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Cell-based chip for the detection of anticancer effect on HeLa cells using cyclic voltammetry

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ABSTRACT

HeLa cells directly immobilized on gold-patterned silicon substrate were used to assess the biological toxicity of anticancer drugs (hydroxyurea and cyclophosphamide). Immobilization of HeLa cells was confirmed by optical microscopy, and cell growth, viability and drug-related toxicity were examined by cyclic voltammetry and potentiometric stripping analysis. The voltammetric behaviors of HeLa cells displayed a quasi-reversible pattern with the peak current exhibiting a linear relationship with cell number. The attached living cells were exposed to different concentrations of hydroxyurea and cyclophosphamide as anticancer drugs, which induced the change of cyclic voltammetry current peak. As the exposed concentration of anticancer drugs was increased, the change of current peak was increased, which indicates the decrease of cell viability. Trypan Blue dyeing was performed to confirm the results of the effect of anticancer drugs on the cell viability which was obtained from cyclic voltammetry assay. The proposed direct cell immobilization method technique can be applied to the fabrication of cell chip for diagnosis, drug detection, and on-site monitoring.

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1. Introduction

The understanding of modeling cell behavior based on only RNA or protein expression levels is difficult, because a cell is a much more complicated system than the sum of its components (Choi et al., 2005). A living cell can properly be described as an electrochemically dynamic system with electron generation and electron transfer on the interface (Bery and Grivell, 1995). Intact living cells have been studied in many electrochemical circumstances such as electron transfer at electroactive centers in cells (Li et al., 1999), open circuit potential at the cell/sensor interface (Woolley et al., 2002a), electric cell–substrate impedance sensing (ECIS) (Xiao et al., 2002; Xiao and Luong, 2003; Arndt et al., 2004), scanning electrochemical microscopy (SECM) to obtain images of the respiratory activity of collagen-embedded living cells (Torisawa et al., 2003; Kaya et al., 2003; Allen et al., 2006), electrochemical impedance spectroscopy (EIS) (Ju and Je, 2005; Choi et al., 2007), and the oxygen electrode (Andreescu et al., 2004; Karasinski et al., 2005; Goro et al., 2007). Cell-based sensor arrays (Brogan and Walt, 2005) and electrical sensing devices have been used for signal-frequency patterns in cell growth media (May et al., 2004), making a cell-based assay an attractive method for the aforementioned investigations. There are currently two types of cell-based assay. One comprises a microfluidic device for the analysis of living cells; of which the example is a microfluidic device fabricated with polydimethylsiloxane (PDMS) consisting of an array of micro-injectors integrated in a base flow channel. This device allows controlled application of drugs to cell cultures (Thiébaud et al., 2002). The second design of microfluidic device uses electrical fields for cell immobilization and analyzes single cell ion channel using the patching-clamping technique. Both microfluidic device designs electrically measure cell viability by detecting changed electrical resistance of a cell membrane within milliseconds when the cells are exposed to a toxic agent (Huang et al., 2003). Cell-based sensor arrays are potentially useful for studying the effects of drugs and cell-external stimuli interactions (Choi, 2005; Yea et al., 2007). In vitro immobilization of living cells is an important process in the fabrication of a cell-based chip (Choi et al., 2004), and the interaction between cell–cell and the adhesion of cells to the chip surface can be a reliable candidate for cellular attachment without loss of viability. Potentiometric stripping analysis (PSA) is a technique that is concerned mainly with the determination of the metals that accumulate on a mercury electrode. PSA monitors the change of potential with time during chemical oxidation of the accumulated metal (Jagner and Graneli,

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1976). Gold electrodes also can be used in PSA experiments (Wang et al., 1995a). Wang et al. have determined trace amounts of RNA by PSA at a carbon paste electrode after adsorptive accumulation (Wang et al., 1995b). The effect of chemicals on breast cancer cells in vitro has been evaluated using PSA (Li et al., 2005) to monitor the change of electrode potential with time during the stripping process. Recently, the voltammetric behavior of HeLa cells has been measured using a graphite electrode as working electrode and suspensions of cells in phosphate buffered saline (PBS). The analysis revealed an anodic peak current but no cathodic peak current. The oxidation peak decreased greatly or disappeared in the second scan in comparison with that of the first scan (Ci et al., 2001). Since the electron-transfer rates for carbon electrodes are often slower than those observed at metal electrodes, a gold electrode potentially offers very favorable electron-transfer kinetics and a large anodic potential range (Joseph, 2000).

