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Fabrication of self-assembled RGD layer for cell chip to detect anticancer drug effect on HepG2 cells

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ABSTRACT

HepG2 cells have been immobilized on nanoscale self-assembled synthetic oligopeptide modified chip surface and subsequently used for anticancer drug screening. Nanoscale controlled self-assembled peptide layer was investigated by AFM (Atomic Force Microscopy). The immobilization of HepG2 cells on nanoscale controlled surface was investigated by using Raman spectroscopy. HepG2 cells were grown on peptide modified gold surface acting as working electrode. The AFM investigation of the oligopeptide modified surface showed excellent agreement with the nanoscale nature of the peptide modification, and the voltammetric response of HepG2 cells on this surface towards an anticancer drug showed a linear relationship with the cell number. As an application, electrochemical detection of anticancer drug effect of HepG2 cells was shown. These results indicate that RGD (Arg-Gly-Asp) peptide self-assembled layer mediated the cell immobilization technique and the voltammetric signal analysis system can be applied to construct a cell chip for diagnosis, drug detection, and on-site monitoring.

1. Introduction

Understanding modeling cell behavior based on only RNA or protein expression levels is very difficult, because a cell is a much more complicated system than the sum of its components [1]. Cell-based sensor arrays [2] and electrical sensing devices have been used for signal-frequency patterns in cell growth media [3]. Microfluidic devices electrically measure cell viability by detecting changed electrical resistance of a cell membrane within milliseconds when it is exposed to a toxic agent [4]. Cell-based sensor arrays are potentially useful for studying effects of drug and cell-external stimuli interactions [5,6]. In vitro immobilization of a living cell is an important process in the fabrication of cell-based chips [7].

Direct immobilization of living cells on gold is possible but is not stable over a period of several consecutive days. Specific biocompatible materials and surface modifications are important in the fabrication of cell-based sensors [3]. This can be a reliable candidate for cellular attachment on special designed surface patterns without loss of viability of surface functionalization based on RGD (Arg-Gly-Asp) interacting with cell adhesion molecules. Especially, a modified RGD peptide terminated with cysteine (Cys) amino acid can be self-assembled on gold (Au) surface [1,4]. However, there has not yet been any demonstration of quantifying the voltammetric signal on the biocompatibly modified surface [8,9].

In this study, cysteine modified oligopeptide which is sequenced as C-R-G-D-R-G-D-R-G-D-R-G-D (C(RGD)$_4$) was introduced for cell immobilization. Self-assembled peptide layer makes a more stable condition for cells than direct immobilization on a metal surface. Immobilized peptides and cells were investigated by Atomic Force Microscopy and Raman spectroscopy. Subsequently, electrochemical assay was used to study the 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. The proposed technique proved that the voltammetric signal obtained from the RGD modified surface could be quantified, and this was applicable to detecting anticancer drug effect.

2. Materials and methods

2.1. Materials

Hydroxyurea and cyclophosphamide were purchased from Calbiochem (Germany). Phosphate buffered saline (PBS) (pH 7.4,

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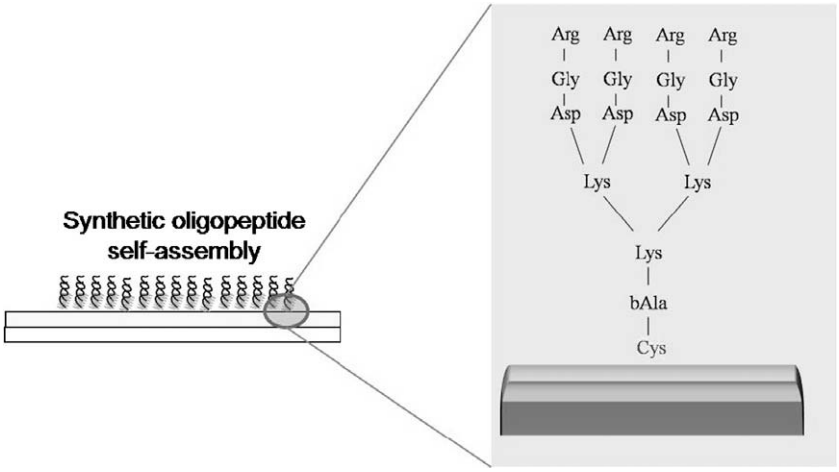

Fig. 1. Schematic diagram of nanoscale controlled surface.

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 used in this study were obtained commercially as reagent grade.

