Microfluidic chip integrated with flexible PDMS-based electrochemical cytosensor for dynamic analysis of drug-induced apoptosis on HeLa cells

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A novel microfluidic platform integrated with a flexible PDMS-based electrochemical cytosensor was developed for real-time monitoring of the proliferation and apoptosis of HeLa cells. The PDMS-gold film, which had a conductive smooth surface and was semi-transparent, facilitated electrochemical measurements and optical microscope observations. We observed distinct increases and decreases in peak current intensity, corresponding to cell proliferation in culture medium and apoptosis in the presence of an anticancer drug, respectively. This electrochemical analysis method permitted real-time, label-free monitoring of cell behavior, and the electrochemical results were confirmed with optical microscopy. The flexible microfluidic electrochemical platform presented here is suitable for on-site monitoring of cell behavior in microenvironments.

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1. Introduction

Apoptosis, or programmed cell death, has been shown to play a crucial role in a number of diseases, especially cancers. Investigating apoptosis and exerting external regulation over it are of great importance in cancer treatment (Kfir-Erenfeld et al., 2010), and selective triggering of apoptosis is an attractive chemotherapeutic strategy (Li et al., 2008). More specifically, monitoring the extent of apoptosis and the speed of its onset has become an important subject for understanding apoptotic machinery and providing good prognostic indicators to guide treatment (Martinez et al., 2010). For this reason, the development of a simple, rapid method to observe apoptosis with high sensitivity is highly desired.

To date, various techniques have been proposed for apoptosis detection, such as electron microscopy (Yasuhara et al., 2003), DNA ladder assay (Suman et al., 2012), comet assay (Ghosh et al., 2012), western blotting (Jiang et al., 2012), TUNEL assay (Ribeiro et al., 2006), and flow cytometry (Riccardi and Nicoletti, 2006). While each of these strategies has distinct advantages, each also has its own severe drawbacks such as poor temporal resolution and lethal cell destruction. Moreover, the necessity of expensive equipment and specialized technicians further limits widespread clinical application of these techniques. Recently, electrochemical techniques have attracted considerable interest for use in detection of apoptosis, owing to their benefits of simplicity, rapid response, and potential for real-time and on-site monitoring (Zhang et al., 2011; Min et al., 2011; Takahashi et al., 2009; Xiao et al., 2008). Among these methods, electrochemical impedance spectroscopy has been proven to be a useful tool since it permits label-free, non-invasive, in-situ monitoring of cells with high sensitivity (Chang and Park, 2010; Qiu et al., 2009; Wang et al., 2011; Zhao et al., 2012). However, electrochemical impedance spectroscopy only detects the changes in signal between cells and an electrode surface, whereas cell–cell interaction cannot be detected (Qiu et al., 2009; Wang et al., 2011; Zhao et al., 2012).

As a promising alternative to electrochemical impedance spectroscopy, an electrochemical method-based chip technique has been developed for investigating cell behavior (Wang et al., 2010; Lee et al., 2010). For instance, Choi’s group used this technique to measure the changes in cell viability upon exposure to different kinds of environmental toxins (Kim et al., 2012; El-Said et al., 2009b, 2010), as well as cell cycle progression (Kafri et al., 2011). In these systems, the electrochemical measurements were performed directly in phosphate-buffered saline (PBS) solution without additional redox probes. However, the electrochemical cell chip method also suffers from some limitations, including inefficient removal of reagents from the chamber and consequent difficulty of chemical removal from the cell monolayer. Furthermore, the preparation of the silicon-based gold working electrodes is
complicated, involving DC magnetron sputtering and sophisticated instrumentation. Also, the cells on the electrode surface could not be observed by optical microscopy owing to the opacity of the silicon substrate. Therefore, a rapid, sensitive electrochemical method is needed to overcome these limitations and permit on-site and visible analysis of cell viability.

Microfluidic, or lab-on-a-chip (LOC), devices are now widely considered as an enabling technology in cancer cell biology because they possess a variety of advantages, including low reagent consumption, multiplexing ability, potential for integration, and efficient downstream analysis (Wlodkowic et al., 2011). Yang et al. (2011) developed a unique microfluidic platform composed of multiple circular channels and parallel branch channels, which was applied for cell apoptosis assay in a high-throughput manner. Bogojivic et al. (2012) introduced the first digital microfluidic device to conduct a fluorogenic apoptosis assay for caspase-3 activity. In our previous report (Wu et al., 2010), a poly(dimethylsiloxane) (PDMS)-based flexible electrochemical device was fabricated using a simple, chemical gold-plating method to construct the working electrode. The prepared gold film on PDMS was semi-transparent and could be observed optically. PDMS has several well-known advantages such as its chemical inertness, flexibility, optical transparency, permeability, and simple machining (Xia and Whitesides, 1998; Sia and Whitesides, 2003), and since the electrodes were patterned on PDMS, the sensor could potentially be integrated within a microfluidic device. The integration of flexible PDMS-based electrodes on a microfluidic chip could be a potential platform for the facile and fast observation of cellular dynamics.