Presently, we used immobilized living HeLa cells to study electrochemically determined viability and growth of living cancer cells, and the action of anticancer drugs on cancer cell growth on a simulative interface for cell adherent growth. Cell–cell interactions and cell–substrate interactions are highly regulated processes that range in time from transient to long lasting, and these interactions play a crucial role in most fundamental cellular functions including motility, proliferation, differentiation, and apoptosis (Blau and Baltimore, 1991; Ruoslahti and Obrink, 1996). Cells attached to the electrode can produce electrochemical signals (Woolley et al., 2002b), which have received considerable attention in the development of biosensors (Burlage and Kuo, 1994). The immobilization of cells on a chip surface through the interaction between gold and mercapto or primary amine groups on the cell membrane also provides an environment similar to a native system, allowing more freedom of orientation of the biomolecules (Liu et al., 2003). This aids in retaining the activity of living cancer cells and prevents cell leakage from the electrode interface. Living HeLa cells immobilized on gold-patterned silicon substrate exhibited a quasi-reversible voltammetric response under the condition of that the cells were adhered and incubated for 2 days before conducting the electrochemical experiments on the cells. PSA was used to study the cell growth with time, evident as a change in the area under the peak with increasing time of cell culture. Anticancer drug affects the growth of HeLa cells, which is significant to the study of anticancer drugs. The anticancer drugs hydroxyurea and cyclophosphamide were selected as models to study the ability of the cell-based chip to monitor the effect on the cell viability using the voltammetric methods. The results were verified with comparison to the well-established Trypan Blue exclusion assay of cell viability. The results demonstrate that 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. The cyclic voltammetry (CV) and PSA methods may provide a simple way to study cell viability, cell growth, and the effect of anticancer drugs on cancer cells.

2. Materials and methods

2.1. Materials

Hydroxyurea and cyclophosphamide monohydrate were purchased from Calbiochem (Germany). Phosphate buffered saline (pH 7.4, 10 mM) solution consisting of 136.7 mM NaCl, 2.7 mM KCl, 9.7 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 was purchased from Sigma–Aldrich (St. Louis, MO, USA). 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 line was cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA), and a 1% concentration of antibiotics (Gibco). Cells were maintained under standard cell culture at 37 °C in an atmosphere of 5% CO_2. The medium was changed every 2 days.

2.3. Cell chip design and fabrication

The chip contained three gold (Au) working electrodes the area of each electrode is 5 mm^2, separated by 2 mm, creating an exposure area for cell attachment of approximately 2.6 mm^2. Gold electrodes 150 mm in thicknesses were patterned on silicon substrate by DC magnetron sputtering. Prior to this, a 50-nm thick layer of titanium (Ti) was established by sputtering to promote the adhesion of Au on silicon. The chamber created had dimensions of was about 2 cm × 2 cm × 0.5 cm (width × length × height). PDMS was used to affix substrates to the chamber. The cells were transferred into the chip at a known cell density by infusion of new culture medium. The number of cells (total and non-viable) was determined by the Trypan Blue dye method (Kun et al., 2006) overlying four 1 mm^2 areas of the counting chamber. For an accurate determination, if the number of cells per mm^2 exceeded 50, the sample was diluted and counted again. This method was replicated four times to obtain an average.

The total (or viable) cell number can be calculated as follows:

$$N_{\mathrm{tot}} = \left(N_4 \times 10^4\right) / (4 \times d) \tag{1}$$

where N_{tot} is the total (or viable) cells per ml, N_4 is the total (or viable) cells number counted in 4 mm^2 and d is the dilution factor. This method was replicated four times to obtain an average cell number.

2.4. Cyclic voltammetry-determined electrochemical behavior of HeLa cells

The cyclic voltammetry experiments were performed using a FAS2 femtostat (Gamry Instruments, Warminster, PA, USA) controlled by general propose electrochemical system software. A homemade three-electrode system comprised a cell-based chip as the working electrode, a platinum wire as the auxiliary electrode, and Ag/AgCl as the reference electrode. Measurements were carried out to study the electrical properties of living cells and the effect of anticancer drugs on their behavior in normal laboratory conditions. PBS (10 mM, pH 7.4) was used as an electrolyte at a scan rate of 0.1 V/s. The schematic diagram of the self-made cell-based chip is shown in Fig. 1.