2.2. Cell culture

HepG2 cell was collected from human liver. The histopathology is hepatoma and its growth pattern is monolayer. The cell line was cultured in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) (from Gibco) and antibiotics (from Gibco, 1%). Cells were maintained under standard cell culture at 37 °C in an atmosphere of 5% CO_2. The medium was changed every three days.

2.3. Cell chip design and fabrication of oligopeptide layers by the SA technique

The chip contains three gold working electrodes, and the area of each electrode is 5 mm^2; they are separated from each other by 2 mm, and the exposure area for cell attachment is about 2.6 mm^2. The gold electrodes of thickness 150 nm are patterned on silicon substrate by DC magnetron sputtering. Prior to this 50 nm thick Ti was sputtered to promote the adhesion of Au on silicon. The chamber created has a volume of about 2 cm × 2 cm × 0.5 cm (width × length × height). PDMS was used to attach the chamber with substrates. A thin film of $C(RGD)_4$ on the gold surface was fabricated by using 0.1 mg/ml of $C(RGD)_4$ solution for at least 24 h [1]. Thereafter, the prepared oligopeptide surfaces were washed with deionized distilled water and dried under N_2 gas (Fig. 1).

2.4. Electrochemical behavior of HepG2 cells by cyclic voltammetry

Cyclic voltammetry (CV) was performed by using a potentiostat (CHI-660, CHI, USA) controlled by general-purpose electrochemical system software. A homemade three-electrode system comprised a cell-based chip as the working electrode, a platinum wire as counter electrode, and Ag/AgCl as reference electrode. Measurements were carried out for studying the electrical properties of living cells and the effect of anticancer drugs on their behavior in normal laboratory conditions. 10 mM PBS (pH 7.4) was used as electrolyte, and the scan rate was 0.1 V s^{-1}.

2.5. Topological analysis by AFM

Surface topography of CRGD-MAP/Au substrate was investigated by Atomic Force Microscopy (AFM, NTEGRA spectra, NTMDT, Russia) in semi-contact mode at room temperature under air condition with an inverted optical microscope. The maximum scan range of the system is 110 μm × 110 μm. The cantilevers used were type NSG10 and had a typical resonant frequency in the range of 190–325 kHz and force constant of 5.5–25.5 N/m. The scan rate was chosen to be 1 Hz. Before measurement, the medium was rinsed off with PBS which was then also used as liquid environment during the experiments.

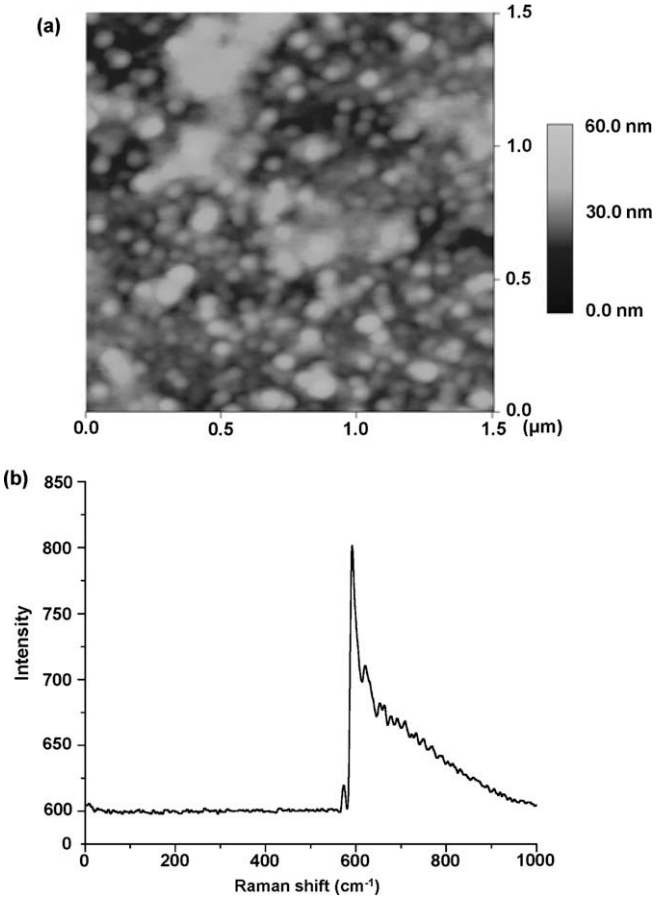

Fig. 2. (a) Atomic Force Microscopy image of $C(RGD)_4$ on gold, and (b) SERS of $C(RGD)_4$ on gold.