Herein, a microfluidic platform integrated with a flexible PDMS-based electrochemical cytosensor was developed for dynamic monitoring of the proliferation and drug-induced apoptosis of HeLa cells. A sandwich-type device consisting of PDMS slabs with a gold film working electrode, flow channel, cell culture chamber, gold film counter electrode, and silver ink reference electrode was fabricated. The gold film electrode was prepared by means of simple chemical gold plating, without metal sputtering. After introducing the cells into the cell culture chamber, cell attachment, growth, and proliferation successively occurred on the gold surface, demonstrating its good biocompatibility. Differential pulse voltammetry (DPV) was then used to monitor the proliferation and apoptosis processes upon exposure to anticancer drugs. These dynamic cell processes were also observed in real-time by optical microscopy, since the gold film was semi-transparent and thus allowed the cells to be observed optically. This flexible, microfluidic electrochemical platform provides a useful tool for the label-free, on-site detection of cell behavior under controlled cellular microenvironments.

2. Experimental

2.1. Materials and reagents

Negative photoresist SU-8 2050 was purchased from Microchem, Newton, CA. PDMS was purchased from Dow Corning (Sylgard 184, Midland, MI, USA). Etoposide was obtained from Nanjing Sunshine Biotechnology Ltd. (Nanjing, China). Chloroauroic acid (HAuCl4·4H2O) was purchased from Shanghai Reagent Co. (Shanghai, China). A PBS solution (pH 7.4) containing 137 mM NaCl, 2.7 mM KCl, 8.7 mM Na2HPO4, and 1.4 mM KH2PO4 was prepared, and a [Fe(CN)6]3–/4– aqueous solution containing 2 mM [Fe(CN)6]3–/4– and 0.1 M KCl was prepared. All other reagents were of analytical grade. All aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore).

2.2. Cells and culture

HeLa (human epithelial carcinoma) cells were cultured in standard 100 × 20 mm2 petri dishes (Corning Inc., Corning, NY) in DMEM medium supplemented with 10% fetal calf serum (HyClone Laboratories Inc., Logan, UT), penicillin (100 µg mL–1), and streptomycin (100 µg mL–1) in a humidified incubator containing 5% CO2 at 37°C. At the logarithmic growth phase, the cells were trypsinized and separated from the medium by centrifugation at 300 g for 5 min, washed twice with PBS, and resuspended in fresh culture medium to obtain a homogeneous cell suspension. The number of cells was counted using a Petroff-Hausser cell counter.

2.3. Microfluidic chip design and fabrication

The sandwich-type chip was composed of three layers of PDMS film. The fluidic layer, which comprised a cell culture chamber (3 mm in diameter) connected by a 300 µm wide flow channel, was fabricated using soft lithography (Xia and Whitesides, 1998) and replica molding. The mold of the fluidic layer had 50 µm thick features fabricated by SU-8 negative photoresist. To obtain the fluidic layer, one tip of a silicon tube was mounted on the inlet of the mold, and then the fluidic layer was prepared by pouring PDMS (15:1 elastomer to crosslinker ratio) onto its mold to a thickness of 2–3 mm and baking the film at 70°C for 2 h. After the PDMS film was cut and peeled off from the SU-8 mold, the silicone tube remained embedded in the PDMS film as a fluid inlet port. To integrate the reference and counter electrodes on the chip, one circular opening of 3 mm diameter was created by puncturing the film downstream of the cell culture chamber with a blunt-tip needle. A fluid outlet reservoir was created by puncturing the film with a needle tip with a diameter of 1.2 mm.

PDMS-gold films were prepared by means of a chemical gold plating method described previously (Wu et al., 2010). In brief, the chemical gold plating solution containing 0.01 g mL–1 HAuCl4, 200 g L–1 KHCO3, and 2% (w/v) glucose (v/v, 2:1:1) was confined in a PDMS frame by two native PDMS thin films for 3 h at 37°C. In this system, two PDMS-gold films were fabricated for use as the working electrode and counter electrode. The reference electrode was prepared by painting silver ink smoothly onto one of the PDMS layers with PDMS-gold film.