3. Results and discussion

3.1. Cyclic voltammetry of HeLa cells at a gold electrode

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 (Bery and Grivell, 1995; Nonner and Eisenberg, 2000). Presently, HeLa cells that were attached to the working electrode were allowed to grow for 2 days before measuring the voltammetric behavior of the cells. The cyclic voltammogram of HeLa cells in the potential range from +0.4 to −0.2 V (versus Ag/AgCl) is shown in Fig. 2a. A quasi-reversible process with a cathodic peak

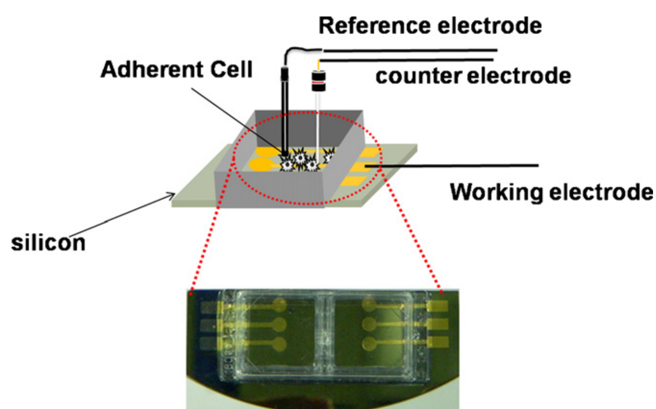

Fig. 1. Schematic representation of the electrochemical detection technique.

at −0.03 V and an anodic peak at +0.218 V was evident on the first scan. Subsequent potential scans moved the peaks to the more positive potential, with the cathodic peak appearing at +0.06 V and the anodic peak appearing at +0.288 V. Cyclic voltammograms at different scan rates of HeLa cells attached to the gold electrode are shown in Fig. 2b. With an increasing scan rate from 25 to 200 mV/s, the potential peaks became more positive, the peak current increased, the separation between the potential peaks at different scan rates $|E_{pc} - E_{pa}|$ exceeded 59 mV, and the peak current ratio at different scan rates $i_{pa}/i_{pc} \neq 1$, which was indicative of a distinct quasi-reversible character of the cell electrode process. These results demonstrated the advantage of the gold electrode in providing electron-transfer kinetics that exceeded those of non-metal electrodes. Also, the rate of electron transfer between the immobilized cells and the electrode was faster than between the suspended cells and the electrode. In the case of cell suspensions, the cell signal becomes weak, decreasing the stability of the cell's voltammetric behavior. Furthermore, the current peak decreases markedly or disappears altogether. In contrast, cell immobilization on chip surface and the resultant improved cell–cell interactions increases the cell signal and stability of the voltammetric response. The voltammetric behavior of HeLa cells was recorded for 50 cycles. A slight peak separation was observed in the second scan as compared to the first. The peaks of the remaining cycles showed no change in potential peaks but there was a slight decrease in peak current. The change in potential between the first cycle and the remainder was related to the high scan rate, but when we applied 50 mV/s there was no change in potential peaks between the first and second cycles. This cyclic voltammetry measurement is evidence of the stable behavior of HeLa cells. We extracted some redox enzymes from HeLa cells using 2D electrophoresis techniques including NADH dehydrogenase (ubiquinine) flavoprotein 2, quinone oxidoreductase-like (QOH-1). From these results we could conclude that the redox enzyme in HeLa cells may be had a relation with the voltammetric behavior. However, more research is needed to conclusively clarify the voltammetric behavior of the cells.

