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Sensitive Detection of MCF-7 Cells Based on Poly Calcein Modified Electrode and Its Applied in Cytotoxicity Assay

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It is highly desirable to develop cost-effective and sensitive cell sensors to evaluate cytotoxicity of anticancer drugs. For the first time, a simple and cheap poly calcein modified electrode(PCA/GCE) prepared by one-step in situ polymerization was applied for study the electrochemical behavior of MCF-7 cells. Since the negative charge and unique properties of calcein can increase sensitivity, the resulting linear scanning of the MCF-7 cells on electrode exhibited two anodic peak that was ascribe to xanthine/guanine(signal) and hypoxanthine/adenine (signal). Meanwhile, the nonlinear exponent relationships between peak current (I_{pa}) and cell concentration (C) in the wide concentration range were firstly demonstrated. Two independent index equation based on two signal were I_{pa} (signal) = 7.458 − 6.415e (-C/52380) (R^2 = 0.986) and I_{pa} (signal) = 4.023 − 3.773e (-C/228700) (R^2 = 0.984) with the low detection limit of 3000 cells mL^{-1}, respectively. Electrochemical responses based on two signals of MCF-7 cells was applied to evaluate cytotoxicity effect of cyclophosphamide (CTX) on MCF-7 cells, the result was in good agreement with that of MTT method. This proposed method could be used to evaluate cytotoxicity of drug.

Keywords: Poly calcein modified electrode; MCF-7 cells; Nonlinear exponent equation; Cytotoxicity

1. INTRODUCTION

Electrochemical method, as a sensitive, rapid and low cost analytical method, has been applied for detection of cell viability, and paid considerable attention, especially in the field of anticancer drugs and environmental toxicology screening [1-5]. For improving the sensibility, stability and repeatability in electrochemical method, a tremendous amount of work was focused on the development of nanomaterials-based modified electrodes, such as colloidal gold nanoparticle, multi-

walled carbon nanotubes, gold nanoparticles-chitosan nanocomposites gel, carbon nanofiber-doped chitosan, reduced graphene oxide-ionic liquid and multi-walled carbon nanotubes-ionic liquid composites modified electrodes [6-12]. However, preparation of these expensive modified electrode bears intrinsic limitations, for instance, requiring multiple steps or pretreatment and special film-coating skills in order to ensure the uniformity and unity of modified film, which is laborious and also inevitably increase difficulty of electrode preparation. So, it is still a challenge to develop a sensitive, simple and cheap sensor of cells [13-15].

Calcein (CA), a cheap and widely used fluorescent dye, possesses rich functional groups -COOH, π-electron backbone and conjugation that are responsible for their unique electronics properties[16, 17]. Polymeric CA film could be obtained from its monomer by the electropolymerization. The polymeric film has one-step fabrication, low cost, excellent repeatability and stability characteristic [18-20]. In order to make the electrochemical analysis of cells more easy and accurate, in this paper, a poly CA modified glassy carbon electrode (PCA/GCE) was fabricated and firstly applied in the study on electrochemical behavior of MCF-7 cells. Two electrochemical signals of MCF-7 cells were detected on PCA/GCE, which were due to xanthine /guanine (signal) and adenine /hypoxanthine (signal Ⅱ), respectively [11].

More importantly, considering the cell concentration is a critical factor for studying cell proliferation capacity [21, 22], the relationships between two signals on PCA/GCE and cell concentration were firstly studied. It is significant to define function relationship between peak current and the cell concentration based on the change rule of more electrochemical signals in a wide range of cell concentrations [23, 24]. However, in our previous work, only in the narrow concentration range, the relationship can be established, and has long been considered as a linear relationship, which restricts the application of electrochemical methods in the area of biomedicine [25, 26]. In recent work, nonlinear exponent relationship based on one signal (xanthine/guanine) of cells was found in the range from 3.0×10^3 to 7.0×10^6 cells mL^{-1} [27]. In this paper, two nonlinear exponent relationship based on two electrochemical signals (xanthine/guanine and adenine/hypoxanthine) were simultaneously established, which provided more indexes for the electrochemical study of cell physiology. Finally, the electrochemical responses based on two signals of MCF-7 cells was applied in cytotoxicity evaluation of cyclophosphamide (CTX) with the same result as MTT method. The electrochemical responses of the cells, based on PCA/GCE, was used to detect cell viability and the toxicity of anticancer drugs, which would be beneficial for application of electrochemical methods in evaluation of potential drugs or toxic hazards

