Sensors & Transducers, Vol. 187, Issue 4, April 2015, pp. 113-119

Sensors & Transducers

http://www.sensorsportal.com

Characterization of Platinum Electrodes and In-situ Cell Confluency Measurement Based on Current Changes of Cell-Electrodes

[1, *]Chin Fhong SOON, [2] Mee Kei FOONG, [1] Mohd Khairul AHMAD, [2] Rosliza MD ZIN, [2] Kok Tung THONG, [2] Kian Sek TEE

[1] Biosensor and Bioengineering Laboratory, MiNT-SRC, Universiti Tun Hussein Onn Malaysia, 86400 Parit Raja, Johor, Malaysia

[2] Faculty of Electrical and Electronic Engineering, Universiti Tun Hussein Onn Malaysia, 86400 Parit Raja, Batu Pahat, Johor, Malaysia

[*] Tel.: +607-4538614, fax: +607-4536060

[*] E-mail: soon@gmail.com

Received: 16 March 2015 /Accepted: 27 March 2015 /Published: 30 April 2015

Abstract: This study aimed at the development of a biosensor to examine the growth confluency of human derived keratinocytes (HaCaT) cell lines in-situ. The biosensor consists of a sputter-coated glass substrate with platinum patterns. Cells were grown on the conductive substrates and the confluency of the cells were monitored in-situ based on the conductivity changes of the substrates. Characterization of the cell proliferation and confluency were interrogated using electrical cell-substrate impedance sensing (ECIS) techniques and current change of cells using a pico-ammeter. The investigation was followed by the electrical characterization of the platinum electrode (PE) using a two probe I-V measurement system. The surface morphology of platinum electrodes were studied using an atomic force microscopy (AFM) and the HaCaT cell morphology was studied using Field-Emission Scanning Electron Microscopy (FE-SEM). The microscopy results showed that the cells coupled and proliferated on the platinum electrodes. For monitoring the conductivity and impedance changes of the cell-electrode in-situ, the cover of a Petri dish was inserted with pogo pins to be in contact with the platinum electrodes. The impedance was sampled using the ECIS technique at a twenty-four hour interval. In our findings, the cell proliferation rate can be measured by observing the changes in capacitance or impedance measured at low ac frequencies ranged from 10 - 1 kHz. In good agreement, the current measured at micro-ampere range by the biosensor decreased as the cell coverage area increased over the time. Thus, the percent of cell confluence was shown inversely proportional to the current changes.

Keywords: Keratinocytes, Electric Cell-Substrate Impedance Sensor (ECIS), Cell confluency.

1. Introduction

Cell confluency is referred to the density of cells or percentage of cell covered area in a culture vessel. The measurement of cell confluency is used to determine the growth phase of cells and suitable time for sub-culturing of cells. In routine microscopy for cell biology, cell confluency is done qualitatively

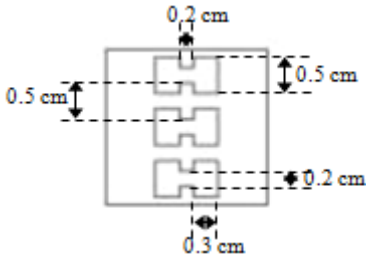
and estimated by approximation of the covered area by the monolayer of cells. Such a measurement technique is inaccurate and introducing high error rate. Hence, microscopy technique has been developed to quantitatively determining the confluency cells. However, the images of cells in a small area obtained from microscopy technique may not be representative for monitoring the confluency of cells and hence, multiple images of cell covered area need to be captured [1-2]. This technique requires further image processing for determining the percentage of cell confluency.

In-situ based measurement methods can provide continuous measurements of living cells, thus it can be efficient in sensing various cellular status. Among the cell-based biosensing, electrical cell-substrate impedance sensing (ECIS) technique is a simple and effective method for computable and live monitoring of cell attachment and spreading activities of cells on a substratum [2]. ECIS biosensor is also capable of sensing and measuring surface-coupling signal of the cell. The morphology of the cells influenced the changes of the impedance measurement [3]. From the previous work that had done by other researchers [3-4], the ECIS technique was used to monitor the cell proliferation, cell adhesion and cell confluency on cell-electrodes. However, characterization of the percentage of cell confluency on a cell-electrodes based on the current changes of the cell-substrate are lacking in previous work [5]. In this study, platinum electrode was chosen due to the attractive physical properties such as high melting point, high density, high electrical resistivity and slightly low electronegativity [6-7]. In this study, the fabrication and characterization of the cell confluency measurement electrode were discussed. In addition, the application in monitoring the cell confluency based on measuring the current change over the cell-substrate was presented.