2.6. Surface-enhanced Raman scattering SERS

Raman spectra were recorded on an NTEGRA Spectra Scanning Confocal Raman Spectrometer (NTMDT, Russia). based on an inverted optical microscope (Olympus IX71). The maximum scanning range, *XYZ*, was 100 μm × 100 μm × 30 μm, and the resolution of the spectrometer was in the *XY* plane 200 nm and along the *Z* axis 500 nm, employing a laser emitting at 785 nm. Scans from 600 to 1700 cm^{-1} were recorded.

3. Results and discussion

3.1. Cyclic voltammetry of HepG2 cells on a gold electrode

HepG2 cells were allowed to be attached and grown on the modified gold electrode (working electrode) for two days before measuring the voltammetric behavior of the cells. Fig. 2a shows an AFM image of a $C(RGD)_4$/gold surface. The SERS of the peptide/gold surface is shown in Fig. 2b. In order to investigate the immobilization of HepG2 cells, we measured the SERS as shown in Fig. 3a,b and c. The figures show the Raman and confocal images of HepG2 cells. Before we measured the cyclic voltammogram of the HepG2 cells, we checked the effect of peptide on the CV of bare gold. From Fig. 4a, we observed that the immobilization of peptide on gold made the background signal increase in the potential range from −0.2 to +0.4 V (versus Ag/AgCl). The cyclic voltammogram of HepG2 cells is shown in Fig. 4b. A quasireversible process with cathodic peak at −0.094 V and anodic peak at +0.17 V were observed on the first scan. The subsequent potential scans after the initial scan moved the peaks to a more positive potential. The cathodic peak appeared at −0.066 V and the anodic peak appeared at +0.18 V. A slight peak separation was observed in the second scan, as compared to the first. The peaks of the rest of the cycles showed no change in potential peaks but there was a slight decrease in peak current. The change in potential between the first and the rest of the cycles was related to the high scan rate, but when we applied 50 mV s^{-1} there was no change in potential peaks between the first and second cycles. This cyclic voltammetry measurement shows the stable behavior of HepG2 cells. The separation between the potential peaks $|E_{pc} - E_{pa}|$ exceeded 59 mV and the peak current ratio $i_{pa}/i_{pc} \neq 1$, which indicated the distinct quasireversible character of the cell electrode process. These results show the advantage of the gold electrode, which offers faster electron-transfer kinetics than nonmetal electrodes; that is, the rate of electron-transfer between cell and electrode is faster. Also, this can be an advantage of using peptide for immobilization of living cells on an electrode, rather than of using the cell suspension in buffer or using direct immobilization. In the case of cell suspension, the cells become weaker, so the voltammetric behavior of the cells would be unstable and the current peak would decrease greatly or disappear. In contrast, in the case of using immobilized cells on a modified electrode, due to the attachment of cells on peptide and the cell–cell interactions, the cells will become stronger and will show a stable voltammetric response.

3.2. Relationship between the peak current and the cell number

In order to investigate the relationship between the peak current and the cell number, HepG2 cells with different density (0.4, 0.6, 0.8, 1, 1.4 and 1.8 × 10^5 cells ml^{-1}) were incubated under the same conditions in many cell chips for 48 h. Fig. 4c shows the cyclic voltammogram for HepG2 cells with different numbers. The peak current increased, with increasing cell number. Fig. 4d shows a linear plot of reduction current peak as a function of cell number.

