Toxicity Studies of Natural Product in Vero Cells Using Impedance Monitoring
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ABSTRACT
Natural products have traditionally been used for medicinal purposes in Asian communities. Toxicity studies typically use animal testing to predict the harmfulness of a particular substance to human health. For this study, in lieu of animal testing, we utilize cell-based biosensors to evaluate the toxicity of natural products. The cell-based biosensors are fabricated on a printed circuit board with copper electrodes and are equipped with PDMS cell culture chambers. Two different electrodes (interdigitated and circular) were designed. Vero cells were used to represent normal healthy cells. The cells are first cultured on biosensors and then are inoculated with natural products, equipped with PDMS cell culture chambers. It is a cost-effective and rapid technique and can act as an alternative for animal testing and live cell imaging technique (Campbell et al., 2007; Tran et al., 2016). Real-time monitoring of cells is significant to study the response of cells towards external treatment or drugs introduction. Numerous research have applied this concept, to note a few examples, studies of toxicity in water (Tan and Schirmer, 2017), drug discovery (Lundstrom, 2017), wound healing (Cui et al., 2017) and cell growth and toxicity (Anh-Nguyen et al., 2016; Mansor et al., 2015a).

In this study, we present a printed circuit board impedance biosensor with copper electrodes based on our previous work (Mansor et al., 2015b) for toxicity studies of natural products on normal healthy cells (vero cells). For comparison, the vero cells are also exposed to Taxol (chemo drug – positive control) and DMSO (negative control). The impedance biosensor operates on the basis that healthy cells adhere to the sensor’s electrodes impeding current flow, resulting in high impedance. A high impedance also correlates to a high cell index, indicating that more cells are adhere on electrode’s surface. Conversely, low impedances reflect that less cells are attached to the electrodes, and this occurs when the cells are dead. Dead and unhealthy cells usually are non-adherent and they slowly detach themselves from the electrodes leading to a decrement of cell index.

MATERIALS AND METHOD
Cell Cultures
Vero cells (ATCC® CCL-81™) (ATCC, Manassas, VA, USA), a type of normal cell line; was grown and maintained to confluence in Dulbecco’s Modified Eagle Medium, DMEM (Gibco, Paisley, UK) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco). The Vero cells were incubated under standard cell culture environment in CO2 incubator at 37°C in an atmosphere containing 5% carbon dioxide, CO2. Once confluent, the cells were detached from the culture flask using accutase and were then resuspended in fresh media. The suspended cells were counted, and a density of $1 \times 10^5$ cells/ml in 100 µL of the media was seeded on each biosensor.

Gelatin Preparation and Cell Adhesion Coating
Gelatin coating is necessary for long term (7-14 days) maintenance of cells. Gelatin coating is performed before seeding feeder cells into the chamber. For preparation of a 1% gelatin, 0.1 grams of gelatin was taken out of the container and put into 100 ml of double distilled water. Next, it is sterilized in an autoclave sterilizer at 121°C for about 15 minutes. In a biosafety cabinet, 0.1 % gelatin was added to each chamber to coat it. After gelatin has been poured into each chamber, it is incubated for a minimum of 30 minutes at a temperature of 37°C and the excess was discarded. Each of the chambers has to be dried for at least 2 hours before cell seeding. Ready chambers were sealed with parafilm for storing purposes.

Natural Product and Toxicity Test
In this study, there are three treatments sampled at 10 µl. Natural product which is the agarwood branch ethanolic crude extract is seeded at final working concentration of IC50 6 µg/ml, Taxol, commercial cancer drug, is seeded at final working concentration of...
IC$_{50}$ 2.3 μg/ml and DMSO, a negative control is seeded at a concentration of a 10% (v/v). All IC$_{50}$ values were based on the response of MCF-7 breast cancer cells for different studies. All treatments were introduced 24 hours subsequent to cell seeding. The response of cells to the drugs was plotted as cell index number in the next section.

Sensor Fabrication

Copper electrodes were designed and fabricated on FR4 board using standard PCB fabrication process of UV exposure, development, etching and finishing. In this study, two different types of electrodes were modelled; interdigitated electrode and circular electrode as shown in Fig. 1. Each board contains 8 sets of identical sensor designs that will be used for experiments. Sensors were kept in a dry place to avoid oxidation and were cleaned with ethanol and PBS. All sensors were exposed to UV light for a night for sterilization process prior to ECM coating and cell seeding.

Experimental Setup

During the experiment, sensors were retained inside the incubator at all times except for impedance measurement. Every 6 hours, the sensors were taken out for data acquisition using AD5933 evaluation board and laptop. Four sets of double experiments were conducted on a single board; cell+natural product (blue box), cell+taxol (red box), cell+DMSO (green box) and control cell without drugs (yellow box).

RESULTS AND DISCUSSION

Cell Index Representation of Cell

Measurement using AD5933 evaluation board provides the magnitude of impedance and phase of the biosensors. Since CI is a much more accurate representation of cellular behaviour (Boyd et al., 2008) that occurs on the surface of electrodes, the impedance and phase is transformed into CI number. CI of cellular growth can be expressed based on the Eq. (1)

$$CI = \max_{i=1,N} \left[ \frac{R_{cell}(f_i)}{R_b(f_i)} - 1 \right]$$

(1)

Where $R_b$ represents the frequency-dependent resistance of control measurement (without cells) and $R_{cell}$ indicates the frequency-dependent resistance of the cells and electrodes. Measured resistances, $R$ were extracted from $Z$ and $\theta$ using Eq. (2) and Eq. (3):

$$|Z| = \sqrt{R^2 + X^2}$$

(2)

$$\theta = \tan^{-1}\left(\frac{X}{R}\right)$$

(3)

Biosensor and T-Flask Comparison

Fig. 1 Left- Details on size and dimension of the circular and interdigitated electrodes. Middle- Design of the PCB mask for PCB fabrication. Right- Fabricated sensors attached together with PDMS chamber and wire soldered for impedance measurement.

Fig. 2 Right- Sensors and T-flask positioned inside incubator. Left- Impedance and phase measurement were taken using AD5933 evaluation board and laptop. Four sets of double experiments were conducted on a single board; cell+natural product (blue box), cell+taxol (red box), cell+DMSO (green box) and control cell without drugs (yellow box).

Fig. 2
This may indicate that the NP suppressed cell growth (causing longer lag phase) and or the NP inhibited cells from attaching to the biosensor. To this end, the impedance-based biosensor shows great potential to be used as a tool to study the toxicity of natural products. Nevertheless, more work needs to be done to provide more data on cytotoxicity testing of different drugs and on different adherent cells to demonstrate the compatibility of the biosensor towards the variation of drug studies.

CONCLUSION

In this study, PCB board was fabricated based on two design, IDT and circular electrode. IDT was observed to have a better sensitivity based on higher CI number compared to a circular electrode design. The impedance-based biosensor was shown to have great potential as a real-time and cost effective tool to study toxicity of natural products.

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