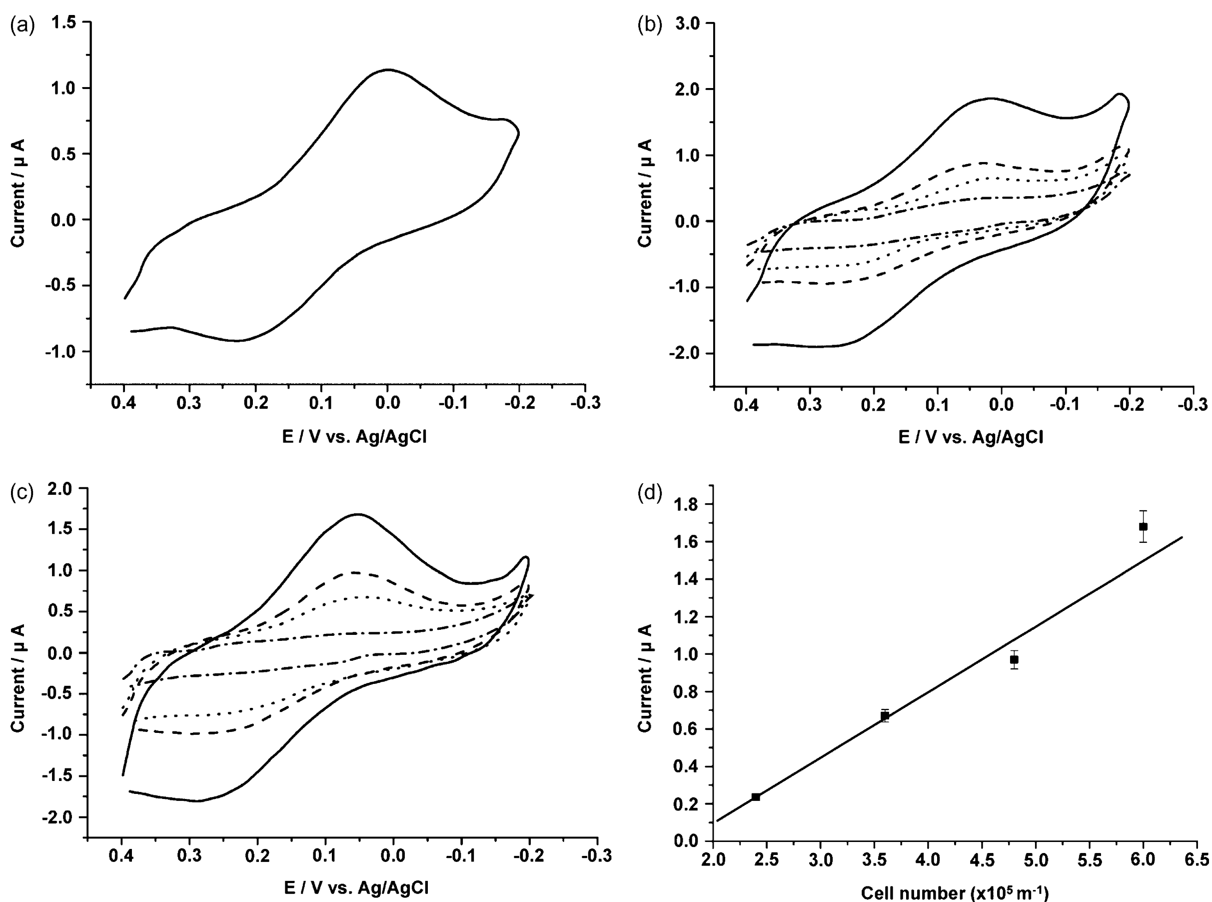

Fig. 2. Electrochemical response (a) of HeLa cells using a scan rate of 100 mV/s, temperature of 37 ± 0.5 °C, and cell number of 5.8×10^5 per ml. (b) Cyclic voltammograms of HeLa cells at different scan rates (–·) 25 mV/s, (···) 50 mV/s, (- - -) 100 mV/s, (–) 200 mV/s. (c) Relationship of peak current with the cell number (–·) 2.4×10^5 cells/ml, (··) 3.6×10^5 cells/ml, (- - -) 4.8×10^5 cells/ml, (–) 6×10^5 cells/ml. (d) Linear plot of reduction current peak as a function of cell number. The scan rate was 100 mV/s and the temperature was 37 ± 0.5 °C. Data are the mean ± standard deviation of three different experiments.

3.2. Relationship between the peak current and cell number

The peak current found to be increased with the increasing cell number (Fig. 2c). Fig. 2d shows a linear plot of the reduction current peak as a function of cell number. The peak current had a positive relationship with the cell number, so under the same conditions we can use the cyclic voltammetry to determine the cell number by measuring the peak current.

3.3. Validation of the CV method

The calibration curves were used for the determination of accuracy of the method (Judith et al., 1998; Kummerle et al., 2003; Tao et al., 2007). Seven calibration curves were constructed; and linear regression analysis was conducted over cell number range from 8×10^4 to 2.08×10^6. The accuracy of the method was expressed as the mean percentage deviation [dev (%)] between nominal and experimental cell number calculated with the established calibration curves. The results shown that the average accuracy was found to be $100.32\% \pm 13.6$ (ranging from 73.2% to 132%, $n = 7$) which provided measurements with accuracy within the recommended ±20% from their nominal values. These results indicated good correlation between the cells number and CV current.

3.4. PSA study of cell growth

To optimize the PSA parameters including deposition time, deposition potential, and stripping current (data not shown), we observed that PSA signal increased as the deposition time increased, while it did not increase appreciably when the time was longer than 400 s. Therefore, we selected 400 s as the deposition time. We examined the influence of different stripping current on PSA signal and observed a decrease in the PSA signal as the stripping current increased. When the stripping current exceeded 100 μA, the stripping time was so short that no obvious signal was evident, while the PSA curves were not smooth when the stripping current was smaller than 6 μA. Therefore, 6 μA was selected as the optimal stripping current.