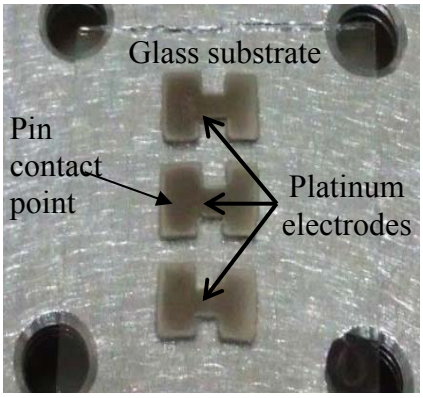
2. Materials and Methods

2.1. Fabrication of Electrodes

The design of electrode was produced using an AutoCAD software and printed on a piece of adhesive paper. The electrode is in an I-shaped pattern with a length and width of 0.8 cm and 0.5 cm, respectively (Fig. 1a). The patterned design was cut and placed on the aluminum sheet to produce a patterned mask. Subsequently, the aluminum mask was then used to cover the glass substrate and the substrate was placed in an autofine coater chamber (JEOL, JFC-1600). In the sputter coater, a current of 40 mA at 125 seconds were set to sputter a thin layer of platinum film functioning as an electrode. There were three electrodes designed on the glass substrate which allowed independent measurements in the same culture (Fig. 1b).



(a)



(b)

Fig. 1. (a) Dimensions of the platinum electrode, and (b) Platinum electrodes on glass substrate.

2.2. Characterization of the Physical and Electrical Properties of Platinum Electrodes

The thickness of the platinum films was determined using an Alpha-Step IQ surface profiler (KLA Tencor Corporation) with a scan length of 1000 μm and scan speed of 50 μm/sec. The thickness measurement of the platinum film was analyzed using the Alpha IQ software installed in a computer and linked to the surface profiler. Subsequently, the electrical conductivity (I-V) characterization was measured using a two-points probe station consists of Keithley's voltmeter (Keithley Instrument, Inc.) linked to an ORIEL Instrument software. In the two-points probe station, the measurement of the resistance and the I-V curve were obtained. The sharp tips of the probes were placed in contact with silver contacts deposited on the terminal ends of the platinum electrodes to avoid damage to the electrodes.

2.3. Surface Morphology Investigation Using Atomic Force Microscopy

An atomic force microscopy (AFM, Park System XE-100) was used to image the topography of the platinum film to investigate the homogeneity of the sputtered coating. The platinum film's magnetic properties were also investigated using the magnetic force microscopy scanning. The platinum films

were scanned in a non-contact mode at a scan rate of 0.8 Hz at an area of 1 μm^2. The roughness of the platinum electrode was analyzed in the XEP software.

2.4. Cell Culture on the Electrodes and FE-SEM Imaging

The human keratinocyte cell lines (HaCaT) were maintained in a 25 cm^2 culture flask containing culture media. Upon reaching confluency, the media were removed from the cell culture flask and the flask was washed three times with Hank's Balanced Salt Solution (HBSS, Sigma Aldrich, UK). After discarding the HBSS solution, 1 ml of trypsin was deposited into the flask and the flask was incubated in a humidified atmosphere containing 5 % carbon dioxide at 37 °C for 5 minutes. Subsequently, the flask was examined in a microscope (Nikon TS-100) to ensure that all the cells were detached from the surface of the culture flask. Dulbecco's modified Eagle's medium (DMEM) medium of 5 mL was then deposited to stop the trypsinization process. The DMEM media contained supplements 10 % Fetal Bovine Serum (Biowest, France), L-Glutamine (2 mM, Sigma-Aldrich, UK), Penicillin (100 units/mL, Sigma-Aldrich, UK), Streptomycin (100 mg/mL, Sigma-Aldrich, UK), Fungizone (2.5 mg/L, Sigma-Aldrich, UK). Next, the cell suspensions were transferred to a centrifuge tube and centrifuged for 5 minutes at 1500 rpm. When this process was completed, the media were discarded and cell was re-suspended in 6 ml of DMEM media. Lastly, the suspensions of cells was deposited into a modified Petri dish and placed inside the incubator. The modified Petri dish has a lid inserted with three pairs of pogo pins with positions corresponding to the terminal ends of platinum electrodes. The temperature of incubator was kept at 37 °C with 5 % carbon dioxide. The proliferation of cells was also monitored at an interval of 24 hours in a phase contrast microscope.

