Biomed Microdevices (2016) 18: 94
DOI 10.1007/s10544-016-0118-3

High-efficient and high-content cytotoxic recording via dynamic and continuous cell-based impedance biosensor technology

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Published online: 19 September 2016

Abstract Cell-based bioassays were effective method to assess the compound toxicity by cell viability, and the traditional label-based methods missed much information of cell growth due to endpoint detection, while the higher throughputs were demanded to obtain dynamic information. Cell-based biosensor methods can dynamically and continuously monitor with cell viability, however, the dynamic information was often ignored or seldom utilized in the toxin and drug assessment. Here, we reported a high-efficient and high-content cytotoxic recording method via dynamic and continuous cell-based impedance biosensor technology. The dynamic cell viability, inhibition ratio and growth rate were derived from the dynamic response curves from the cell-based impedance biosensor. The results showed that the biosensors has the dose-dependent manners to diarrhetic shellfish toxin, okadiac acid based on the analysis of the dynamic cell viability and cell growth status. Moreover, the throughputs of dynamic cytotoxicity were compared between cell-based biosensor methods and label-based endpoint methods. This cell-based impedance biosensor can provide a flexible, cost and label-efficient platform of cell viability assessment in the shellfish toxin screening fields.

Keywords Cell-based impedance biosensor · Dynamic and continuous recording · Cell viability · Cell growth rate · Okadiac acid

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1 Introduction

Toxicity in foods, therapeutic drugs, and other products endangers the human health, leading to other various serious diseases, even death, so the toxicity testing is necessary to assess these chemicals. Conventionally, these assessments are performed by the living animals (Alexander 1995; Olson et al. 2000; Pfohl-Leszkowicz and Manderville 2007; Prassopoulou et al. 2009), however, animal analytical methods are inaccurate due to many false negatives and false positives, which only used the death of animals for the toxicity evaluation. Moreover, animal methods were limited by regulatory constraints and ethical considerations (Botana et al. 2010; Browne et al. 2000; Kilkenny et al. 2009), so the alternatives are demanded in toxicological studies.

In vitro toxicity testing was recently suggested by toxicologists, who believed the *in vitro* analytical methods were more effective than the animal methods. These *in vitro* methods were usually performed on cultured mammalian cells or bacteria with sensitive responses to toxic compounds (Bulich 1979; Heinlaan et al. 2008; Riss and Moravec 2004). In the cell-based bioassays, cell viability or cytotoxicity were main

indicators for In-vitro toxicology. Proteases were utility biomarkers to measure the cell viability due to they can only keep active in live cells (Niles et al. 2007). MTT-based bioassay (MTT, XTT, MTS) was commonly used to validate the cell viability using colorimetric reactions of live cells (Goodwin et al. 1995; Mosmann 1983; Tominaga et al. 1999). Furthermore, ATP which was the essential element in the luminescent reactions was set as a marker of cell viability (Crouch et al. 1993; Fan and Wood 2007; Riss and Moravec 2004). These colorimetric-based methods can preferably reflect compound-induced cytotoxicity or other cellular responses. However, all of these methods were label-base endpoint bioassay, which significantly hampered the dynamic monitoring of the cell viability during the compound tests, therefore, the information of compound toxicity were incomplete by the endpoint cell viability or cytotoxicity.

In recent decades, the micro/nanofabrication and detection technologies are developed to create varieties of biosensor platforms, which served the recognition and detection of trace molecules (Lee et al. 2009; Wan et al. 2015; Wang 2006). Meanwhile, cell-based biosensor is a unique biosensor which takes the cells as sensitive elements, and reflects the toxicity or efficacy of compounds by the extracellular physiological studies (Liu et al. 2014; Wang and Liu 2009). The extracellular physiological mainly contains cellular metabolism (e.g., consumption of glucose and oxygen, production of acidic wastes by Light-addressable potentiometric sensor and field effect transistor-based microphysiometers (Eklund et al. 2006; Fanigliulo et al. 1996; Hu et al. 2013; Poghossian et al. 2009)), cellular growth (attachment, proliferation and apoptosis by electric cell-substrate impedance sensing platform (Giaever and Keese 1984; Giaever and Keese 1993; Zhou et al. 2013)), and extracellular potential of some electrogenic cells (e.g., neurons and cardiomyocytes by micro/nanoeletrodes (Heer et al. 2004; Nisch et al. 1994; Xiao et al. 2010; Xie et al. 2012)). In these cell-based biosensors, the cell-based impedance sensor platform was proven to have the similar results with MTT-based bioassay to test the cell viability in previous work (Zou et al. 2016, 2015). Nevertheless, the dynamic information of impedance responses were usual ignored for the toxicity assessment. Cell viability information at some time points were only used as MTT-based cell viability assays (e.g., cell viability at 24 h after compound treatment). Thus the dynamic and continuous advantages of the cell-based biosensor technology were incomplete utilized in the toxicity assessment.

