iMEDiTEC 2017

ORIGINAL PAPER

Toxicity Studies of Natural Product in Vero Cells Using Impedance Monitoring

Hamdan T.H [a], Mansor A.F.M [a], Abbas P [b], Nordin A.N [a,*], Hashim Y.Z.H.Y [b],

[a] Department of Electrical and Computer Engineering, Kulliyyah of Engineering, IIUM Kuala Lumpur, Malaysia
[b] Department of Biotechnology Engineering, Kulliyyah of Engineering, IIUM Kuala Lumpur, Malaysia

* Corresponding author: anisnn@iium.edu.my

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, Taxol (chemo drug – positive control) and DMSO (negative control). Impedances of these biosensors were then recorded at six-hour intervals for 80 hours to determine the growth of the cells. It was found that compared to Taxol, natural products have a very low toxicant values.

INTRODUCTION

A diverse family of Natural products (NPs) have shown to be remarkably useful in many applications such as in biomedicine and agriculture (Bhanot *et al.*, 2011; Demain, 2014; Giddings and Newman, 2013; Katz and Baltz, 2016; Newman and Cragg, 2012). Natural products originating from plants have been utilized in various aspects of medicines for thousands of years. Of focus is the agarwood tree, which has been traditionally used for its medicinal properties in South East Asian communities, Chinese, Tibetan, Unanai and Ayurvedic medicine (Hashim *et al.*, 2016). The pharmacological activities of agarwood has been tested and this plant material has been reported to exert various bioactivities, including anti-cancer, anti-inflammatory, anti-microbial and anti-oxidant (Hashim *et al.*, 2014).

Electrical cell sensing method is a well-known technique for real-time monitoring of cellular conditions that is non-invasive, simple and based on impedance measurements of cells. 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 CO_2 incubator at 37°C in an atmosphere containing 5% carbon dioxide, CO_2. 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 $1x10^5$ 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 IC_{50} 6 µg/ml, Taxol, commercial cancer drug, is seeded at final working concentration of

eISBN 978-967-0194-93-6 FBME

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.

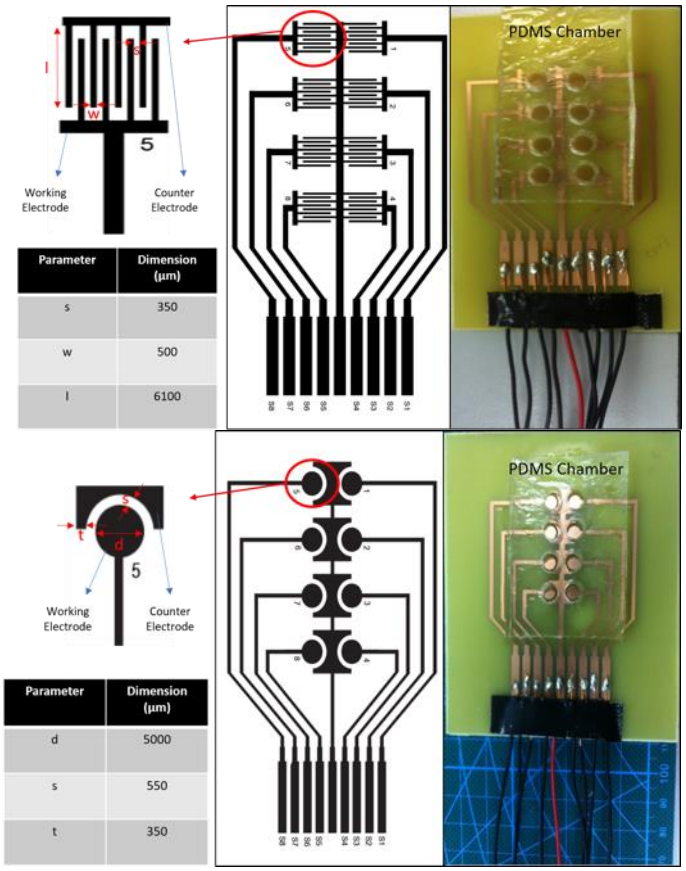

Fig. 1 *Left*- Details on size and dimension of the circular and interdigitated electrodes. *Middle*- Design of the PCB mask for PCB fabrication. *Right*- Fabricated sensors attached together with PDMS chamber and wire soldered for impedance measurement.

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 which is connected to a laptop. Impedance and phase of the cells at the specific time were measured using an AC frequency of 180Hz. The impedance and phase was converted into resistance and Cell Index (CI). As a point of reference, the conventional method of cell culture using T-flasks were also performed and microscopic cell counting was done on cells inoculated with natural products. A total of 13 flasks were prepared and cell-counting were performed every 6 hours for impedance measurements. Fig. 2 shows the setup of the experiment.