The construction process of the sandwich-type device is shown in Fig. 1. To construct the chip, the fluidic layer (the middle layer in
Fig. 1) was irreversibly bonded on the PDMS-gold film layer (the bottom layer) using oxygen plasma treatment (Harrick Scientific Corporation, Ossining, NY) to form a sealed device. The cell culture chamber on the fluidic layer was aligned to the gold film on the PDMS-gold film layer so that the fluidic layer insulated most of the gold film but left a working electrode with a diameter of 3 mm confined in the cell culture chamber. Area of the gold film outside the fluidic layer was led out to the data acquisition system. Then the PDMS layer with the gold and silver films (the top layer) was bonded to the fluidic layer with 3 M Scotch tape. The gold and silver film confined in the hole on this layer served as the counter and reference electrode, respectively.

2.4. Apparatus

Electrochemical measurements were carried out with a CHI 660A electrochemical workstation (Shanghai Chenhua, Shanghai, China). The images of the cells at each time point for electrochemical measurement were taken using a Nikon TE-2000-U inverted optical microscope. SEM images were acquired on a scanning electron microscope (SEM, LEO 1530VP).

2.5. Electrochemical measurements

The microfluidic chip was primed with 75% ethanol (v/v) and vacuumized to wet the PDMS channel and sterilize the device. Cyclic voltammetry (CV) was used to characterize the electrochemical behavior. DPV was performed to monitor the proliferation and apoptosis processes upon exposure of the cells to an anticancer drug, etoposide. Prior to each electrochemical measurement, PBS buffer (10 mM, pH 7.4) was used to wash the cells in the culture chamber for 5 min. Electrochemical measurements were carried out using the same PBS solution as the electrolyte. The PBS solution was thoroughly bubbled with high-purity nitrogen before use and the chip was placed in a nitrogen atmosphere during measurements. After each electrochemical measurement, the culture medium with or without the anticancer drug was introduced into the chip and then the cell contained chip was placed in an incubator (5% CO2, 37 °C).

2.6. Viability assay

Separate chips with the same number of cells were incubated under the same conditions. After 24 h of culturing, culture medium containing 100 μM etoposide was introduced into the chips. At each selected time point, an electrochemical measurement was performed on one of the chips, and then the cells on the chip were harvested. The viability of the harvested cells was determined by counting live cells under a microscope after trypan blue staining. The values were expressed as a percentage of the number of cells counted in a control chip that was not exposed to etoposide.

3. Results and discussion

3.1. Characterization of the device

The three electrodes in the present study were located on two separate PDMS layers. The working electrode was on the bottom layer, and the counter and reference electrodes were on the top layer. This arrangement deviates from our previous study, where three electrodes were assembled on one PDMS film. Furthermore, the electrochemical measurements conducted here were performed in a microenvironment different from our previous measurements in bulk solution (Wu et al., 2010).

The surface properties of the gold film were investigated by SEM. As shown in Fig. 2A, the gold film on the PDMS was a continuous layer with a compact and smooth surface composed of Au nanoparticles, which was beneficial for electrochemical testing to make good electrical contact. We acquired a CV of the gold planar electrode confined in the cell culture chamber of the device in 0.5 M H2SO4 (Fig. S1). Three anodic peaks and a cathodic peak appeared in the CV profile, corresponding to surface gold oxidation and the consequent reduction of gold oxides, respectively. The CV profiles of ten independent chips exhibited similar features, i.e., the relative standard deviation of 3.1% from the cathodic currents, indicating the excellent reproducibility. The electrochemical behavior of the device was also investigated using [Fe(CN)6]3−/4− as a redox probe. As shown in Fig. 2B, the electrode showed a pair of well-defined redox peaks with a ΔE_p of 69 mV at a scan rate of 10 mV/s. ΔE_p increased with increasing scan rate, and the peak current ratio (Ipa/Ipc) was equal to 1.0. These electrochemical behaviors showed that the kinetics of electron transfer was sufficiently fast to maintain the surface concentration of redox-active species at the values required by the Nernst equation. The anodic or cathodic peak current increased linearly with scan rate in the range of 10–500 mV/s (Fig. 2B inset), which suggested that the reaction was a surface-controlled process.