In this work, we focused on assessing the compound-induced cytotoxicity by the dynamic and continuous cell-based impedance recording. The cell viability, half maximal inhibitory concentration (IC_{50}), and growth rate were derived from the dynamic and continuous dose-dependent cell growth curves. The dynamic compound-induce cytotoxicity information of cell-based impedance recording was calculated at different time points or at different phases during the whole compound experiments. Therefore, comparing to the traditional MTT-based study, this dynamic analytical methods will significantly provide more toxicity information with lower experimental throughputs.

2 Experimental and method

2.1 Dynamic and continuous impedance biosensor recording

To monitor the cell growth status, interdigitated electrodes (IDEs) based impedance sensor array was utilized to dynamically and continuously measure the impedance value, which is related with the number, attachment, and morphology of cells on the IDEs surface. The impedance sensor array contain 16 IDEs, which consisted of many electrode branches in each IDE pair (Fig. 1(a)). Usually, the impedance of IDEs array was measured by the multiplex impedance reader (Fig. 1(b)), which applied a low amplitude sine voltage (30 mV, 10 kHz) at one electrode of IDEs and sampled the current signals at the match one of IDEs.

A human hepatoma cell line (HepG2) was employed to establish the cell-based biosensors. Based on the impedance sensor recording system, the cell growth status can be dynamically and continuously monitored. The impedance responses of these biosensors were induced by cell plating on the sensor array. Initially, the impedance will gradually increase due to the cell attachment and proliferation. Then the compound cytotoxicity bioassays were performed after 1-day culture. Usually, the impedance of biosensors will decrease and present a dose-dependent manner to cytotoxic compounds. Taking the advantages of dynamic and continuous biosensor measurement technology, all the impedance signals which were related to the cell viability and compound cytotoxicity can be recorded by impedance reader system. Moreover, the detailed information (E.g. cell viability, cell growth rate) can also be analyzed and derived from the dynamic and continuous signals (Fig. 2). The dynamic IC_{50} can be calculated from the dynamic relationship of cell viability and compound concentration, while the cell growth rate can also be obtained from the each experimental phases.

2.2 Cell culture

HepG2 cells were purchased from the American Type Culture Collection (ATCC) and were cultivated in 25 cm^2 plastic flasks (Corning) by DMEM with 10 % (*v*/v) fetal bovine serum (FBS) and 1.0 % (*w*/*v*) penicillin and 1.0 % (w/v) streptomycin in a 37 °C and CO_2 incubator. The culture medium was refreshed every 2 days. To establish a cell-based biosensor, 0.25 % (w/v) Trypsin-0.53 mM EDTA solution was added into flask to harvest the cells, and 190 μL suspension medium with 2×10^4 cells was added in each well of impedance biosensor before measurement.

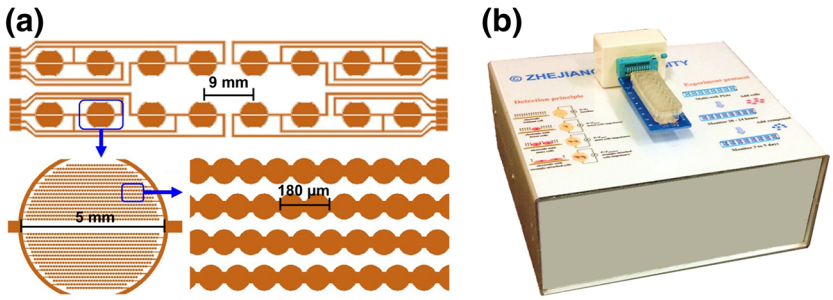

Fig. 1 **a** Interdigitated electrode-based impedance sensors. **b** Multiplex impedance reader

2.3 Reagent preparation

Mussel was collected from the shellfish beds of Gouqi Island in China in the spring and summer of 2015. The preparation of shellfish extracts were administrated according to the protocol in the previous work (Ledreux et al. 2012; Prassopoulou et al. 2009). Then 1 g shellfish extracts were dissolved in 5 mL methanol. Okadaic acid (OA) from Sigma was added in the shellfish extract solution to generate the OA solutions with different doses. All the solutions were filtered with 0.22 μm membrane filter and stored at 4 °C. To decrease the cytotoxicity, the final methanol were diluted to 1 % (*v*/v) in the cell-based bioassay.