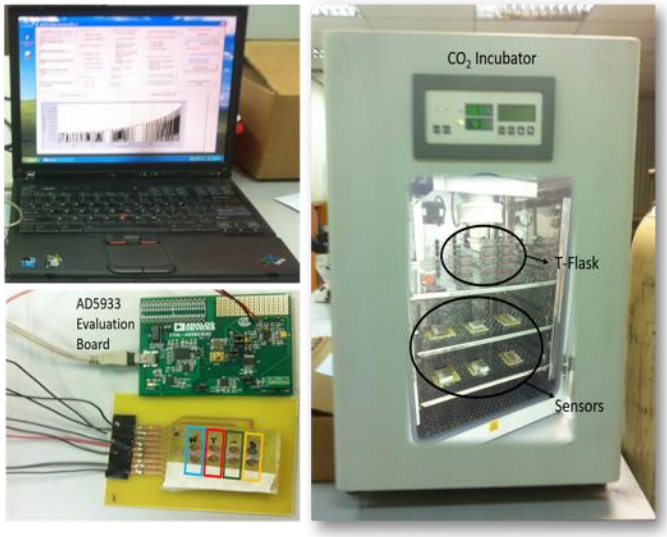

Fig. 2 *Right*- Sensors and T-flask positioned inside incubator. *Left*- Impedance and phase measurement were taken using AD5933 evaluation board and laptop. Four sets of double experiments were conducted on a single board; cell+natural product (blue box), cell+taxol (red box), cell+DMSO (green box)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

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)

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

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 θ using Eq. (2) and Eq. (3):

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

$$\theta = \tan^{-1}\left(\frac{X}{R}\right) \quad (3)$$

Biosensor and T-Flask Comparison

eISBN 978-967-0194-93-6 FBME

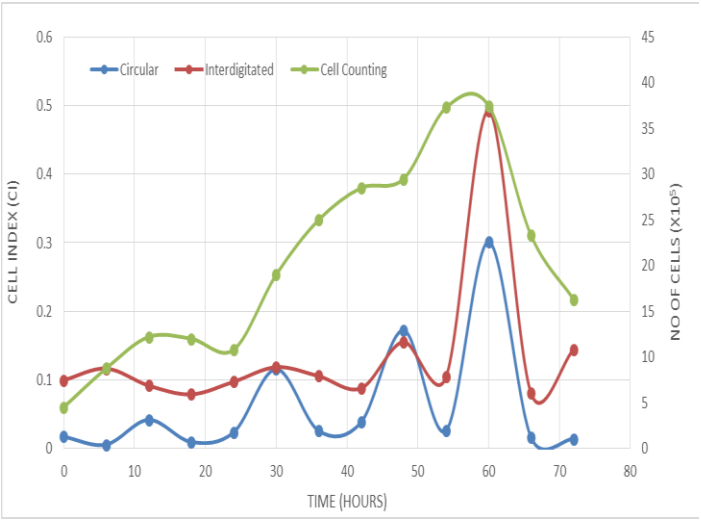

Fig. 3 Comparison of cell counting using T-Flask method and Cell Index using biosensors.

Fig. 3 shows the comparison of monitoring NP exposure on vero cells using two techniques, conventional method of trypan blue exclusion and impedance biosensor. A similar graph trend can be seen from both methods where the highest number of cells counted were shown at time 60 hours that correlates to the highest CI number measured. Fluctuation of CI in several measurements is due to the limitation of growth due to limited surface area. For this case, the T-flask offers a higher surface area for adhesion compared to biosensors where dead cells need to detach before there is space for the next adhesion process. These results indicate that the biosensor can be used to predict the response of cells towards drugs exposure. The IDT electrode design has shown higher CI values ranging from 0.08 to 0.5 CI compared to circular electrode ranging from 0.01 to 0.3 CI suggest a higher accuracy representation of cellular activities thus can be concluded as a better sensitivity of both design.

Cytotoxicity Evaluation of NP, Taxol and DMSO

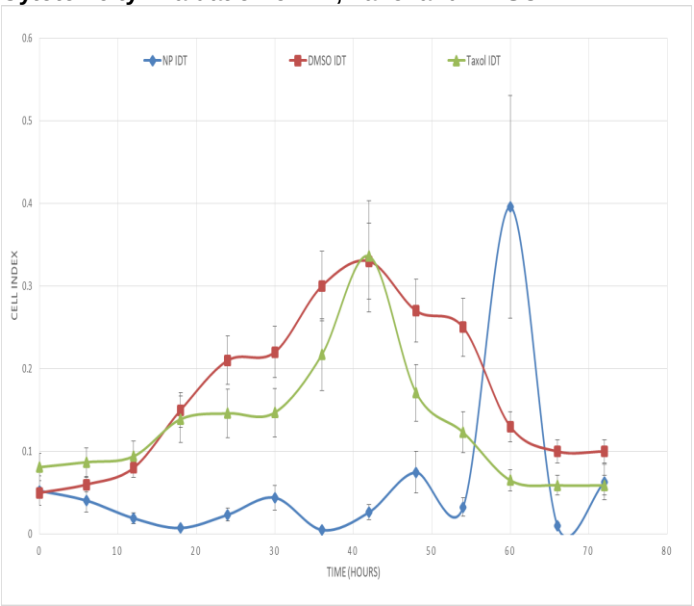

Fig. 4 Cytotoxicity of NP, Taxol and DMSO on vero cells using IDT electrode sensor.

