Int. J. Electrochem. Sci., 7 **(2012)** 8848 - 8858

International Journal of
ELECTROCHEMICAL
SCIENCE
www.electrochemsci.org

Quantification of Cell Number in 3-Dimensional Cell Culture Construct by Impedance Measurement using Microfluidic Technology

Kin Fong Lei[1, 2, *, #], *Min-Hsien Wu*[3, #], *Che-Wei Hsu*[1], *Cheng-Yuan Lin*[3]

[1] Graduate Institute of Medical Mechatronics, [2]Department of Mechanical Engineering,
[3] Graduate Institute of Biochemical and Biomedical Engineering,
Chang Gung University, Taoyuan, Taiwan, R.O.C.
[*]E-mail: kflei@mail.cgu.edu.tw
[#]These authors contribuate equaly to the work.

Received: 25 July 2012 / *Accepted:* 17 August 2012 / *Published:* 1 September 2012

This paper presents a technique to determine cell number in 3D cell culture construct quantitatively. A measurement platform embedded with on-chip vertical electrodes has been developed and is capable of onsite impedimetric detecting cell number without sacrificing the cultured cells. *In vitro* 3D cell culture has been interpreted for faithfully representation of the *in vivo* cellular behavior in living tissues. However, monitoring of the cell number in 3D cell culture construct is normally based on the detection of the indicative cellular components, e.g., DNA assay, which requires sacrificing the cultured cells and hampers the subsequent cellular assays. In this study, the total impedance of the cells/agarose construct can be measured across the vertical electrodes and the cell number in the construct can be determined. The impedance amplitude of the construct was proportional to the cell number in the construct. Breast cancer cells in cell numbers of 10^4, 10^5, and 10^6 cells/ml were estimated using the proposed method. The proposed technique provides a fast and easy method compared with the conventional cell analysis techniques. It has a high potential to be integrated into a microfluidic chip for on-chip cell analysis during 3D cell culture process.

Keywords: Microfluidics; Electrical impedance; Cell culture; Cell number.

1. INTRODUCTION

Animal cell cultures have been widely used as *in vitro* cell-based models for the study of the physiology and biochemistry of cells [1], or of the cellular response to the tested substance such as drug [2], toxin [3], or biomaterials [4]. Cell culture is an operation of culturing cells outside the biological tissue from which they are harvested. Conventionally, cells are cultured as a monolayer on a surface of a cell culture vessel (e.g. Petri dish or multi-well microplate), which is also referred to as

two-dimensional (2D) cell culture. The 2D cell culture is widely used because of its simplicity in terms of operations and observations of cellular behavior [5]. Because cells inhabit environments with very specific three-dimensional (3D) features in animal tissue [6], however, the conventional 2D cell culture may not well mimic the native cellular microenvironments [7]. In order to faithfully explore the *in vivo* cellular behaviors *in vitro*, cells are encapsulated in a 3D polymeric scaffold material for the culture. The 3D culture scheme is more representative of the behavior of cells in living tissues and thus, provides a more physiologically-meaningful culture condition for cell-based assays [6, 8]. In 3D cell culture practice, the cellular responses such as cell metabolic activity, cell number, and cell viability are commonly investigated to study the cellular activities under various culture conditions explored. Among them, the quantification of cell number in a 3D cell culture can provide us with the information about cell proliferation. The commonly used analytical techniques for the measurement of cell number are based on indirect detection of some indicative metabolites synthesized by the cultured cells [1, 9, 10], or direct detection of cellular quantities [2]. The latter method can count the cell number microscopically, or measure the turbidity of the cell suspension optically. Nevertheless, these operations are normally labor-intensive and time-consuming. More importantly, they may not be practical in the 3D cell cultures because the cells in such culture model are virtually encapsulated in a 3D scaffold, by which the cultured cells are difficult to observe microscopically. Therefore, in 3D cell culture model, the cell number is normally quantified by the measurement of cellular components such as DNA [1]. In this case, the cultured cells need to be lysed in order to release the DNA molecules. As a result, this technique could hamper the use of the cultured cells for further cellular assays. Moreover, the measurement of cell number based on the DNA assay is also a time-consuming task. In order to determine the cell number in a 3D cell culture construct, a both effective and efficient detection scheme is crucial in need.