2.4 Scanning electron microscopy characterization

HepG2 cells were treated by the shellfish extract with different concentrations of okadaic acid (OA), while the control ones were initially fixed by 2.5 % glutaraldehyde in 0.01 M PBS, pH 7.4 at 4 °C for 2 h. Subsequently, they were washed by PBS and fixed in 1 % (*w*/*v*) of osmium tetraoxide at 4 °C for 1 h. Finally, the cells were dehydrated by a series of ethanol solutions from 30 % to 100 % for 15 min in each grade. Scanning electron microscopy (SEM) analyze was performed on SU-70 (Hitachi).

2.5 Cell viability assay using cell counting kit-8

Cells were seeded in 96-well plates and cultured for 24 h, and then the cells were treated with OA for 24 h. Subsequently, the cell culture medium were replaced by 100 μL fresh medium with 10 μL CCK8 reagent in each well, and 96-well plate was placed into the incubator for 0.5 h. Finally, the absorbance was measured by a microplate reader at 450 nm (sample) and 600 nm (reference).

3 Results and discussion

3.1 Cytotoxicity assessment by dynamic cell viability monitoring

To monitor the cell viability, IDEs-based impedance biosensors were applied in the compound experiments. Okadaic acid (OA), which inhibit protein phosphatases, specifically serine/

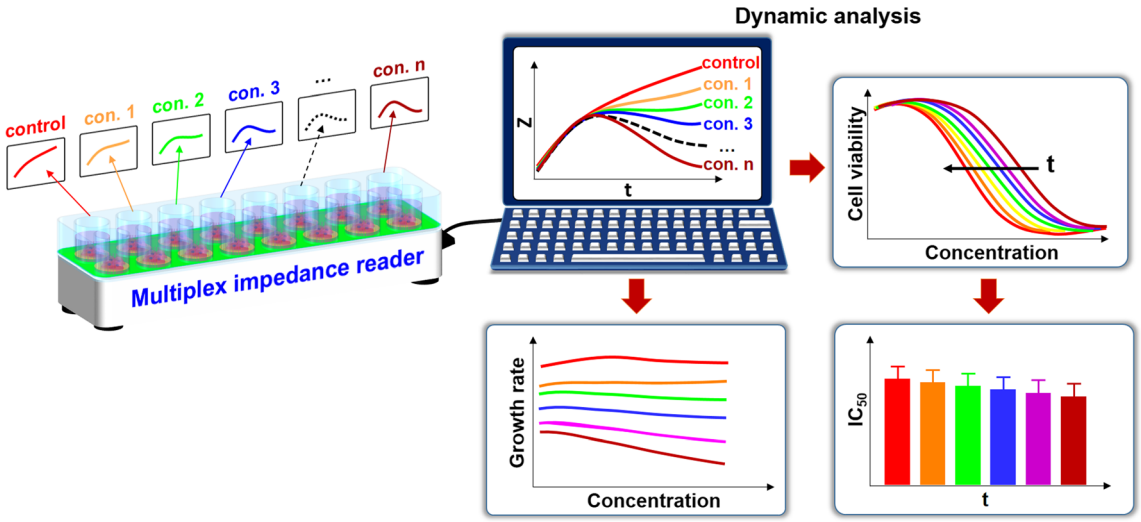

Fig. 2 Dynamic and continuous cytotoxicity assessment by cell-based impedance biosensor system. Cell growth and compound-induced cytotoxicity can be evaluated by the dynamic impedance recording. The continuous impedance-based cell growth curves present the dose-dependent responses to cytotoxic compound, while compound-induced cytotoxicity can be assessed by cell viability-concentration curves at different time point and cell growth rate-concentration curves at different phases, which are both derived from the dynamic and continuous impedance curves