2. MATERIALS AND CHEMICALS

2.1. Chemicals

Human breast cancer (MCF-7) cells were obtained as a gift from Jilin University (Changchun, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) and calcein were purchased from Sigma Chemical Co.. CTX was purchased from Aladdin industrial Co.. Phosphate buffer saline (PBS,

pH 7.4) containing 136.7 mmol L^{-1} NaCl, 2.7 mmol·L^{-1} KCl, 9.7 mmol L^{-1} $Na_2HPO_4·12H_2O$, 1.5 mmol·L^{-1} KH_2PO_4 was used in the experiments. All other chemicals were of analytical grade and used as received.

2.2. Cell culture and collection

MCF-7 cells were cultured in DMEM medium (Hyclone) containing 10% fetal calf serum, 100 μg mL^{-1} penicillin (Gibco) and 100 μg mL^{-1} streptomycin (Gibco) in an incubator (5% CO_2, 37℃). After the medium in the culture dishes was removed, the cells in the culture dishes were washed with the sterile pH 7.4 PBS for three times and then suspended in the sterile pH 7.4 PBS to obtain the MCF-7 cell suspension.

Finally, the fragmented MCF-7 cell suspension was obtained by heating the MCF-7 cell suspension in 50℃ water bath for 30 min. To study the cytotoxic effects of cyclophosphamide on MCF-7 cells, CTX were added to the cell culture medium. The same culture conditions were kept for both the control and CTX treatment groups. Cell concentrations were determined by a CBC DRM-700 cell counting plate (China).

2.3. Preparation of the PCA/GCE

Prior to modification, GCE was polished with 0.05 μm alumina slurry and sonicated in ethanol and distilled water continuously. The electrode was pretreated electrochemically by scanning in 0.5 mol L^{-1} H_2SO_4 solution between 0.5 and 1.5 V, until a stable background current was obtained. The polymeric film was formed by scanning from -1.4 to 1.8V at 100 mV s^{-1} for 20 cycles in 0.2 mol L^{-1} PBS solution containing 0.01 mol L^{-1} KNO_3 and 2.5×10^{-3} mol L^{-1} CA. Finally, the modified electrode was washed with doubly distilled water, and then air-dried.

2.4. Electrochemical measurements

Cyclic voltammogram (CV) and linear scanning voltammetry method (LSV) were carried out using a CHI 760B workstation (CHI Instrumentation, Shanghai, China). A three-electrode configuration was employed for the electrochemical measurements, where PCA/GCE served as the working electrode, Ag/AgCl and Pt served as the reference and counter electrode, respectively. The electrochemical behavior of MCF-7 cells was studied in the potential range from 0.0 V to 1.2 V at a scan rate of 50 mV s^{-1}. Each experiment measurement was repeated for three times. The viability of the cells treated with drugs was calculated as follows:

$$\text{Viability} = [(i_{p,\,control} - i_{p,\,exp})/i_{p,\,control}]100\% \quad (1)$$

Here, $i_{p,\,control}$ is the peak current of the cells in the absence of drug treatment and $i_{p,\,exp}$ is the peak current of the cells treated with drugs.

2.5. MTT assay

MCF-7 cells (1×10^5 cells mL^{-1}) in 200 μL of either medium alone or medium containing CTX at various concentrations was added to each well of a 96-well plate, and the process was referred to literature [28].

3. RESULTS AND DISCUSSION

3.1. SEM of PCA/ ITO

SEM was used to characterize the morphologies of PCA. Considering the too large size of the GCE for the morphological characterization, CA was electropolymerized to ITO substrate with the same method as PCA/GCE mentioned above. Figure 1(A) showed a smooth surfaces, indicating it is an unmodified ITO. As shown in Fig.1(B), the PCA film showed a rough texture and scracked surface, this slot structure may be caused by the rapid oxidation of CA, indicating the increase of the carbonyl and carboxylic groups with high electron density in CA polymer, which may results in an improvement in conductivity and response current.