In the past decade, electrical impedance measurement has been proposed to quantify the biological substances (e.g. cells) [11]. The rationale behind is to utilize a pair of electrodes as an electrical transducer to detect the impedance change across the electrodes, causing by the existence of biological substance. Based on this, detection of a wide variety of biomolecules such as enzymes [12], antibodies and antigens [13-15], DNA [16], and cells [17-19] has been successfully demonstrated. For example, the detection of *Legionella* bacteria was demonstrated using a pair of indium tin oxide (ITO) interdigitated microelectrodes on glass substrate [19]. Analyte-specific antibody, i.e., anti-*Legionella* IgG, was first immobilized on the electrode surface for the capture of bacteria specifically. Then, bacterial concentration could be determined by the impedance change across the electrodes. Also, the detection of *Escherichia coli* O157:H7 has been realized using the similar method [20-22]. With the recent advance in microfluidic technology, moreover, microfluidic biochip for the detection and identification of bacteria was proposed [23]. The bacteria suspended solution was injected to the microfluidic chamber and cell concentration in the solution can be determined by the impedance change of the chamber. Furthermore, cellular behaviors, e.g., number, growth, and morphology, have also been successfully detected through the impedance change across the interdigitated electrodes [24, 25]. Although reports in literature have demonstrated the use of impedance measurement for cell number detection, most of these studies were for the quantification of cell number in 2D cell culture

format. To the best of our knowledge, utilization of electrical impedance measurement for quantifying cell number in 3D cell culture construct has not yet been well explored.

In this work, a measurement platform is proposed for the use of determining cell number in 3D cell culture construct. The measurement platform mainly consists of a culture chamber embedded with a pair of vertical electrodes located at its opposite sidewalls. In this study, the impedance of the 3D culture construct tested was measured via the vertical electrodes. By referencing the measured impedance to the standards, the cell number in the construct can be determined. In this paper, the design and fabrication of the measurement platform is first described. Equivalent circuit diagram of the 3D cell culture construct is then discussed. Finally, electrical characterization of the 3D construct with and without cells encapsulated was performed to demonstrate the feasibility of using the proposed technique for the cell number detection in a 3D cell culture construct. As a whole, the proposed scheme provides an effective and efficient manner to detect the cell number in a 3D cell culture setting without sacrificing the cultured cells. The characteristic feature is found valuable for any high throughput 3D cell culture-based assays (e.g., drug or toxin testing). Moreover, the presented mechanism is also promising to be integrated in a microfluidic system for the on-chip cellular analysis.

2. EXPERIMENTAL METHODS

2.1 Design and fabrication of the measurement platform

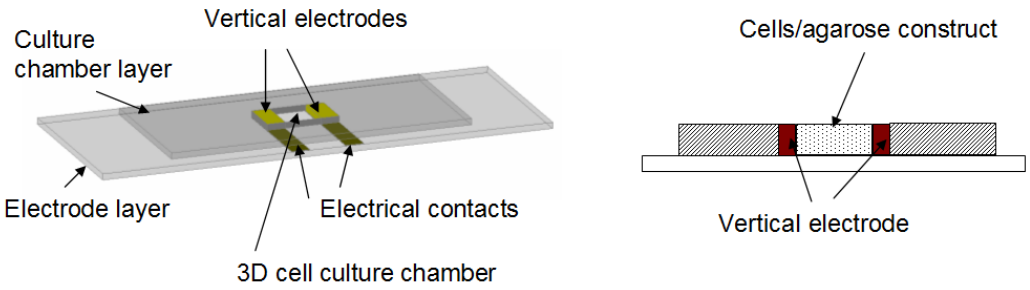

Figure 1. Illustration of the measurement platform. Vertical electrodes are located at the opposite sidewalls of the culture chamber and connected by electrical contacts for impedance measurement. Cells/agarose construct is loaded in the chamber for 3D cell culture.

