was 0.1 V/s and the temperature was 37 ± 0.5 °C. (d) CV of HeLa cells on PANI/ITO at different scan rate(—) 20, — —) 50, (•••) 100, (—•—•) 150 and (— •• —) 200 mV/s. Inset linear plot of reduction current peak as a function of scan rat. Data is shown as the mean ± standard deviation of three different experiments.

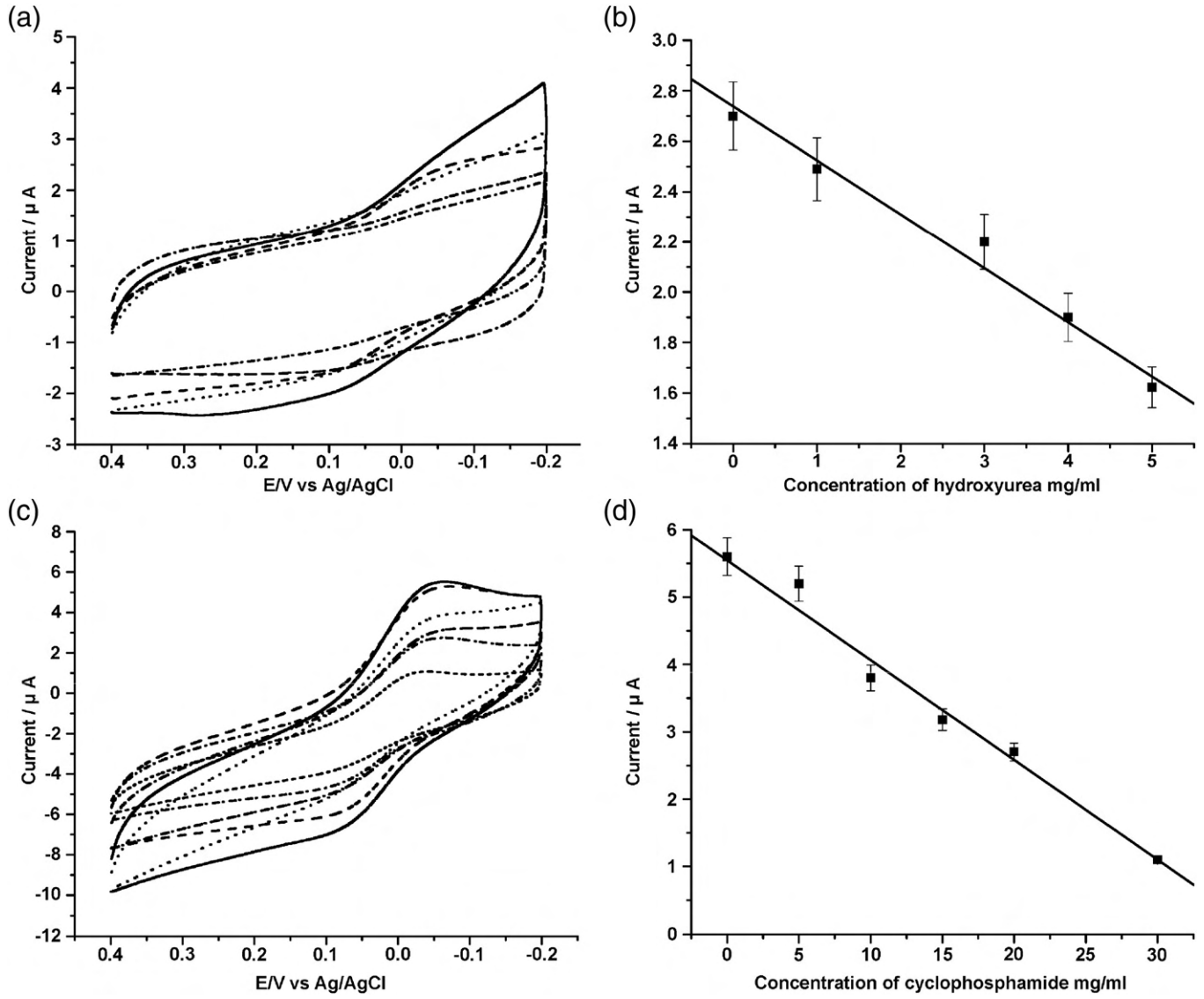

Fig. 5. (a) CV of HeLa cells treated with varying concentrations of hydroxyurea (—) 0, (— —) 1, (•••) 3, (—•—•) 4 and (— •• —) 5 mg/ml. (b) Linear plot of reduction current peak as a function of anticancer concentration of hydroxyurea. (c) CV of HeLa cells treated with varying concentrations of cyclophosphamide (—) 0, (— —) 5, (•••) 10, (—•—•) 15, (— •• —) 20, (- - -) 30. (d) Linear plot of reduction current peak as a function of the concentration of cyclophosphamide. The cell number was 5.8×10^5 per ml, the scan rate was 0.1 V/s, and the temperature was 37 ± 0.5 °C. Data is shown as the mean ± standard deviation of three different experiments.

hydroxyurea on CV response of HeLa cells. Fig. 5b shows the corresponding linear plot between the reduction current peak and hydroxyurea concentration. It was observed that as the concentration of hydroxyurea increases the peak current decreases drastically. Similar behavior was obtained when we study the effect of varying concentrations of cyclophosphamide (Fig. 5c). The corresponding linear plot between the reduction current peak and cyclophosphamide concentration is shown in Fig. 5d. These results indicate that the decrease of current peak depended on the anticancer drugs concentration, which related to the decreased cells viability and proliferation.

4. Conclusions

The present study demonstrates the cell-based chip design, which is quick and easy to do, is useful not only as a good substrate for the culture of HeLa cells but also as an electrode for measuring cellular electrochemical properties, and permits the assessment of cell viability. Also, the results establish the generality of cyclic voltammetry for use as direct electrochemical detection technique to monitor cell growth, viability and the effect of anticancer drugs on the cell viability. In this work the advantageous features of using transparent ultrathin PANI film on ITO surface, which shows redox activity at neutral pH. Anticancer drugs display significant influence on the CV of immobilized living HeLa cells.

Acknowledgments

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