3.2. CV behavior of HeLa cells

It has been reported that cells attached to an electrode can produce electrochemical signals (Woolley et al., 2002), which differ with differing cell types. To investigate the electrochemical
behavior of HeLa cells in the present microfluidic device, a cell suspension (10 μL, 5.0 × 10⁵ cells mL⁻¹) was introduced into the device and allowed to grow for 24 h before measuring the voltammetric behavior of the cells. The CV signal in the potential range from −0.3 V to +0.4 V was monitored and a representative scan is shown in Fig. 3A, curve b. HeLa cells in the device exhibited a quasi-reversible voltammetric response, with a very strong cathodic peak at −0.076 V and a weaker anodic peak at +0.10 V. These peaks are likely attributable to redox enzymes in the HeLa cells, such as NADH dehydrogenase (ubiquinine) flavoprotein 2, which is quinine oxidoreductase-like (El-Said et al., 2009b). In contrast, no redox peaks were observed for a control device without immobilized cells (Fig. 3A, curve a).

3.3. Relationship between the peak current and cell number

To further investigate the influence of cell number on the DPV peak current, different concentrations of HeLa cells were introduced into separate devices and incubated under the same conditions.

After incubation for 12 h, electrochemical measurements were performed. The peak current increased with increasing cell concentration (Fig. S2), and Fig. 3B shows the corresponding linear relationship. The exhibited relationship illustrates that the peak current intensity was dependent on the number of cells attached to the electrode, and thus it is possible to calculate the number of cells in a device from device’s observed peak current.

3.4. Real-time monitoring of HeLa cell proliferation

To investigate the relationship between the peak current and the proliferation of HeLa cells in a microfluidic device, the electrochemical responses of cells at different time points were recorded after being introduced into the device. DPV signals of HeLa cells are shown in Fig. 4A after incubation for 6, 12, and 24 h. The results show a well-defined cathodic peak current at −0.01 V at different cell incubation times (Fig. 4A, curves a–c). The peak current increased with increasing incubation time until 48 h, which was related to the cellular proliferation. Incubation times longer than 48 h did not result in further signal improvement, indicating that saturation of cell densities on the surface was achieved at 48 h (Fig. S3A). Furthermore, the increase of the cell numbers was consistent with the variance tendency of peak current intensity in the proliferation within 24 h (Fig. 4A, inset). The results showed that the cell number greater than 7.5 × 10⁵ was undetectable in proliferation. Meanwhile, optical images were acquired after each electrochemical measurement was taken. As shown in Fig. 4B, the cells attached on the PDMS-gold film were still round after culturing for 6 h, indicating incomplete adherence. With increasing incubation time, however, the cells began to grow and spread (Fig. 4C). After culturing for 24 h in the chip, the cells roughly formed a confluent layer on the PDMS-gold film in the chamber (Fig. 4D). The optical images taken at 48 and 60 h incubation showed that cell growth increased on the upper side of the confluent layer in the chamber (Fig. S3B and C) and thus the numbers of cells attached on the surface were unchanged at the time points, in accordance with the electrochemical data. These results suggest that proliferation of the cells occurred quickly on the PDMS-gold film in the device and that the viability of the cells was high within 60 h incubation, due both to the timely changing of the culture medium in the device and to the good biocompatibility of the PDMS-gold film. The optical microscope findings were in good agreement with the electrochemical results, and further substantiated the accuracy of the electrochemical measurement. These results demonstrate that the chip not only was a good platform for cell culturing but also was a sensitive cytosensor for on-site and real-time monitoring of cellular behavior.

3.5. Real-time monitoring of HeLa cell apoptosis upon exposure to anticancer drug

In various cancer treatment methods, selective induction of apoptosis is an attractive chemotherapeutic strategy (Su et al., 2011). In this respect, understanding the extent of apoptosis and the speed of its onset is important for providing good prognostic indicators to guide treatment. To further test the performance of the present device, the electrochemical signal was recorded in real time in the apoptosis of HeLa cells induced by etoposide. Etoposide, a well-known inducer of apoptosis, can prevent re-ligation of the DNA strands, which causes errors in DNA synthesis and promotes apoptosis of cancer cells. A cell suspension (10 μL, 5.0 × 10⁵ cell mL⁻¹) was introduced into each of two separate devices. After culturing for 24 h, the cells in one of the devices were continuously incubated with culture medium containing 100 μM etoposide, whereas the cells in the other device were incubated with blank culture medium as a control. The DPV signal
Fig. 4. Real-time monitoring of HeLa cell proliferation on the chip. (A) DPV measurements at different time points in the cell proliferation process. (a–c) Represent the DPV responses of the cells after being incubated in the device for 6 h (a), 12 h (b), and 24 h (c). Inset: the number of cells at the time points. (B–D) Photo images of HeLa cells after being incubated in the device at different time points corresponding to (a–c) in (A), respectively. Scale bars, 100 μm.