The measurement platform with a culture chamber was designed. In the culture chamber, a pair of vertical electrodes is embedded at the opposite sidewalls. The measurement platform consists of two layers: a culture chamber layer and an electrode layer, as illustrated in Figure 1. The culture chamber layer is a layer of 1 mm polydimethylsiloxane (PDMS) material with a rectangular opening (7×2×1 mm^3) for accommodating the 3D cells/agarose construct and the vertical electrodes. The volume of culture chamber (5×2×1 mm^3) is designed to define the loading of 3D cells/agarose construct quantitatively. The electrode layer, a glass substrate, consists of a pair of vertical electrodes (1×2×1 mm^3) with their electrical contacts to the external measurement equipment. The vertical electrodes are located at the opposite sidewalls of the cell culture chamber. The electrical property of the 3D

cells/agarose construct can be measured by an impedance analyzer (HP 4192A, Hewlett-Packard) via the vertical electrodes. Parallel electric field generated by two opposite vertical electrodes penetrates through the 3D cells/agarose construct when performing the impedance measurement. The cell number in the construct causes the impedance change and can be determined. This method provides a fast and easy measurement without sacrificing the cultured cells.

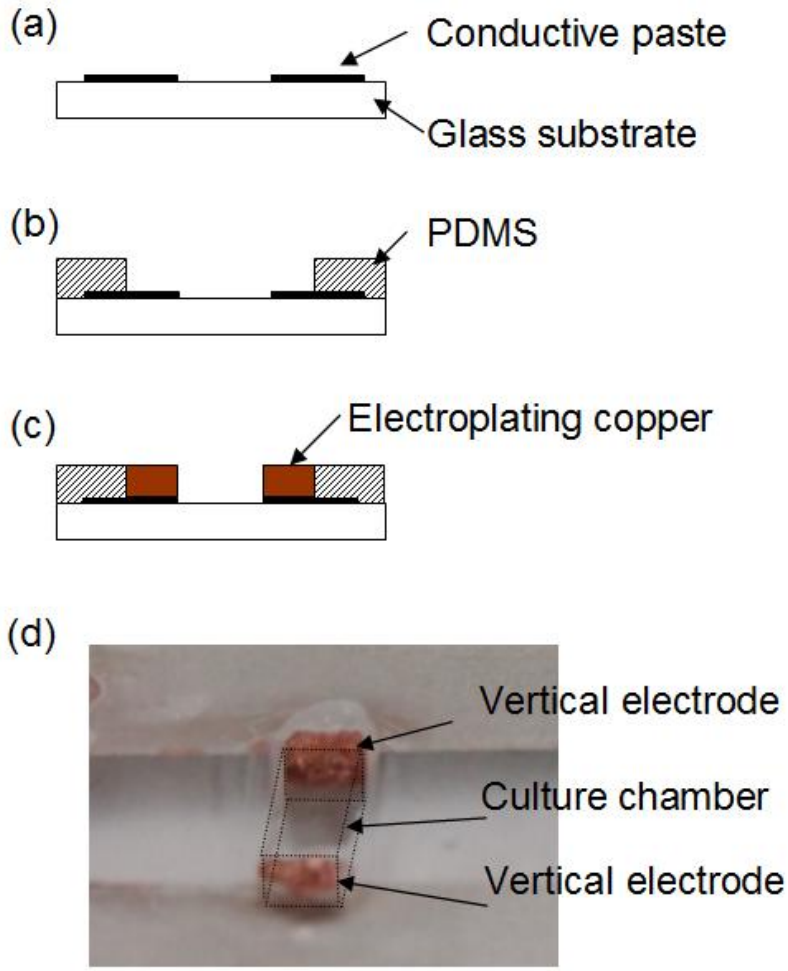

Figure 2. Fabrication process of the measurement platform. (a) Definition of the seed conductive layer on a glass substrate. (b) Bonding of the PDMS culture chamber layer with rectangular opening. (c) Copper electroplating to fabricate a pair of vertical electrodes located at opposite sidewalls. (d) Photo of the on-chip vertical electrodes located at opposite sidewalls of the culture chamber. The dash lines show the location of the culture chamber.