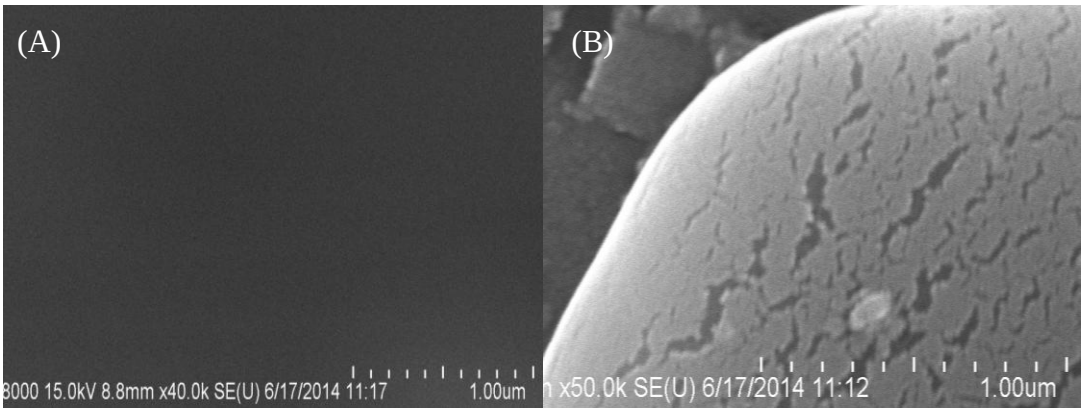

Figure 1. SEM of PCA/ ITO

3.2. Electrochemical characterizations of PCA/ GCE

Fig. 2 showed CVs of GCE and PCA/GCE obtained in 5 mmol L^{-1} $Fe(CN)_6^{3-/4-}$ solution containing 0.1 mol L^{-1} KCl, respectively. The redox peak current obtained on PCA/GCE increased significantly and corresponding peak-to-peak separation (ΔE_p) decreased than that obtained on GCE, indicating PCA can effectively increase the electron transfer rate of $Fe(CN)_6^{3-/4-}$ due to its upstanding electric conductivity.

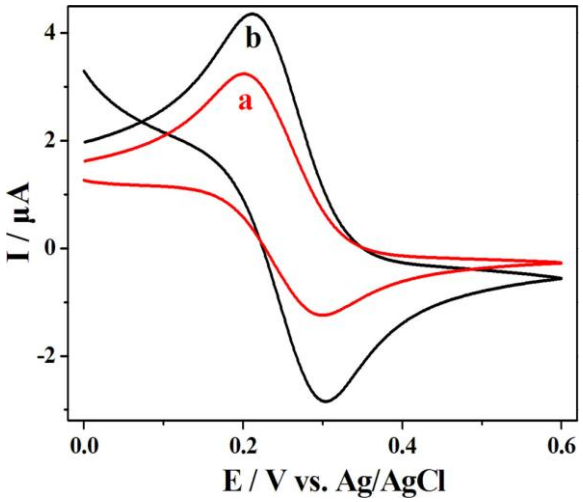

Figure 2. CVs of $[Fe(CN)_6]^{3-/4-}$ on GCE (a) and PCA/GCE (b)

3.3. Voltammetric behavior of MCF-7 cell suspension on PCA/GCE

Fig. 3 showed the LSVs of MCF-7 cells on GCE and PCA/GCE in 0.20 mol L^{-1} PBS (pH 7.4), respectively. Only one anodic peak at 0.72 V was observed (Fig. 3a) on bare GCE, while on PCA/GCE, the anodic peak at 0.70 V was dramatically stronger and shifted negatively, meanwhile, a new anodic peak at 1.05 V emerged. The two anodic peaks were due to the oxidation of xanthine/guanine and adenine/hypoxanthine released from MCF-7 cells, respectively (Fig. 3b). The results indicated the PCA film possessed excellent electrocatalytic activity toward oxidation of purine bases. The reasons could be as follows: First, PCA film with the π-π * stack has excellent electron conductivity; Second, carbonyl and carboxylic groups to act as the acceptor and the anchoring group could enhance the electrostatic accumulation of the positively charged bases[29].