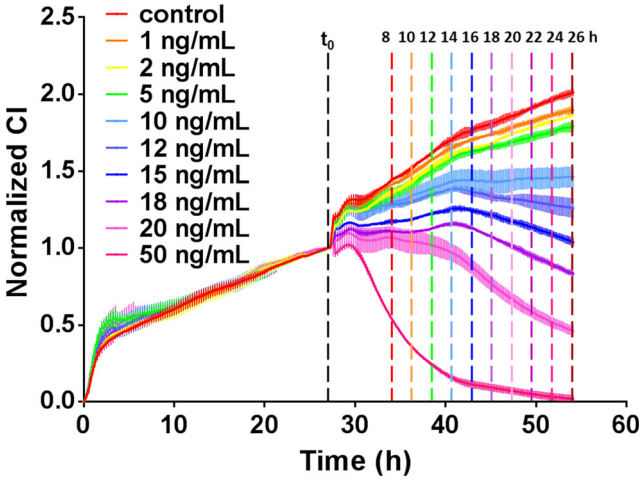

Fig. 3 Dynamic and continuous cell growth recording before and after OA treatment (1–50 ng/mL). t_0 is the time of compound addition (*dark dash line*) and cell responses to compound at typical time points are indicated by other colorized *dash lines*

threonine phosphatases inhibition (Dounay and Forsyth 2002), was selected as the toxic compound to test the dynamic analysis function of cytotoxicity. To improve the consistency of cell-based impedance biosensor for compound test, cell index (CI) and normalized CI were usually introduced. CI is defined the ratio of impedance changes $\triangle Z$ and background impedance Z_0 of impedance sensor, which is proposed for easy comparison between each well. The normalized CI is ratio of CI_t at the time t and CI_{t0} at the normalized time t0 (usually the time point of compound addition), which facilitates to compare the compound effects. Figure 3 showed the impedance responses induced by cells cultured on the IDEs array. Initially, the CI values dramatically increased due to the cellular attachment in the first 2 h. Then the CI gradually increased by the cellular proliferation. The compound OA with different doses from 1 to 50 ng/mL were added into cell-based impedance biosensors at t_0 = 27 h. Subsequently, CI values of treated cell-based impedance biosensors were smaller than that of control group, indicating the compound-induced cytotoxicity with dose-dependent manners. The CI values significantly dropped to a low level in the presence of high-dose compound. Based on the dynamic and continuous recording, the CI values at each recording points can be derived from each group. As shown in Fig. 3, the t_0 is the time point of compound addition. Some time points were selected to presents that the impedance biosensor method provides dynamic information. To calculate the cell viability, the normalized CI values should be derived from the cell growth curves at one time frame in Fig. 3. The cell viability curve can be then calculated based ratio of treatment group normalized CI values and control group normalized CI value. (Fig. 4(a)). Further, the dynamic IC_{50} then were calculated from these dynamic cell viability curves by Graphpad software, and the IC_{50} values decrease from 31.39 ± 1.13 ng/mL (10 h) to 16.21 ± 1.06 ng/mL (26 h) indicating the cytotoxicity changes in a flexible way (Fig. 4(b)).

To obtain these dynamic cell growth curves, cell viability curves and IC_{50} values, only 36 wells of cell-based impedance biosensors were required. If it is administrated by label-based endpoint methods, experimental throughputs should reached at least 300 wells, considering to obtain each point by 3 individual wells in cell viability curves of Fig. 4(b). Moreover, the protocols of endpoint method should be repeatedly followed at each time points, which are time and labor-consuming. Therefore, it has been demonstrated that cell-based impedance biosensor can dynamically and continuously assess the cytotoxicity of compound with lower cost, throughputs, and complexity.

3.2 Cytotoxicity assessment by dynamic cell growth rate monitoring

The dynamic cell growth rate can also derived from the impedance responses of cell-based impedance biosensors as well as the dynamic cell viability, which presented dose-dependent cytotoxicity. There were four obvious cell growth phases (I, II, III, and IV) in each group (Fig. 5(a)). Phase I was generated the attachment stage of cells as mentioned above. Phase II was the proliferation stage of cells without compound. Phase III was the early stage in the presence of compound. Phase IV was the late stage in the presence of compound. All of cell growth rates in these phases can be calculated from cell growth curves by linear fitting using Graphpad software.