The measurement platform consists of a culture chamber layer and an electrode layer. The fabrication process is schematically illustrated in Figure 2. The culture chamber layer was a 1 mm thick PDMS (Sylgard® 184, Dow Corning, USA) layer with a rectangular opening and was fabricated by standard soft lithography. In brief, the PDMS layer was prepared by thoroughly mixing the PDMS pre-polymer and curing agent in a weight ratio of 10:1. Then, the mixture was degassed under a vacuum chamber, and followed by pouring onto the polymethylmethacrylate (PMMA) mold fabricated

by micro-machining technique. After curing at 100 °C for 1 hour, the PDMS layer was obtained by peeling off from the mold carefully. For the fabrication of the electrode layer, a pair of electrodes was first printed on a glass substrate by using conductive paste. The electrodes were used for the seed layer of electroplating process and the electrical contacts to the external measurement equipment. Then, the culture chamber layer was bonded to the glass substrate with appropriate alignment. Copper electroplating with the current density of 0.2 A/cm^2 was performed for around 16 hours. Copper was grown from the seed layer to the upper surface of the culture chamber. Therefore, a pair of vertical electrodes located at the opposite sidewalls of the culture chamber was fabricated. A photograph of the vertical electrodes located at opposite sidewalls of the culture chamber is shown in Figure 2(d).

2.2 Impedance characterization of the 3D cell culture construct

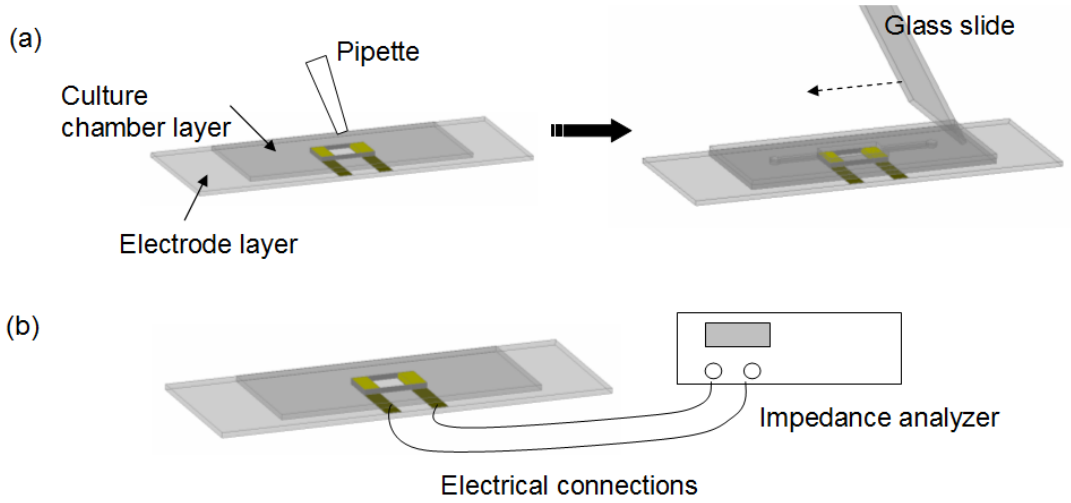

Figure 3. (a) The process of the loading of cells/agarose construct in the culture chamber. Cells/agarose suspension was applied to the chamber by manual pipetting. The redundant suspension was removed by spreading horizontally using a glass slide. (b) Illustration of electrical connection of the measurement platform and the impedance analyzer.