Fig. 5. Real-time monitoring of HeLa cells after treatment with 100 μM etoposide at different time points. (A) DPV responses of HeLa cells in the device after treatment with 100 μM etoposide for 0 h (a), 4 h (b), 12 h (c), 24 h (d), and 36 h (e). (B) Relationship between the peak currents and the time exposure to etoposide or blank culture medium as a control. Error bars represent one standard deviation for three parallel experiments. (C–F) Photo images of HeLa cells after treatment with 100 μM etoposide for 4 h (C), 12 h (D), 24 h (E), and 36 h (F). Scale bars, 100 μm. The images (C–F) correspond to the DPV responses of (b–e) in (A), respectively.
responses at different time points for the two devices are shown in Fig. 5A. Contrary to the cell proliferation process, in which the peak current response increased with increasing incubation time, the peak current response decreased with increasing incubation time for the etoposide-treated cells, which could be attributed to the loss of cell viability owing to apoptosis triggered by the etoposide. The sigmoid curve obtained between the peak current and the incubation time indicated that the apoptosis was a time-dependent event (Fig. 5B). The relative standard deviation (RSD) of the control experiments ranged from 4.4% to 9.6%, whereas the RSD of the drug-treated experiments ranged from 4.0% to 11.1%, as obtained at different time points by measuring the same concentrations of cells on three chips prepared independently under identical experimental conditions. These results demonstrate that the proposed protocol has an acceptable precision and reproducibility. The optical images taken during apoptosis (Fig. 5C–F) further confirmed the data from the electrochemical measurements. The results showed that the pseudopod of the cells retracted and turned round gradually with increasing exposure time to etoposide up to 12 h. After exposure to etoposide for 36 h, almost all of the cells were detached, and some of the dead cells were washed away from the chamber in their final stage of apoptosis (Fig. 5F).

To confirm the electrochemical and optical microscopy results for the anticancer drug's effect on cell viability, electrochemical measurements first were performed at each selected time point. Then, the viability of the cells in the chamber was determined by counting the number of live cells under a microscope after trypan blue staining. The experimental results are provided in Table 1. Obviously, the relative viability of the cells decreased with increased incubation time. After treatment with etoposide for 36 h, only 17.4% of the cells were still viable. The agreement of the electrochemical measurements and cell counting assay results implies that the present strategy is feasible for studying cell behavior in vitro.

### 4. Conclusions

The work reported here provides a microfluidic platform integrated with a flexible PDMS-based electrochemical cytosensor for label-free, on-site monitoring of the proliferation and the drug-induced apoptosis of HeLa cells. A sandwich-type device consisting of PDMS slabs with a gold film working electrode, flow channel, cell culture chamber, gold film counter electrode, and silver ink reference electrode was fabricated. Good biocompatibility and electrochemical properties of the gold film electrode facilitated cell culturing and subsequent electrochemical responses. This microfluidic platform was successfully applied to monitor the proliferation and apoptosis of the cells. Meanwhile, the semi-transparency of the gold film permitted optical microscopy observation of the cell morphology on the surface, which substantiated the electrochemical measurements. Such a platform is an advantageous tool for real-time monitoring cell behavior, and is promising in various biomedical applications such as pharmaceutical studies and toxicological analyses.

### Acknowledgments

We greatly appreciate the support of the National Basic Research Program of China (2011CB933502), the International S&T Cooperation Projects of China (2010DFA42060), and National Natural Science Foundation of China (Grants 21121091 and 21020102038).

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.07.025.

### References


### Table 1

<table>
<thead>
<tr>
<th>Incubation time with etoposide (h)</th>
<th>Current (µA)</th>
<th>Cell viability (%)</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.271 ± 0.011</td>
<td>100.0 ± 3.8</td>
</tr>
<tr>
<td>4</td>
<td>0.233 ± 0.012</td>
<td>93.9 ± 6.3</td>
</tr>
<tr>
<td>12</td>
<td>0.174 ± 0.019</td>
<td>70.8 ± 7.7</td>
</tr>
<tr>
<td>24</td>
<td>0.148 ± 0.016</td>
<td>45.0 ± 4.8</td>
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<tr>
<td>36</td>
<td>0.093 ± 0.003</td>
<td>17.4 ± 0.6</td>
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