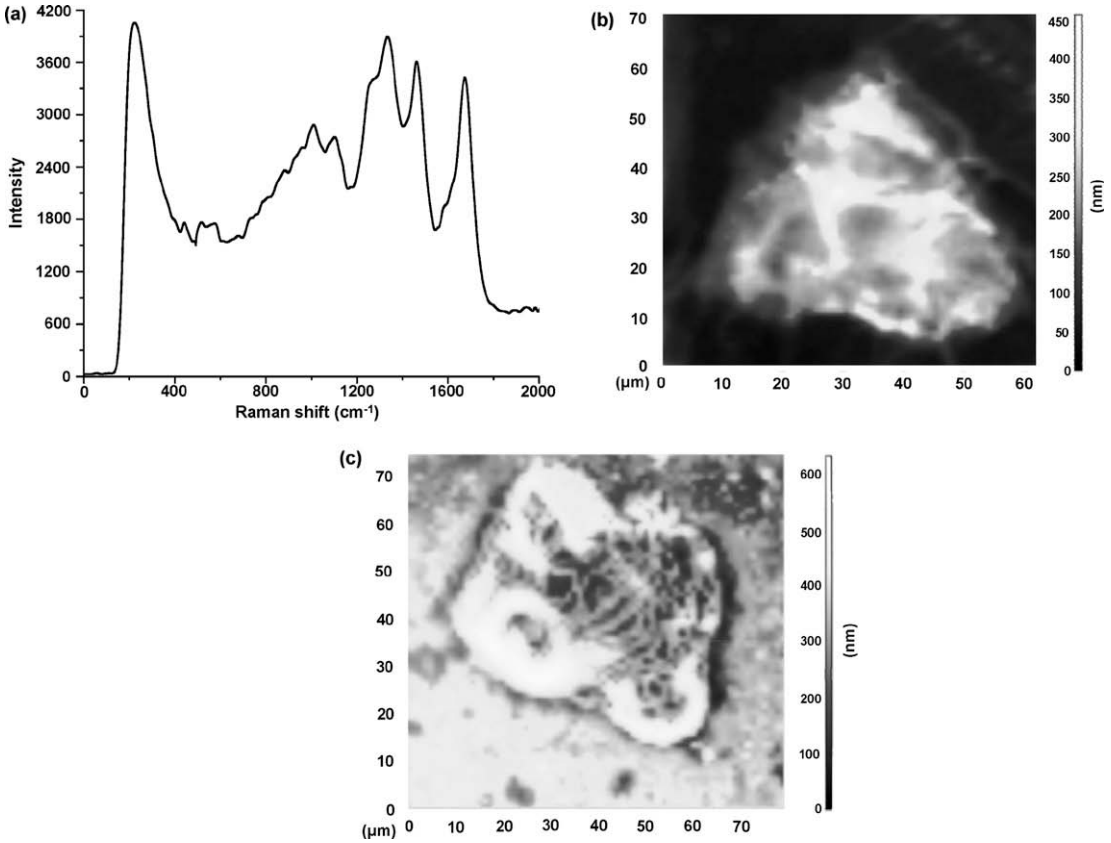

Fig. 3. (a) Raman spectroscopy of HepG2/$C(RGD)_4$ on gold, (b) Raman image of HepG2/$C(RGD)_4$ on gold and (c) Raman spectroscopy of HepG2/ $C(RGD)_4$ on gold.