For the morphological study of cells in a field emission-scanning electron microscopy (FE-SEM, JEOL 7600), cells adhered to the platinum electrode was fixed and dehydrated. After rinsing the platinum electrode with HBSS Solution three times, the cells were fixed with 1 % formaldehyde for 6 minutes. Cells were dehydrated in a serial dilution of ethanol at different concentrations of 25 %, 50 %, 75 % and 90 % for 5 minutes each and allowed to air dried. Subsequently, the cells were sputtered coated using a palladium target and the sample substrates were mounted to the stub using double sided carbon tape. Then, the samples were placed in the vacuum chamber in FE-SEM ready for imaging using the secondary electron image (SEI) mode. The accelerating voltage was set at 7 kV with magnifications of 300× and 1000× for the analysis of keratinocytes cell morphology on the platinum electrode.

2.5. Cell-substrate Impedance Measurement

Fig. 2 shows a schematic diagram of experimental setup for cell-electrode impedance investigation. The impedance analyzer (Solartron Electrochemical Test System 1260A) was connected to a potentiostat (Solartron Electrochemical Test System 1280C) and the computer. The Solartron Impedance/ Gain-phase Analyzer support a huge frequency sweep range from 10 μHz to 32 MHz with an accuracy of 0.1 %. The impedance measurements of the cell-electrode were taken every 24 hours over a wide frequency range from 10 Hz to 100 kHz. The cell impedance was analyzed using a Zplot® software bundled to the Impedance/Gain-phase Analyzer (Solartron 1260A).

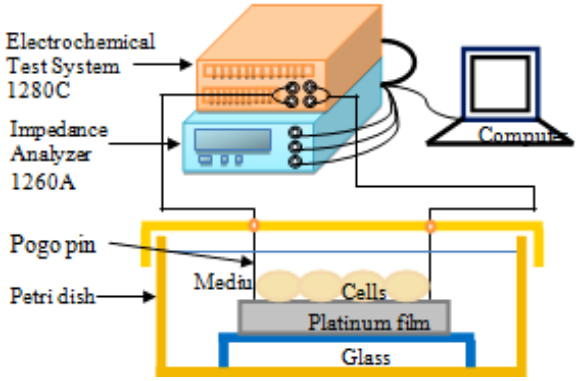


Fig. 2. Experimental setup for cell impedance investigation.

2.6. Cell Confluency Characterization Using Pico-Ammeter

The cell confluency was characterized based on the current changes of the cell in a setup as shown in Fig. 3. The current flow within the cells cultured on the platinum electrodes were analyzed by the PocketPico Reader™ software linked to a PocketPico pico-ammeter (PocketPico P110 1XPP02B).

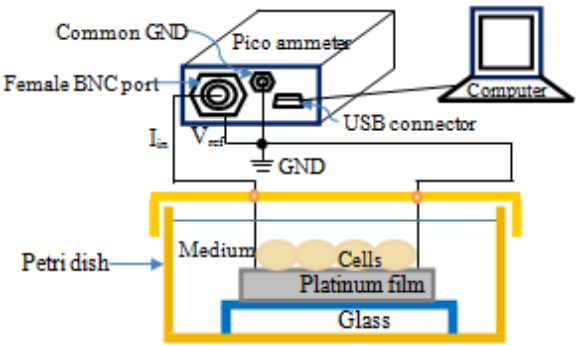


Fig. 3. Experimental setup for cell confluency measurement.

The PocketPico pico-ammeter capable of measuring a very low electrical current range from

20 pA to 2 mA with an accuracy of ±0.5 %. Electrical current of cells were taken every 24 hour interval through the BNC port as analog input. A female BNC-crocodile clip cable was used as the connector between the Pico-ammeter and pogo pins attached to the Petri dish. The current changes of the cell-electrode were measured using the pico-ammeter.