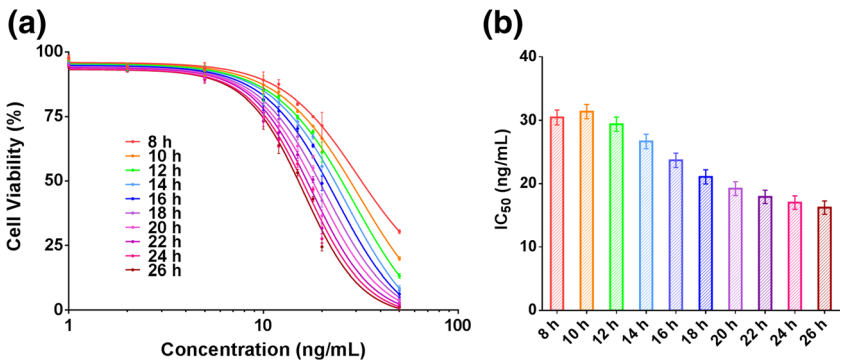

Fig. 4 The OA-induced cytotoxicity assessment by cell viability. **a** Cell viability curves derived from the cell growth curves from 8 to 26 h after compound addition. **b** IC_{50} calculated from cell viability curves at different time points. All the average and standard deviation values are calculated by three individual wells

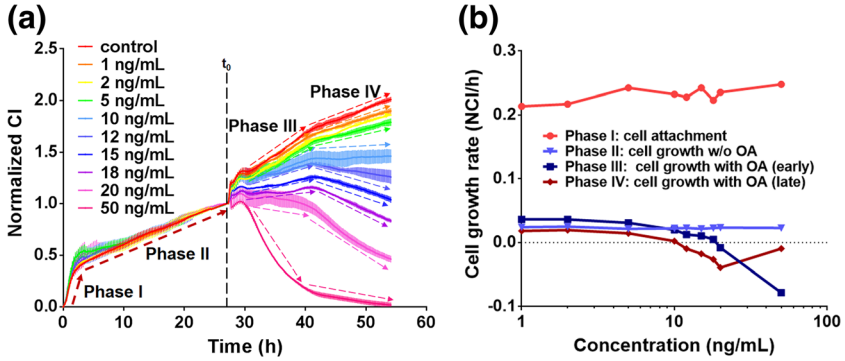

Fig. 5 The OA-induced cytotoxicity assessment by cell growth rate. **a** Dynamic and continuous cell growth recording with 4 obvious phases in the absence and presence of OA. t_0 is the time of compound addition (*dark dash line*) and cell growth status in different phases under different dose compounds were indicated by colorized *dash lines* with *arrow*. **b** Cell growth rate curves in each phase derived from the cell growth curves. All the average and standard deviation values are calculated by three individual wells

Figure 5(b) showed cell growth rate in each phases of Fig. 5(a) under different dose compound. In the phase I, the growth rates had a large difference between each group which is probably due to random cell positions and different attached status on the impedance sensor in each well. In the phase II, the growth rates are similar between different concentrations due to the similar cell proliferation status without compound effect. Cell-based impedance biosensor can monitor cell viability during whole cell growth and easily exclude the cells with bad status before the compound assays, which was seldom perform in the endpoint assay. The growth rate in Phase III and IV showed a dose-dependent manner from positive to negative due to the joint effect of cell proliferation and compound cytotoxicity. The cell growth rate in the late phase reduced to a low level due to the rapid death of cell in the case of a higher dose (50 ng/mL). In addition, the cell morphology under different dose OA was characterized by scanning electron microscopy (SEM). It was obviously seen that the cells attached on the surface and connected with other cells in the good status in the control group at 24 h (Fig. 6, left image), while the cells turned small and round under higher doses (Fig. 6, middle and right), which were matched with the responses of cell-based impedance biosensors to cytotoxic compounds.

3.3 Comparison of cell viability test of cell-based impedance biosensor and CCK-8

To compare the cell viability between cell-based impedance biosensor and conventional MTT-based bioassay, Cell Counting Kit-8 (CCK-8), an improved MTT kit, was used to determine the cell viability by using WST-8 to produces a water-soluble formazan dye upon bioreduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 solution can be added directly to the cells without pre-mixing of components as conventional MTT kits. WST-8 is bioreduced by cellular dehydrogenases to an orange formazan product that is soluble in tissue culture medium. The amount of formazan produced is directly proportional to the number of living cells. Cell Counting Kit-8 allows sensitive colorimetric assays for the determination of the number of viable cells in the proliferation and cytotoxicity assays, and the detection sensitivity is higher than MTT, XTT or MTS. As shown in Fig. 7, compared to the cell viability results of CCK-8, cell-based impedance biosensor can also reflect the similar signal changes which are induced by changes of cell growth degree/cell spreading area on electrode surfaces in the presence of toxin treatment.