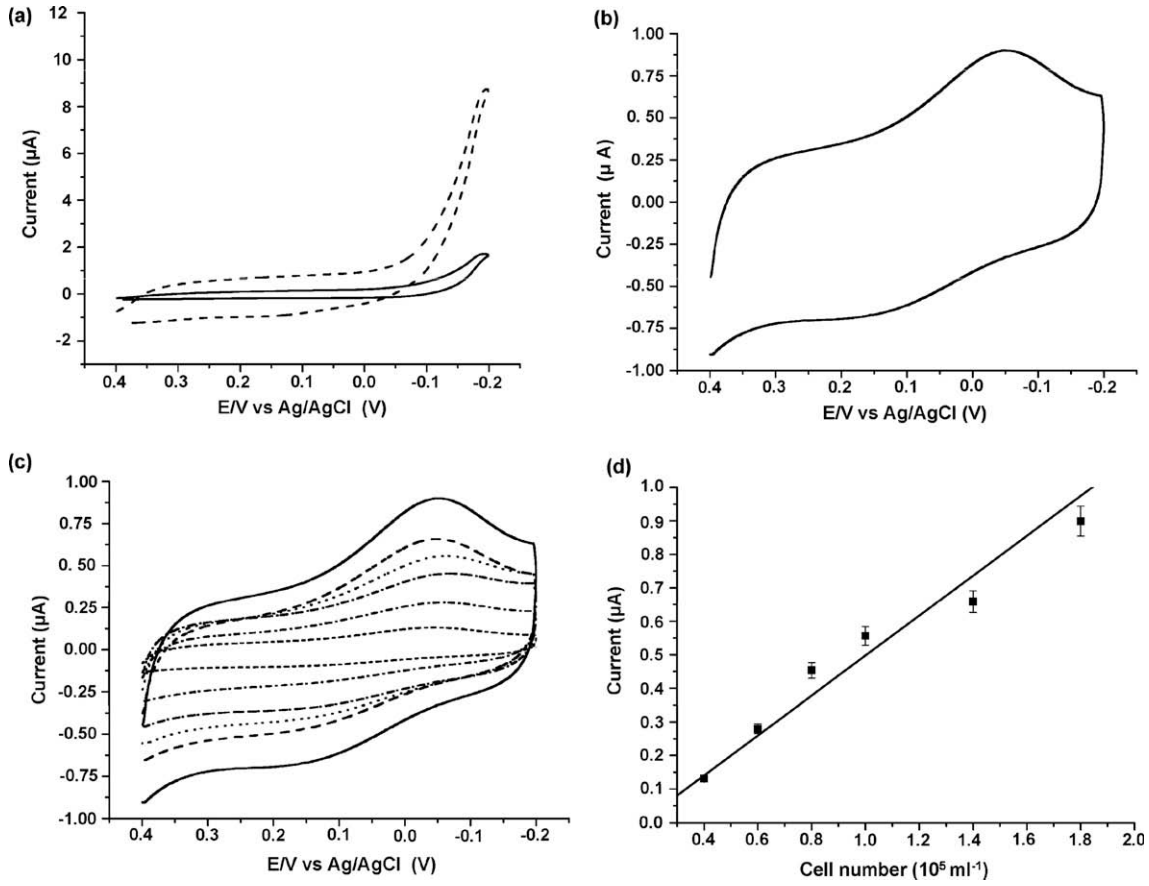

Fig. 4. Electrochemical response: (a) (—) bare gold and (— —) RGD/gold, the scan rate was 100 mV s^{-1} and the temperature was 37 ± 0.5 °C. (b) HepG2 cells; the scan rate was 100 mV s^{-1}, the temperature was 37 ± 0.5 °C, and the cell number was 1.8×10^5 ml^{-1}. (c) Relationship of peak current with cell number: (—) 4×10^4, (— —) 6×10^4, (•••) 8×10^4, (—•—•) 1×10^5, (— •• —) 1.4×10^5, and (----) 1.8×10^5 cells/ml. The scan rate was 100 mV s^{-1} and the temperature was 37 ± 0.5 °C and (d) linear plot of reduction current peak as a function of cell number. Data are mean S.D. of three different experiments.

This result indicated that the peak current had a positive relationship with the cell number, so under the same conditions we can use cyclic voltammetry to determine the cell number by measuring its peak current.

3.3. Voltammetric study of the effect of anticancer drugs on HepG2 cell

Hydroxyurea depletes the pools of deoxynucleotide triphosphate (dNTP) [10] by inhibiting ribonucleotide reductase. Isabelle et al. showed that the presence of hydroxyurea could lead to DNA damage in HepG2 cells after 5 h exposure [11]. Cyclophosphamide is a cytotoxic nitrogen mustard derivative widely used in cancer chemotherapy. Cyclophosphamide can cross-link with DNA, which leads to strand breakage and induces mutations [12,13]. Its clinical activity is associated with a decrease in aldehyde dehydrogenase 1 (ALDH1) activity [14,15].

Inoculated HepG2 cells with the same number of cells were allowed to attach and grow on a working electrode 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 hydroxyurea on the cyclic voltammetry response of HepG2 cells. It was observed that as the concentration of hydroxyurea increases, the peak current decreases drastically. Fig. 5b shows the corresponding linear plot between the reduction current peak and the log of hydroxyurea concentration. The effect of varying concentrations of cyclophosphamide on CV response of HepG2 cells also gave similar results to those obtained with hydroxyurea (Fig. 5c). The corresponding linear plot between the reduction current peak and the log of cyclophosphamide concentration is shown in Fig. 5d. These results indicated that the decrease of the current peak depended on the concentration of the anticancer drugs. The reducing peak current was related to the decreasing viability and proliferation of the HepG2 cells. 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. These results indicated that the decreasing current peak depended on anticancer drug concentration, which indicated that CV could be used as an anticancer drug sensitivity test.

4. Conclusions

A nanoscale controlled biosurface using synthetic oligopeptide was fabricated, and HepG2 cells were successfully immobilized on the fabricated surface. Also, the results showed that the gathering and quantifying of a voltammetric signal was possible on the peptide modified biosurface. The voltammetric signal of HepG2 cells on the modified surface was linear with increasing cell number. We applied this result to detect anticancer drug effect using hydroxyurea and cyclophosphamide. The voltammetric signal decreased as the exposed concentration of drug increased. The proposed cell immobilization technique using a self-assembly technique and a voltammetric signal analysis system would be applicable to diagnosis, drug detection, and on-site monitoring.