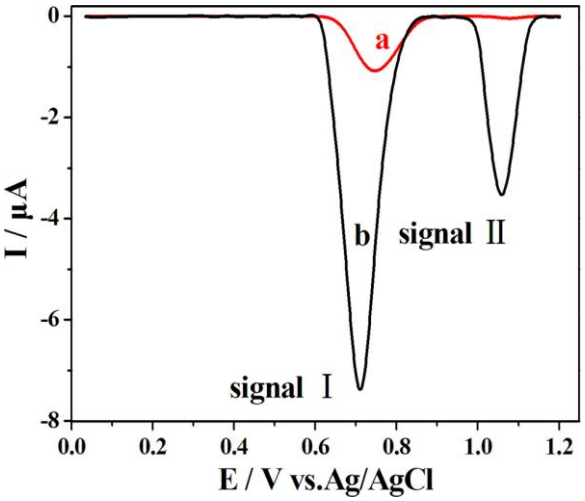

Figure 3. Baseline-corrected LSVs of fragmented MCF-7 cell suspension on GCE (a) and PCA/GCE (b). Cell concentration: 5.0×10^6 cells mL^{-1}; scan rate: 50 mV s^{-1}.

3.4. Influence of pH on the voltammetric behavior of MCF-7 cell suspension

The effect of pH (5.4-8.5) on the voltammetric behavior of MCF-7 cell suspension was shown in Fig. 4. The results indicated that both the peaks potential (E_{pa}) and peaks current (I_{pa}) of MCF-7 cells depended on the solution pH. The potential for two signals shifted negatively with the increase of pH (Inset A: a and Inset B: a). Linear relationships between the E_{pa} and the solution pH can be established as E_{pa} = -0.058 pH + 1.146 (R^2 = 0.995) for xanthine /guanine and E_{pa} = -0.068 pH + 1.576 (R^2 = 0.999) for adenine /hypoxanthine, respectively. The slopes of 58.0 and 68 mV pH^{-1} implied that the electron transfer was accompanied by equal numbers of protons and electrons in the electrode reaction process[30].

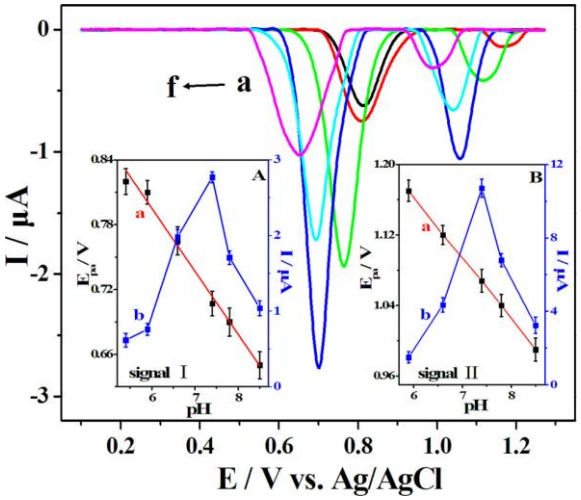

Figure 4. Baseline-corrected LSVs of fragmented MCF-7 cell suspension at different pH of (a) 5.4, (b) 6.0, (c) 6.6, (d) 7.4, (e) 7.8 and (f) 8.5. Inset A: Effect of pH on the (a) potential and (b) current response of MCF-7 cell suspension (signal Ⅰ). Inset B: Effect of pH on the (a) potential and (b) current response of MCF-7 cell suspension (signal Ⅱ). Cell concentration: 1×10^5 cells mL^{-1}.

When pH varied between 5.4 and 8.5, the peak currents for signal Ⅰ and signal Ⅱ increased firstly to the maximum value at pH 7.4, and then decreased (Inset A: b and Inset B: b). In addition, the peak current for signal Ⅱ was not almost observed under pH 5.4 due to low electroactivity of PCA/GCE toward oxidation of adenine /hypoxanthine. This phenomenon could be explain as follows : firstly, the form of carboxylic groups of PCA molecules changed from $-COO^-$ to -COOH under acidic condition, which reduced the electrostatic interaction between carboxylic groups and purine. Second, there exists a keto-enol tautomerism equilibrium in purine, and enol form of purine is the major structure under physiological pH and is more easily oxidized, while the keto of purine under alkaline condition and protonated purine under acidic condition are difficult to be oxidized[31].Thus, pH 7.4, in which the existence form of purine was coincident with that in physiological condition, was used in the subsequent experiments.