Moreover, the dynamic cell viability bioassays were efficiently performed with low resource consumption and easy

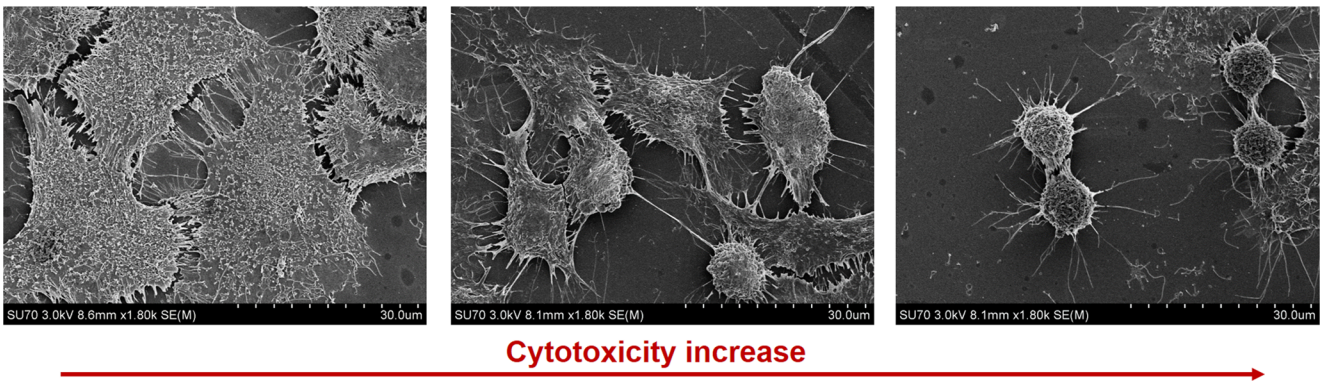

Fig. 6 SEM images of HepG2 at the end of experiments (Left: control, middle: 15 ng/mL OA, right: 50 ng/mL OA)

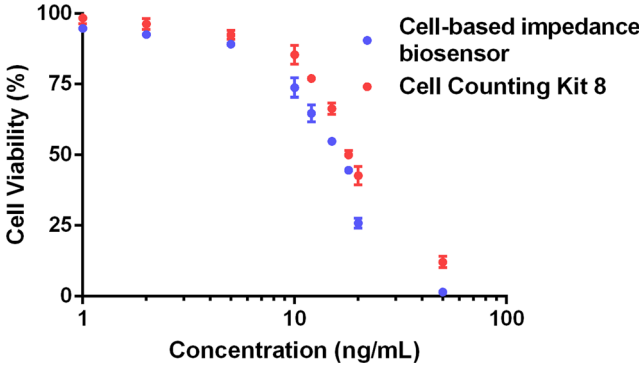

Fig. 7 The comparison of cell viability determined by cell-based impedance biosensor and Cell Counting Kit-8 after 24 h OA treatment

operation by cell-based impedance biosensor. To obtain these dynamic cell viability curve, only 36-well experimental throughputs were required. If the same experiments were performed by label-based endpoint methods, throughputs should reached at least 360 wells, considering to obtain each rate calculated by linear fitting of 3 individual wells from cell viability curves. To obtain the cell growth rate, cell-based impedance biosensor method was cost and labor-efficient, comparing to the endpoint method. Furthermore, the cell growth curves can be divided into more growth phases to study the compound-induce cytotoxicity without additional extra throughputs. It can be conveniently achieved by dynamic and continuous recording method of cell-based impedance biosensor.

4 Conclusion

In this study, we focused on performing the dynamical and continuous cytotoxicity analysis by cell-based impedance recording. The dynamic cell viability, IC_{50}, and cell growth rate were derived from the dynamic cell growth curves. Moreover, experimental throughputs for dynamic information were discussed between the biosensor methods and the label-based endpoint methods. The dynamic and continuous compound assessment of cell-based biosensor will be a promising and utility platform for the toxin and drug assessment.

Acknowledgments This work was supported by NSFC (No.61320106002, No.81501553), High-level Project of Cooperation Improvement of Sino-American and Oceania Region of Ministry of Education of China (No.20152026), and China Postdoctoral Science Foundation (No. 2015 M570511).

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