Different deposition potential values were investigated. With the increase of deposition potential, the PSA signal decreased and no obvious PSA signal was observed when the deposition potential was applied at 0.3 V. On the other hand, at −0.2 V the PSA curve was not smooth and the baseline was much higher compared to 0.0 V. The applied potential of 0.0 V was thus preferred. HeLa cells with the same density (1.1×10^5 cells/ml) were cultured under the same conditions in many culture dishes. After 24 h, fresh culture medium was supplied to each dish and the cells were cultured continuously; the initial time for PSA measurement was recorded when the fresh culture medium was added. The PSA signals were measured by either facilitating cell attachment to the working electrode and taking the PSA signals at different times during cell culture (Fig. 3a) or harvesting the cells and using PBS suspensions of the cells for the measurements (Fig. 3b). In both approaches, the PSA signals of HeLa cells showed an oxidation peak at about 0.26 mV. Fig. 3c shows the relationship between the relative area under the peak of PSA signal and the cell culture time. The area under the

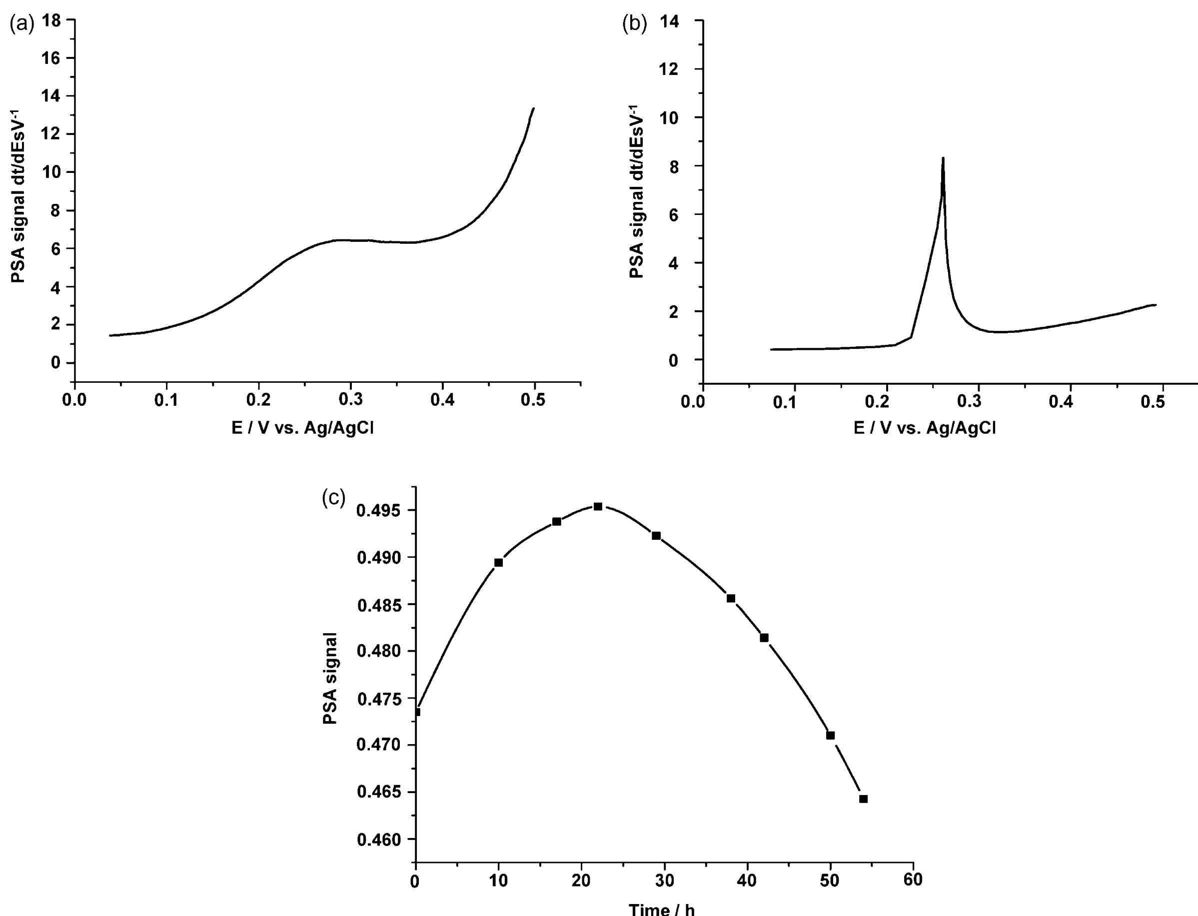

Fig. 3. PSA study of (a) HeLa cells attached with bare gold electrode and (b) freshly suspension HeLa cells on bare gold electrode. (c) Growth curve of HeLa cells described by the relative baseline corrected PSA signal. Experimental conditions: deposition potential, 0.0 V; deposition time, 400 s; stripping current, 6 μA. Cell density: 1.1×10^5 cells/ml. The initial time was recorded when the cells were allowed to settle and grow for 24 h.