3.5. Influence of accumulation time and potential on the voltammetric behavior of MCF-7 cell suspension

Preconcentration of electroactive species on the electrode surface can improve the sensitivity of detection. The effect of accumulation time on the peak current of the cells was shown in Fig. 5A. Both oxidation peak currents of xanthine/guanine and adenine/hypoxanthine increased with increasing accumulation time from 0 to 200 s, which indicated that electroactive species released from cells at the electrode surface were rapid adsorption. After 200 s, peak currents almost reached a maximum value owing to the saturated adsorption of the electroactive species. As the accumulation time go on increasing, peak currents do not raise anymore, and even reduce. As a result, 200 s was chosen as the optimal accumulation time in further experiments.

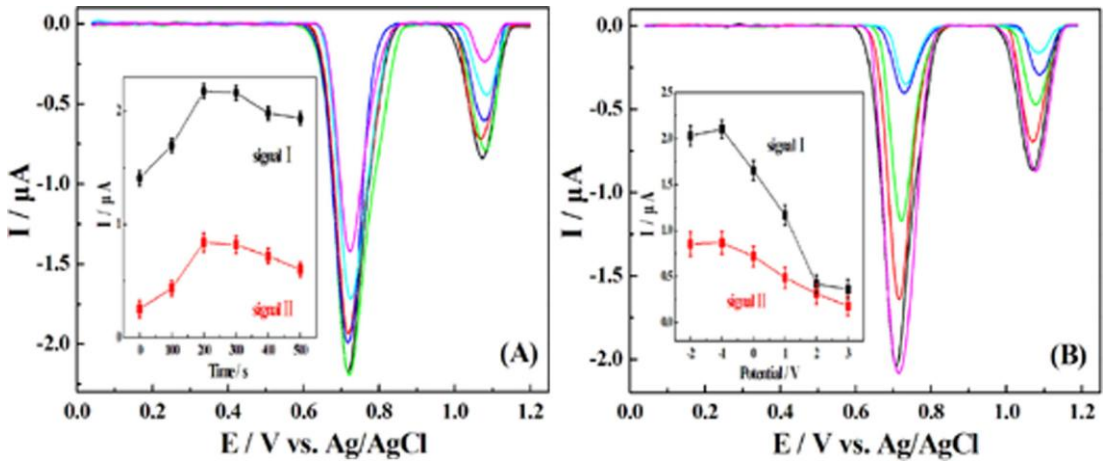

Figure 5. (A) Baseline-corrected LSVs of fragmented MCF-7 cell suspension on different accumulation time of (a) 0 s, (b) 100 s, (c) 200 s, (d) 300 s, (e) 400 s and (f) 500 s. Inset A: The dependence of peak current of MCF-7 cells on the accumulation time; (B) Baseline-corrected LSV of fragmented MCF-7 cell suspension on different accumulation potential of (a) 0.3 V, (b) 0.2 V, (c) 0.1 V, (d) 0 V, (e) -0.2 V, (f) -0.1 V. Inset B: The dependence of peak current of MCF-7 cells on the accumulation potential. 3.5×10^5 cells mL^{-1}.

The effect of accumulation potential on the peak current of cells was also examined over the potential range of -2 ~ 3 V. As shown in Fig.5B, the better peak current for two signals were achieved over the potential range of (-0.2 ~ 0 V) due to the increase of the accumulation rate, because of the more favorable alignment of the molecules in the electric field of the electrode solution interface. However, the peaks currents of xanthine/guanine and adenine/hypoxanthine decreased by changing accumulation potentials to more positive values, indicating that the cells was no longer strongly adsorbed. Hence, accumulation potential of -1 V was chosen in the subsequent work.

3.6. The dependence of the peak current on cell concentration

The dependence of the peak current on cell concentration in 0.2 mmol L^{-1} PBS (pH 7.4) was investigated using LSV. As shown in Fig. 6, the intensity of the peak current of MCF-7 cell suspension

was directly related to the cell concentration. With increasing concentration of MCF-7 cells, the peak current increased nonlinearly. In the concentration range from 3.0×10^3 to 7.0×10^6 cells mL^{-1}, the peak currents (I_{pa}) for two signals showed exponent relationships with cell concentration (C), and followed two independent index equations $I_{pa} = 7.458 - 6.415e$ (-C/52380) ($R^2 = 0.986$) for signal Ⅰ and $I_{pa} = 4.024 - 3.711e$ (-C/228780) ($R^2 = 0.983$) for signal Ⅱ with a detection limit of 3.0×10^3 cells mL^{-1}, respectively. However, in the previous study, the relationship between peak current and concentration of cancer cells has always been considered as linear in a narrow concentration range[25, 26]. In this study, two nonlinear exponent equations based on two electrochemical signals were simultaneously established in the wide concentration range, which provided more indexes for the study of cell physiology by electrochemical methods.