5. Results and Discussions

Fig. 4 shows the result of a line profile for the platinum electrode coated on the glass substrate. The thickness of the platinum film at approximately 1.38 µm was obtained by determining the difference between two distinct level of the platinum film and glass.

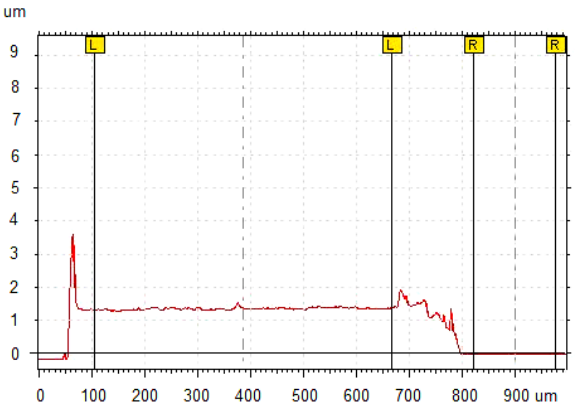


Fig. 4. Surface profile of the platinum film coated on glass substrate.

The results of the linear I-V graph of the platinum cell-electrodes show that the electrical resistance of the platinum electrode is 278.43 ± 6.80 Ω (mean ± SD, N=50). The standard deviation of the resistance is within an 5 % of tolerance. The resistivity of the platinum films (ρ) at 0.21 Ω-mm was calculated using the following formula:

$$\rho = R\frac{A}{L}, \qquad (1)$$

where A is the diameter of the silver contact point at 1 mm multiply by the thickness (L) of the platinum film at 1.5 µm. The conductivity of the platinum film was determined at 4.76 S/mm.

Fig. 5(a) presents the 3D view of surface morphology of the platinum cell-electrode at 1 µm^2 with a roughness, R_a of 1.82 nm. The white spots in Fig. 5(a) represent the high region of the platinum film's surface and the dark spots represent the low region of the surface. During the sputtering process, the platinum film was not coated directly onto the glass substrate but sputter-coated onto the glass substrate. Hence, the result demonstrated that the platinum electrode have a rough surface at nano-metric scale. The roughness is considerably low.

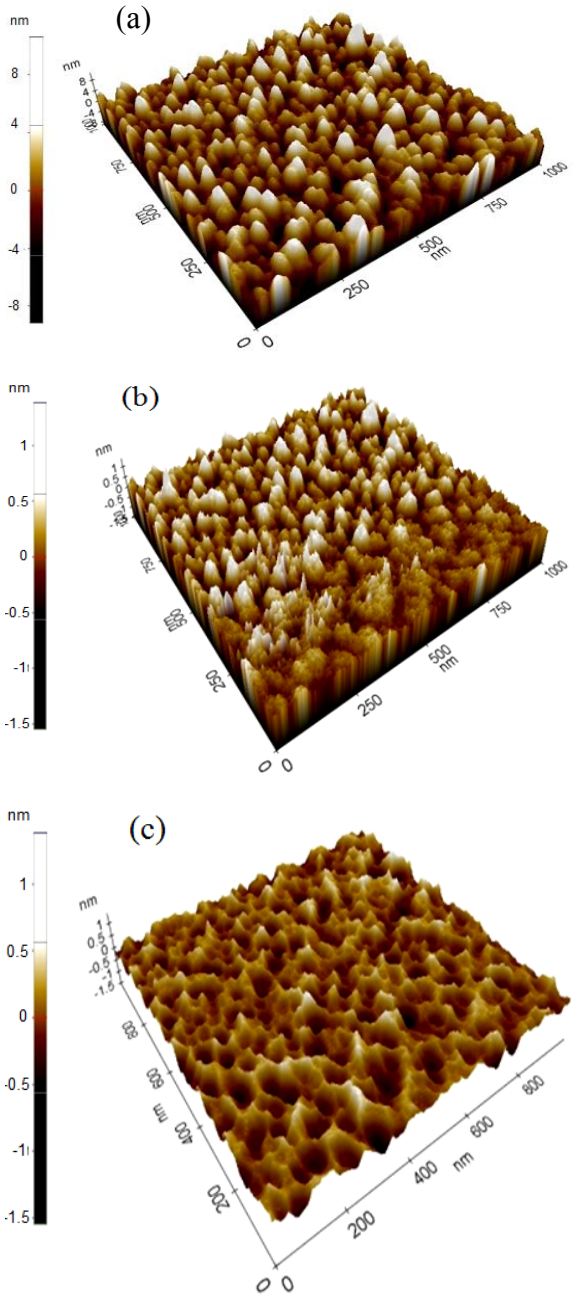
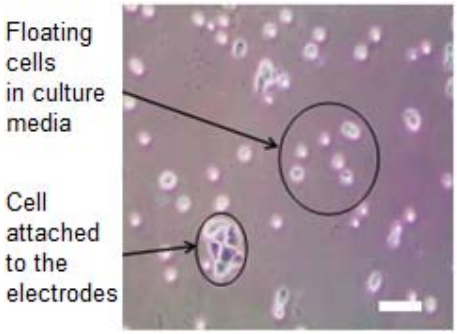