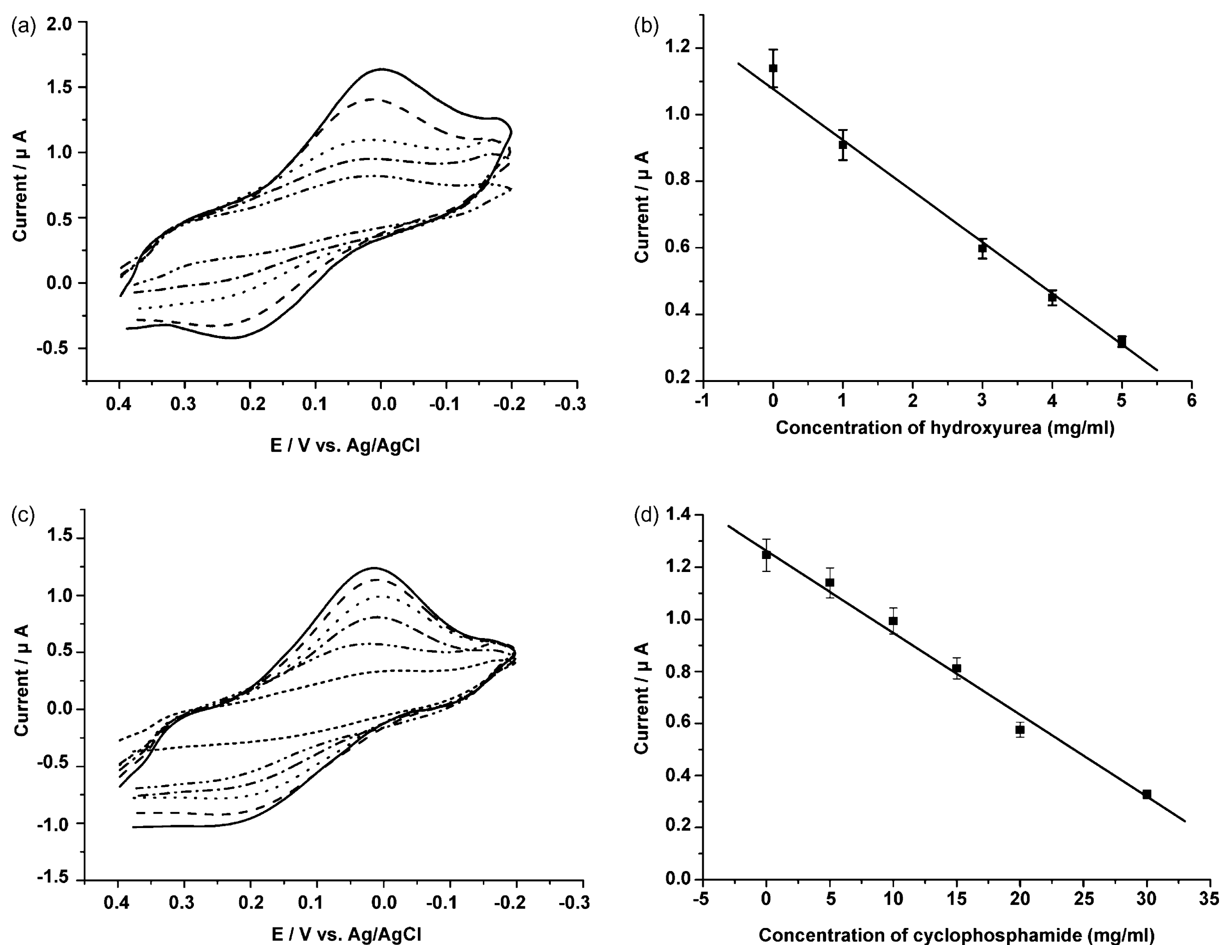

Fig. 4. (a) Cyclic voltammetry of HeLa cells treated with varying concentrations of hydroxyurea (– ·· –) 5 mg/ml, (–·–·) 4 mg/ml, (···) 3 mg/ml, (– –) 1 mg/ml and (–) 0 mg/ml. (b) Linear plot of reduction current peak as a function of anticancer concentration of hydroxyurea. (c) Cyclophosphamide concentration (- - -) 30 mg/ml, (– ·· –) 20 mg/ml, (–·–·) 15 mg/ml, (···) 10 mg/ml, (– –) 5, (–) 0 mg/ml. (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 100 mV/s, and the temperature was $37 \pm 0.5\,^{\circ}$C. Data are the mean ± standard deviation of three different experiments.