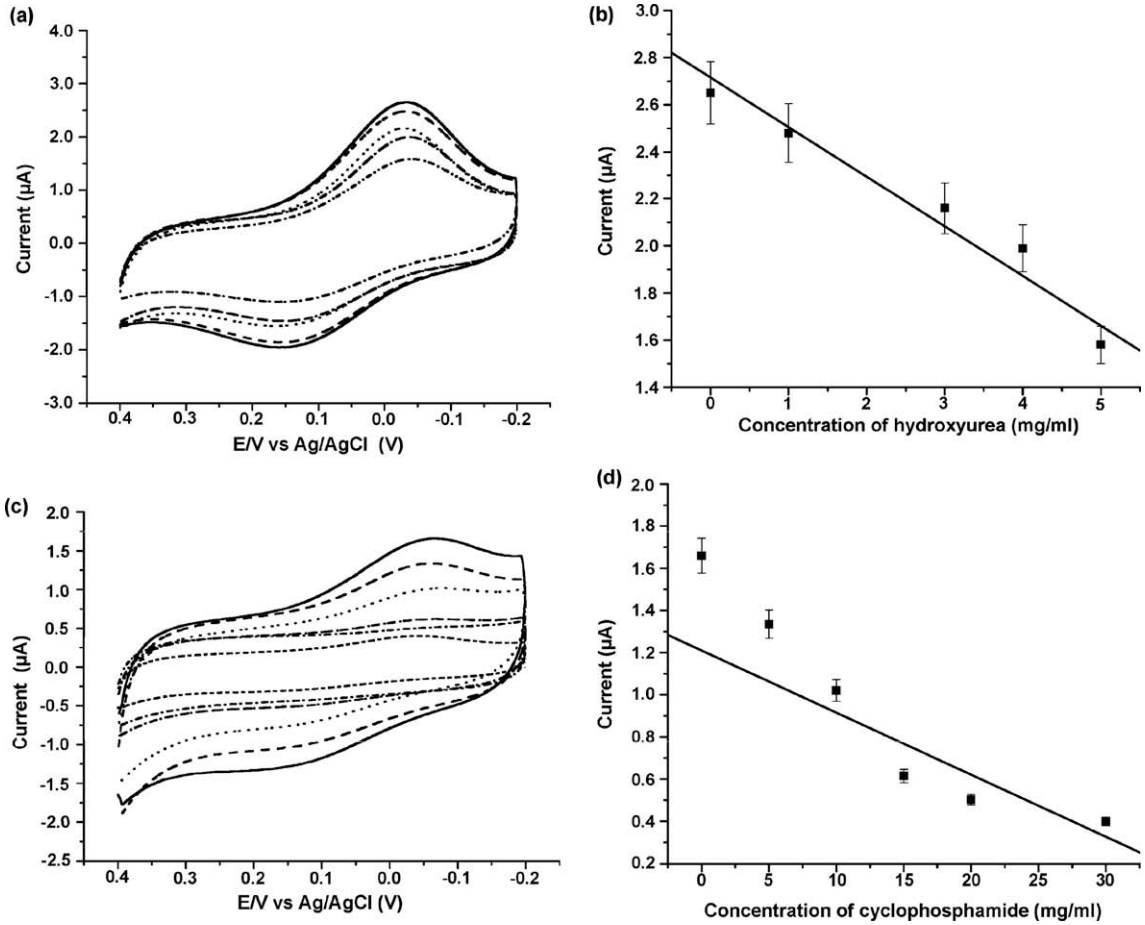

Fig. 5. Cyclic voltammetry of HepG2 cells treated with varying concentrations of (a) hydroxyurea; (—) 0, (— —) 1, (•••) 3, (—•—•) 4, and (— •• —) 5 mg/ml. (b) Linear plot of reduction current peak as a function of log concentration of hydroxyurea. The cell number was 1.4×10^6 ml^{-1}, the scan rate was 100 mV s^{-1}, and the temperature was 37 ± 0.5 °C. Data are mean S.D. of three different experiments, (c) cyclophosphamide concentration; (—) 0, (— —) 5, (•••) 10, (—•—•) 15, (— •• —) 20 and (----) 30 mg/ml. (d) Linear plot of reduction current peak as a function of anticancer concentration of cyclophosphamide. The cell number was 1.4×10^6 ml^{-1}, the scan rate was 100 mV s^{-1}, and the temperature was 37 ± 0.5 °C. Data are mean S.D. of three different experiments.

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