Fig. 5. (a) Surface topography with roughness of R_a=1.82 nm, (b) magnetic surface topography at R_a=0.223 nm and the magnetic vortice of (c) R_a= 0.146 nm.

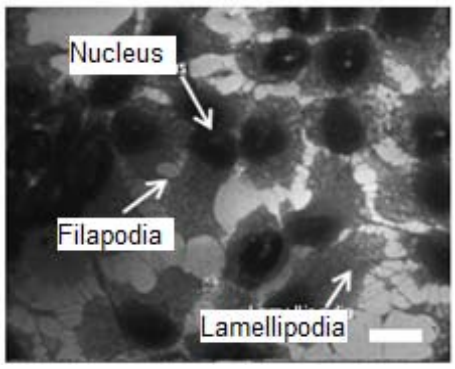
The magnetic force microscopy (MFM) of platinum is a technique used to determine the magnetism of the electrode, in which high magnetism of a substrate is believed to have influence to the proliferation of cells [8]. Fig. 5(b) shows the 3D view of magnetic structure of the platinum film with roughness, R_a equal to 0.223 nm. Fig. 5(c) shows the result of the surface morphology superimposed with

the morphology of the magnetic surface. The difference of roughness of 0.146 nm was obtained. The large dark holes shown in Fig. 5(c) represents the magnetic vortices on surface of platinum cell-electrode. Thus, the result indicates that the platinum film has a high coercivity, but low strength of magnetic-field [8]. The magnetism of the platinum needs to be investigated because the surface roughness and magnetic field can influence cell adhesion and cell proliferation. The results indicates that the surface and magnetism properties of the platinum films presented minimum magnetic field that may not affect cell adhesion and cell proliferation.

In order to study the cell attachment and cell growth on the platinum cell-electrode, the biocompatibility of platinum cell-electrode was analyzed. The cells were deposited to the platinum cell-electrode in a Petri dish and kept in the 37 ºC CO_2 incubator for optimal growth. Fig. 6(a) shows the attachment of cells after 24 hours in phase contrast images. The cells continued to grow and proliferate on the platinum electrodes after 7 days of culture (Fig. 6). From the FE-SEM microscopy image, it can be shown that the human derived keratinocytes (HaCaT) cell lines show affinity to the platinum cell-electrodes and platinum is suitable to be used as cell-electrode material in which platinum does not have any harm to the cells [9-10].



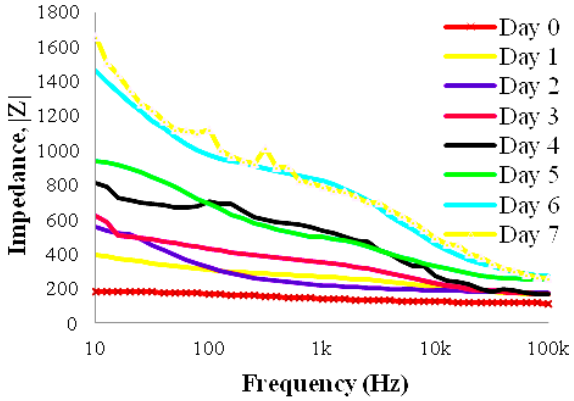
(a)



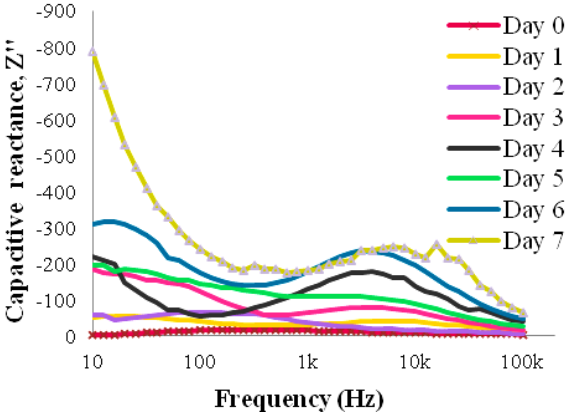
(b)

Fig. 6. (a) Phase contrast microscopy of HaCaT cells after 24 hours of culture on the glass and (b) FE-SEM micrograph of cells on the platinum electrode after 7 days of culture. (Scale bar: 10 µm).