peak increased gradually as the culture time increased up to 24 h and then declined sharply. The initial increase of PSA signal with the culture time was due to the proliferation of the cells. In cell culture, nutrients are important in maintaining cell viability; when the cells are cultured continuously in the presence of CO_2 for an extended time without replenishment by fresh nutrient, the viability of cells will be affected. Thus, the decreasing PSA signal is related to decreased cell viability. These results indicate that the baseline corrected PSA signal can represent cell viability and so be used to monitor cell growth in both cases if cells are attached to an electrode or in suspension.

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

Hydroxyurea is an effective inhibitor for DNA synthesis in HeLa cells, and the continued synthesis of RNA and protein in the presence of hydroxyurea leads to a state of unbalanced growth (Hatse et al., 1999; Miri et al., 2005). Cyclophosphamide is a cytotoxic nitrogen mustard derivative widely used in cancer chemotherapy; it cross-links with DNA leading to strand breakage, and induces mutations (Ji et al., 1993; Jurgen et al., 1998). Its clinical activity is associated with a decrease in aldehyde dehydrogenase 1 (ALDH1) activity (Ren et al., 1999; Rua et al., 2000). To investigate the effect of anticancer drugs on the cyclic voltammetric response of HeLa cells, the inoculated 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. 4a shows the effect of different concentrations of hydroxyurea on cyclic voltammetry response of HeLa cells, it was observed that as the concentration of hydroxyurea increases the peak current decreases drastically. Fig. 4b shows the corresponding linear plot between the reduction current peak and hydroxyurea concentration. The effect of varying concentrations cyclophosphamide on the CV response of HeLa cells was also studied; similar results to those obtained with hydroxyurea were

Table 1
The effect of anticancer drugs on HeLa cells by CV-mediated peak current measurement and Trypan Blue-determined cell viability

Anticancer drugs	Dosage (mg/ml)	Current (μA)	Cell viability (%)
Hydroxyurea	Control	1.139356	100
	1	0.909192	88.37
	3	0.59764	65.41
	4	0.45086	52.71
	5	0.318508	44.18
Cyclophosphamide	Control	1.246	100
	5	1.14	87.13
	10	0.994	78.32
	15	0.812	70.23
	20	0.576	62.71
	30	0.328	48.18

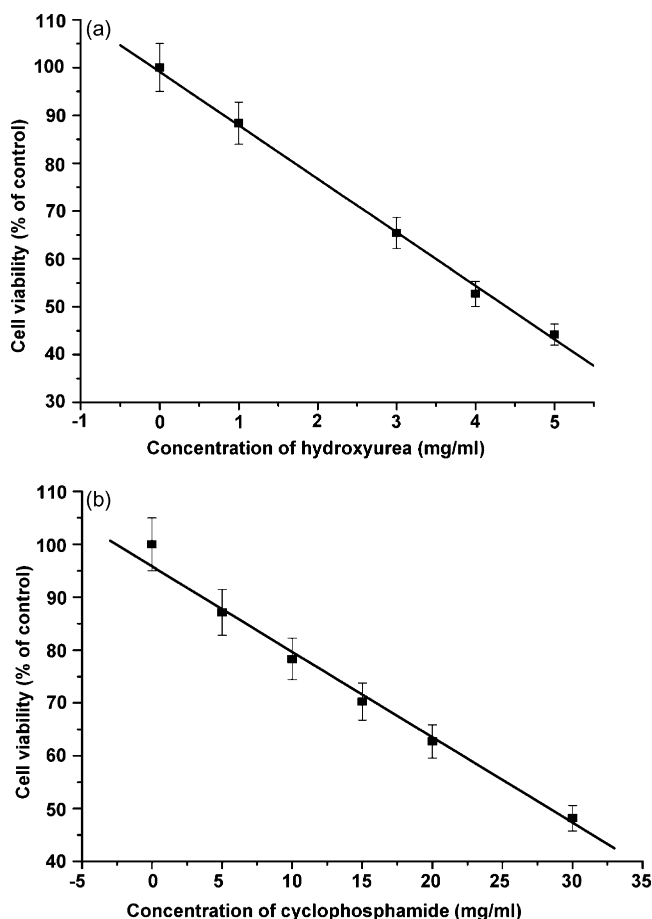

Fig. 5. Variance of cell viability with concentration of (a) hydroxyurea and (b) cyclophosphamide. The cell number was 5.8×10^5 per ml, and the temperature was $37 \pm 0.5\,^{\circ}$C. Data are the mean ± standard deviation of three different experiments.