In addition, the repeatability of PCA/GCE was investigated, at cell concentrations of 1×10^5 cells mL^{-1}, relative standard deviations (RSD) of 3.5% and 4.2% (n = 6) were obtained for signal Ⅰ and signal Ⅱ, respectively. When the modified electrode was stored for twenty days, peaks currents of signal Ⅰ and signal Ⅱ decreased 15% and 20%, respectively.

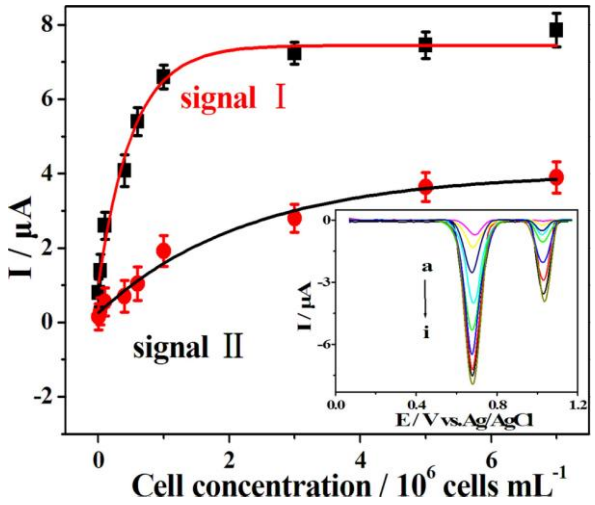

Figure 6. Relationship between the peak current of MCF-7 cells and cell concentration. Inset: Baseline-corrected LSVs of fragmented MCF-7 cell suspension on PCA/GCE with the different cell number: (a) 3.0×10^3, (b) 3.0×10^4, (c) 1.0×10^5, (d) 4.0×10^5, (e) 6.0×10^5, (f) 1.0×10^6, (g) 3.0×10^6, (h) 5.0×10^6 and (i) 7.0×10^6 cells mL^{-1}.

3.7. Effect of CTX on MCF-7 cells viability

CTX, an anticancer drug with a broad spectrum of activity, was selected as a drug model for studying cytotoxicity on MCF-7 cells based on PCA/GCE. Fig. 7 showed two signals of MCF-7 cells exposed to CTX with different concentrations (0~400 nmol·L^{-1}) for 30 h. The change of both two current peaks had negative linearity with the concentrations of CTX. Peak current reduced significantly with the increase of CTX concentrations at the range from 0 to 100 nmol·L^{-1}, and then reduced slowly from 100 to 300 n mol·L^{-1}, almost was stable when CTX concentrations reach 300 n mol·L^{-1}. That is, as the concentration of CTX reached 300nM, the MCF-7 cells had nearly died out, and further increases in the CTX concentration therefore had no significant effect on the

electrochemical signals from the MCF-7 cells. The dose-dependent relationships of CTX were in agreement with a previous study[32].

The decrease in the signal after treatment with the toxicant suggested a reduction in the viability of the cells and may be caused by the inhibition effect of anticancer drugs for DNA synthesis and further leading to loss of cell viability. These sequential inhibition activities of anticancer drugs finally lead to the cell death. It was therefore concluded that the voltammetric signals intensities from PCA/GCE were reliable for accurate detection of the cytotoxicity effects of CTX on target cells.