Fig. 7(a) shows the bode plot of magnitude impedance over a wide range of ac frequency from 10-100 kHz. From the plot, it was observed that the impedance magnitude decreased with an increase in frequency. At low frequency range, the impedance increased as period of culture increased. This is due to the increase of cell number and an enlarged cell covered area on the electrode leading to the impedance to increase. However, the impedance presented not much differences over different culture period at high frequency range. Our findings suggest to measure the impedance of cell at low frequencies ranging from 10 – 1 kHz.



(a)



(b)

Fig. 7. The bode plot of the (a) magnitude impedance, |Z| and (b) Capacitive reactance, X_c over different frequencies.

Fig. 7(b) shows the capacitive reactance, X_c over different frequency ranged from 10 - 100 kHz. The plot demonstrates that the capacitive reactance decreased with an increase in frequency at the range of 10 – 1 kHz. The longer the cell incubation time, the higher is the capacitive reactance (Fig. 7b). At higher ac frequency, the capacitive reactance of cell-electrode was relatively small and the cell-electrode behaved like a capacitor. Thus, the low capacitive cell membrane was permeable to current flows directly through the cell. At low ac frequency, the cell turned more capacitive and the impedance increased due the

insulating cell membrane. The high insulating cell was impermeable to current flows. Therefore, the current may flow underneath cells and route crossing intercellular spaces through a junction resistance. These impedance changes can be related to the cell proliferation rates as indicated in Fig. 7b [4, 11]. The slight increase of capacitive reactance at higher range of frequency from 1 kHz to 10 kHz may be due to the leakage membrane capacitance during ions active transport in cell membrane and cell mobility [4, 5, 11]. At 100 kHz and beyond, the cell-electrode behaved like a short circuit. In our findings, the cell proliferation rate can be measured by observing the changes in capacitance or impedance measured at low ac frequencies.

In addition to the impedance measurement using the bulky ECIS instrumentation, this work extended to determine the current change associated with the cell confluency on platinum electrode using a portable pico-ammeter. Fig. 8 shows the current measured for platinum electrode cultured with cells over a period of 168 hours expressed in mean ± SD. The current of the cell-electrode decreased non-linearly with the prolonged incubation time. The current were then correlated with the cell confluency measurement as shown in Fig. 9. The insets of the phase contrast micrographs in Fig. 9 indicate the cells grown at different confluency. In comparison, the cell covered area was determined by capturing multiple images of cells and the percentage of covered area was computed using ImageJ software. With an increase in cell confluency, it can be observed that the current decreased gradually over the time (Fig. 9). This is due to the increase in cell-electrode impedance with respect to time as revealed by the result presented in Fig. 7. The enlarged covered area of cell monolayer formed the resistance to the current flew through the electrode and thus, limit the current flew. The log currents of 1.25 nA and 4 nA measured corresponded to the 80 % and 5 % of cell confluency, respectively.

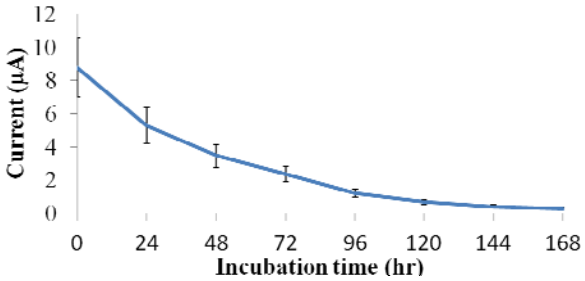


Fig. 8. The relationship of cell-electrode current and incubation time.