evident (Fig. 4c). The corresponding linear plot between the reduction current peak and cyclophosphamide concentration is shown in Fig. 4d. These results indicate that the decrease of current peak depended on the concentration of the anticancer drugs. The reduction in peak current was likely related to the decreased viability and proliferation of the HeLa cells, considering that cell–cell interactions that affect drug sensitivity have been found in vivo and in vitro for cells grown under specific experimental conditions. Therefore, the voltammetric response of immobilized living cancer cells could be used to monitor the change of cell physiological viability, which can provide a simple way to study the function of anticancer drugs in cancer cell growth.

3.6. Counting assay

The effect of anticancer drugs on the cell viability was determined by the exclusion of Trypan Blue. The chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, cells that exclude the dye are viable (Freshney, 1987). The test involves an initial supply of the anticancer drugs for 24 h followed by removal of the culture medium and rinsing of the cell wells with PBS. The rinsed cells were harvested using 200 μl of single strength trypsin for 2 min and adding 1.8 ml of PBS to each well. To make appropriate dilutions of cell suspension we used 0.1% Trypan Blue. The transparent living cells could be easily distinguished from blue colored dead cells that could be counted in a hemocytometric chamber under light microscope. The cell viability was calculated using the following equation:

$$\text{Relative activity (\%)} = \frac{N_{\text{tot}} - N_{\text{blue}}}{N_{\text{tot}}} \quad (2)$$

where N_{tot} represents the total cell number and N_{blue} represents the number of blue cells. Considering the cell viability for control cell as 100%, the results for the effect of hydroxyurea and cyclophosphamide on the viability of HeLa cells obtained from the cell counting assay (Fig. 5a and b) it was apparent that as the drug concentration increased the cell viability decreased. We obtained effective concentration (EC_{50}) values of 4.355 mg/ml for hydroxyurea and 28.75 mg/ml for cyclophosphamide.

3.7. Comparison of the proposed electrochemical anticancer drug sensitivity test with the cell counting assay

To confirm the cyclic voltammetric results for the anticancer effect on cell viability, we used the counting assay test to check the effect of the same concentrations of anticancer drugs on the same number of HeLa cells under the same conditions. The results for the effect of anticancer on the viability of HeLa cells obtained from the cell counting assay (Fig. 5a and b) were compared with those of electrochemical (CV) based assay (Fig. 4a and c). Summary of these values in Table 1 highlights the effect of two anticancer drugs with different drug concentrations, and verifies the good agreement of the cytotoxicity effects of the two drugs when compared with counter assay method. Due to its accuracy and stability, this method can be used as an in vitro test for anticancer drugs sensitivity.

4. Conclusions

The present study shows the feasibility of detection of the anticancer drugs effect using immobilized HeLa cells, and the applicability of this approach to a cell chip platform. Also, the results establish the generality of cyclic voltammetry and potentiometric stripping analysis for use as direct electrochemical detection techniques to monitor cell growth and viability and the effect of anticancer drugs on cell viability. Based on the principles of the cell–cell interactions and the interaction between gold and mercapto or primary amine groups on the cell membrane provides an environment similar to a native system and allows more freedom in orientation of the biomolecules, which helps retain the activity of living cancer cells and prevents cell leakage from the electrode interface. The immobilized living cells exhibit a quasi-reversible voltammetric response and the peak current has a positive relationship with cell number. The cytotoxicity of two anticancer drugs with different inhibition mechanisms has been investigated by using CV technique, which shows that anticancer drugs display significant influence on the electrochemical response of immobilized living HeLa cells. PSA technique was used for study the growth of living HeLa cells suggesting a new technique to monitor electrochemically the viability of living cancer cells and the action of anticancer drug on cancer cell growth on a simulative interface. The electrochemical results also agree with the counting assay test. The proposed direct cell immobilization method technique may be applicable to diagnosis, drug detection, and on-site monitoring.

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