To investigate the impedance characterization of the 3D cell culture construct using the measurement platform, experiments were carried out and the process is shown in Figure 3. Before cell loading, the measurement platform was sterilized using 70% (w/v) ethanol for 30 minutes. Breast cancer cells (cell line: OECM1) were encapsulated in 2.0% (w/v) agarose gel (low-gelling temperature agarose, Sigma, Taiwan) to prepare the cells/agarose suspension. The suspension was then loaded into the culture chamber by manual pipetting. The redundant suspension was removed by spreading horizontally using a glass slide. By this process, the volume of the 3D cells/agarose construct can be quantitatively loaded to the culture chamber. Impedance of the construct could be quantitatively investigated by a fixed volume of such sample. Impedance measurement was conducted directly using the on-chip vertical electrodes. Potential of 0.1 V was applied and the impedance magnitude was

measured from 5 to 100 kHz with an HP 4192A Impedance Analyzer. The duration of data acquisition required around 30 seconds. Parallel electric field penetrated through the cells/agarose construct and the cell number in the construct could be estimated by the impedance change.

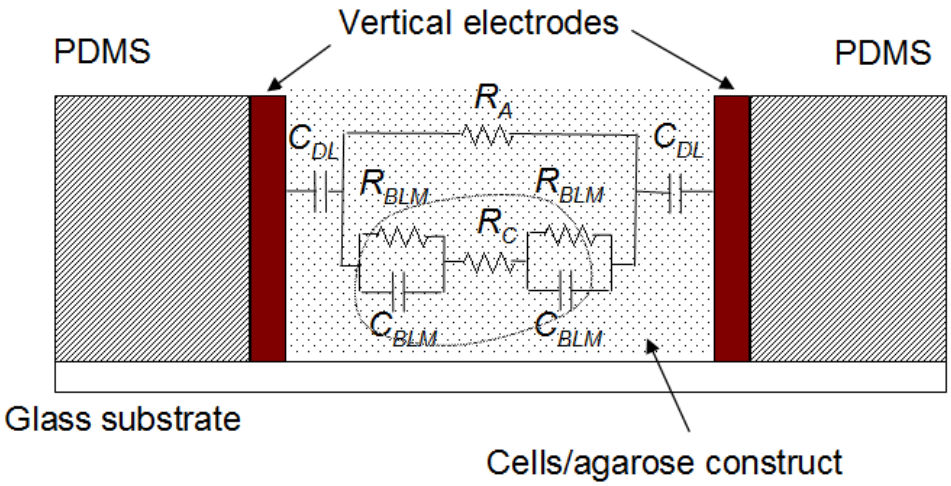

Figure 4. Equivalent circuit of the cells/agarose construct across the vertical electrodes. C_{DL}: double layer capacitance; R_A: resistance of agarose scaffold and culture medium; C_{BLM}: capacitance of the cell membrane; R_{BLM}: resistance of the cell membrane; R_C: resistance of the cell.

The equivalent circuit diagram of the cells/agarose construct across the vertical electrodes is proposed and shown in Figure 4. The circuit diagram is based on the model from [26]. In the figure, C_{DL} is the double layer capacitance between the electrode and the electrolyte, R_A is the resistance of agarose scaffold and culture medium, C_{BLM} is the capacitance of the cell membrane, R_{BLM} is the resistance of the cell membrane, and R_C is the resistance of the breast cancer cell. In 3D environment, cells are encapsulated in the agarose scaffold and uniformly distributed in the construct. In this circuit diagram, the electrical property of cells is represented by a unit of cell model, i.e., C_{BLM}, R_{BLM}, and R_C. Hence, the circuit diagram is then proposed to be interpreted as having two parallel branches. One branch represents the impedance of agarose scaffold and another branch represents the impedance of the cells. The total impedance of the 3D cell culture construct is represented by the combination of these branches according to the measuring frequency.

3. RESULTS AND DISCUSSION

3.1 Electrical characterization in salt solution

The agarose scaffold was first electrically characterized in NaCl electrolytes in various concentrations. Agarose gel without cells was loaded into the culture chamber based on the above described procedure in Section 2.2. NaCl electrolytes in the concentration of 10^{-2}, 10^{-1}, and 1 M, culture medium (DMEM with 1000 mg/l glucose, 25 mM HEPES, without sodium bicarbonate; pH 7.5), and DI water for the control measurement were applied respectively to the agarose scaffold.