7. Conclusions

The platinum electrodes were fabricated using sputter coating technique and presented small surface roughness of 1.82 nm. The AFM results revealed that the platinum electrode is lowly magnetizable and hence, this has little influence to the cell adhesion. In addition, cell affinity to the electrode as revealed by FE-SEM indicated that the platinum electrodes are suitable to be used for cell culture and measurement. The capacitive reactance and impedance of the cell-electrode clearly increased with the cell proliferation on the platinum electrode at low frequency range (10 – 1 kHz), suggesting that the current measurement for cell confluency can be determined. An in-situ biosensor used to characterize the percentage of cell confluency was successfully developed based on the current change of platinum electrodes grown with the presence of cells.

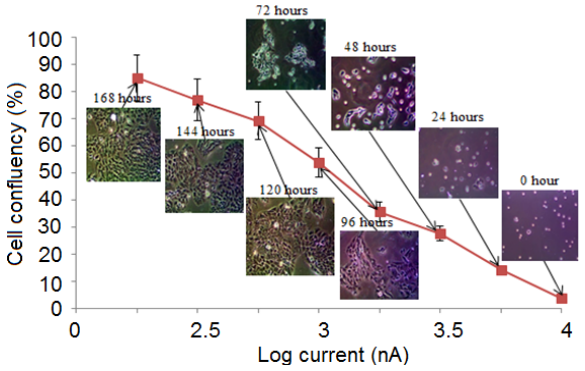


Fig. 9. Percentage of cell confluency over the current change of cell-electrode.

Acknowledgements

The authors are grateful to the research financial support (Science Fund Vot No. 0201-01-13-SF0104 and FRGS grant Vot No. 1482) awarded by Malaysia Ministry of Science and Technology (MOSTI) and Malaysia.

References

[1]. S. Busschots, *et al.*, Non-invasive and non-destructive measurements of confluence in cultured adherent cell lines, *MethodsX*, Vol. 2, 2015, pp. 8-13.

[2]. G. Topman, *et al.*, A Method for Quick, Low-Cost Automated Confluency Measurements, *Microscopy and Microanalysis*, Vol. 17, 2011, pp. 915-922.

[3]. A. N. Nordin, *et al.*, Printed Circuit Board Cultureware for Analysis of Colorectal Carcinoma Cells using Impedance Spectroscopy, in *Proceedings of the IEEE EMBS Conference on Biomedical Engineering and Sciences (IECBES)*, 2012, pp. 574-578.

[4]. D. Das, *et al.*, Electrical Characterization of Suspended HeLa Cells using ECIS based Biosensor, in *Proceedings of the 6th International Conference on Sensing Technology Kolkata*, 2012, pp. 734-737.

[5]. C.-M. Lo, *et al.*, Impedance Analysis of MDCK Cells Measured by Electric Cell-Substrate Impedance Sensing, *Biophysical Journal*, Vol. 69, 1995, pp. 2800-2807.

[6]. J. Martin, Effects of relativistic motion of electrons on the chemistry of gold and platinum, *Solid State Sciences*, Vol. 7, 2005, pp. 1464-1474.
[7]. M. Schuettler, Electrochemical properties of platinum electrodes in vitro: comparison of six different surface qualities, in *Proceedings of the Conference of the IEEE EMBS, Cité Internationale*, Lyon, France, 2007.
[8]. P. Glijer, *et al*., Magnetic force microscopy (MFM) studies of micromagnetic structures of high coercivity CoCrPt/Cr and CoCrPtB/Cr thin filmsd CoCrPtB/Cr thin films, *IEEE Transactions on Magnetics*, Vol. 31, 1995, pp. 2842-2844.
[9]. N. A. Kolpakova, *et al*., Platinum Test in Biological Objects, in *Proceedings of the VI International Scientific and Practical Conference of Students, Post-graduates and Young Scientists Modern Techniques and Technology (MTT'2000)*, Tomsk, 2000, pp. 205-207.
[10]. W. Franks, *et al*., Impedance Characterization and Modeling of Electrodes for Biomedical Applications, *IEEE Transactions on Biomedical Engineering*, Vol. 52, 2005, pp. 1295-1302.
[11]. H. R. Siddiquei, *et al*., Electrical Cell-substrate Impedance Sensing (ECIS) based Biosensor for Characterization of DF-1 Cells, in *Proceedings of the International Conference of the IEEE Engineering on Computer and Communication Engineering (ICCCE)*, 2010, pp. 1-4.