Cytotoxic evaluation of NP, Taxol and DMSO were performed on IDT electrode design sensor as it has better sensitivities compared to the circular design. From the results in Fig. 4, both Taxol and DMSO showed a similar trend indicating that Taxol did not cause toxicity to cells at concentration tested. Meanwhile, NP showed slower growth between 0-60 hours and the maximum CI was achieved at 60 hour. 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.

ACKNOWLEDGEMENT

This work was financially supported by the International Islamic University Malaysia and Malaysia Ministry of Science Technology and Innovation Malaysia (SF16-004-0073). The Vero cells stock was kindly given by Assoc. Prof. Dr. Raha Ahmad Raus, Department of Biotechnology Engineering, IIUM.

REFERENCES

Anh-Nguyen, T., Tiberius, B., Pliquett, U., and Urban, G. A. (2016). An impedance biosensor for monitoring cancer cell attachment, spreading and drug-induced apoptosis. *Sensors and Actuators A: Physical*, *241*, 231-237.

Bhanot, A., Sharma, R., and Noolvi, M. N. (2011). Natural sources as potential anti-cancer agents: A review. *International journal of phytomedicine*, *3*(1), 09.

Boyd, J. M., Huang, L., Xie, L., Moe, B., Gabos, S., and Li, X.-F. (2008). A cell-microelectronic sensing technique for profiling cytotoxicity of chemicals. *Analytica chimica acta*, *615*(1), 80-87.

Campbell, C. E., Laane, M. M., Haugarvoll, E., and Giaever, I. (2007). Monitoring viral-induced cell death using electric cell–substrate impedance sensing. *Biosensors and Bioelectronics*, *23*(4), 536-542.

Cui, Y., An, Y., Jin, T., Zhang, F., and He, P. (2017). Real-time monitoring of skin wound healing on nano-grooves topography using electric cell-substrate impedance sensing (ECIS). *Sensors and Actuators B: Chemical*, *250*, 461-468.

Demain, A. L. (2014). Importance of microbial natural products and the need to revitalize their discovery. *Journal of industrial microbiology & biotechnology*, *41*(2), 185-201.

Giddings, L.-A., and Newman, D. J. (2013). Microbial natural products: molecular blueprints for antitumor drugs. *Journal of industrial microbiology & biotechnology*, *40*(11), 1181-1210.

Hashim, Y. Z. H.-Y., Kerr, P. G., Abbas, P., and Salleh, H. M. (2016). Aquilaria spp.(agarwood) as source of health beneficial compounds: A review of traditional use, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, *189*, 331-360.

Hashim, Y. Z. H.-Y., Phirdaous, A., and Azura, A. (2014). Screening of anticancer activity from agarwood essential oil. *Pharmacognosy research*, *6*(3), 191.

Katz, L., and Baltz, R. H. (2016). Natural product discovery: past, present, and future. *Journal of industrial microbiology & biotechnology*, *43*(2-3), 155-176.

Lundstrom, K. (2017). Cell-impedance-based label-free technology for the identification of new drugs. *Expert Opinion on Drug Discovery*, *12*(4), 335-343.

Mansor, A., Ibrahim, I., Voiculescu, I., and Nordin, A. (2015a). *Screen printed impedance biosensor for cytotoxicity studies of lung carcinoma cells.* Paper presented at the International Conference for Innovation in Biomedical Engineering and Life Sciences, 122-126.

Mansor, A. F. M., Nordin, A. N., and Ibrahim, I. (2015b). *Cytotoxicity studies of lung cancer cells using impedance biosensor.* Paper presented at the Smart Sensors and Application (ICSSA), 2015 International Conference on, 1-6.

eISBN 978-967-0194-93-6 FBME

Newman, D. J., and Cragg, G. M. (2012). Natural products as sources of new drugs over the 30 years from 1981 to 2010. *Journal of natural products*, *75*(3), 311-335.

Tan, L., and Schirmer, K. (2017). Cell culture-based biosensing techniques for detecting toxicity in water. *Current Opinion in Biotechnology*, *45*, 59-68.

Tran, T. B., Baek, C., and Min, J. (2016). Electric Cell-Substrate Impedance Sensing (ECIS) with Microelectrode Arrays for Investigation of Cancer Cell–Fibroblasts Interaction. *PloS one*, *11*(4), e0153813.

eISBN 978-967-0194-93-6 FBME