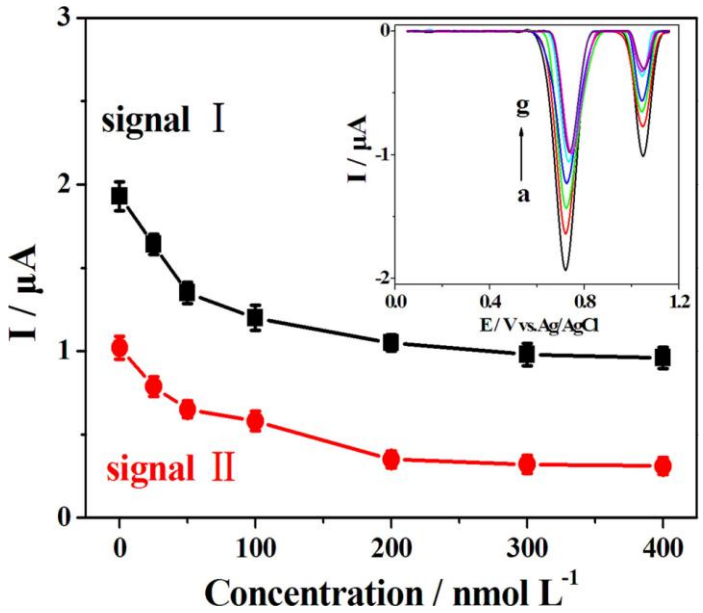

Figure 7. Dependence of peak current of MCF-7 cell suspension on CTX concentration by the electrochemical method. Inset: Baseline-corrected LSVs of MCF-7 cells treated with (a) 400, (b) 300, (c) 200, (d) 100, (e) 50, (f) 25, (g) 0 nmol·L^{-1} CTX. Drug incubation time: 30 h; cell vaccination concentration: 2.0×10^6 cells mL^{-1}.

Cytotoxicity curves for the 30h exposure of the MCF-7 cells to CTX obtained by the electrochemical method were shown in Fig. 8. The viability of MCF-7 cells decreased as the concentration of CTX increased. To prove the credibility of electrochemical method, the MTT assay was performed as comparison and viability inhibition effect of CTX on MCF-7 cells was also of dosage-dependence. The cytotoxic tendency was in accordance with the electrochemical result, indicating the two-signal electrochemical method based on PCA/GCE was reliable. Moreover the maximum inhibitory rates based on electrochemical signal Ⅰ and signal Ⅱ were 58% and 69%, the value obtained from MTT was 59%. The IC_{50} values of 159.12 μmol/L and 130.45 μmol/L from the signal Ⅰ and signal Ⅱ as the index was lower than the value 160 μmol/L obtained from MTT assay, and signal Ⅱ seems to have greater cytotoxicity inhibition and lower IC_{50}. These result confirmed that electrochemical method based on PCA/GCE for cytotoxicity test of CTX was more sensitive than the traditional MTT assay to some extent. Besides, electrochemical method was simple and rapid.

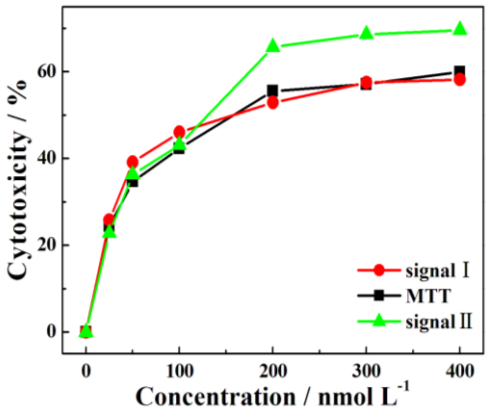

Figure 8. Cytotoxicity curves for the 30 h exposure of the MCF-7 cells to CTX obtained based on electrochemical method and MTT assay.

4. CONCLUSIONS

PCA/GCE was constructed and applied for studying voltammetric behavior of MCF-7 cells. Two maximum voltammetric signals of MCF-7 cells was obtained under the condition of accumulation time 200s, accumulation potential -0.1V and pH 7.4. Two independent nonlinear exponent relationships between peak current and cell concentration were first found with the low detection limit of 3000 cells mL^{-1}. The proposed PCA/GCE was applied in cytotoxicity test of CTX, and the dose-dependent relationship was in agreement with that obtained by the MTT assay. The IC_{50} values of 159.12 μmol/L and 130.45 μmol/L from the signal Ⅰ and signal Ⅱ as the index was lower than the value 160 μmol/L obtained from MTT assay, suggesting a reliable and more sensitive technique to electrochemically monitor the cytotoxicity effects of CTX on cells. The preparation of the polymer modified electrode was such simple, easy and cheap so that is believed to be of great value for cytotoxicity study of anticancer drug.

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