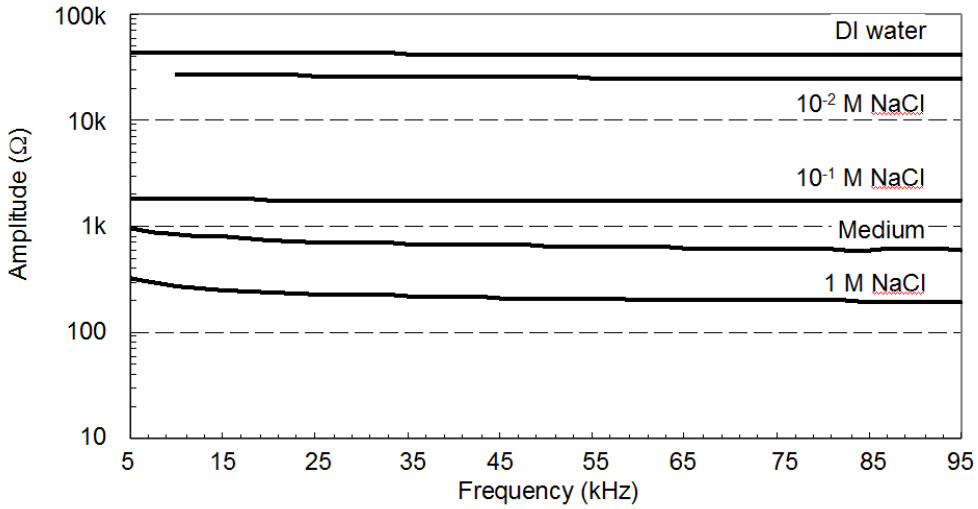

Figure 5. Impedimetric spectroscopy of the agarose scaffold across the vertical electrodes under NaCl electrolytes, culture medium, and DI water.

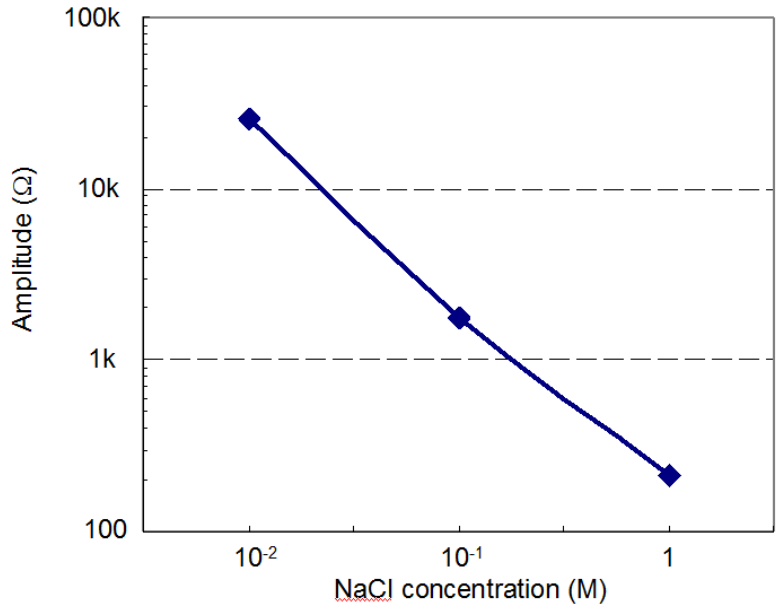

Figure 6. Total resistance of the agarose scaffold R_A soaked in NaCl electrolytes in the concentration from 10^{-2} to 1 M. R_A is from the stable impedance amplitude at the frequency range from 15 kHz to 100 kHz.

After the agarose scaffold fully absorbed the solution, the total impedance of the agarose scaffold across the vertical electrodes was measured at the potential of 0.1 V and the frequency range from 5 to 100 kHz. NaCl electrolytes, culture medium, and DI water were respectively measured under the same conditions. The electrical characterization of the agarose scaffold was investigated and the measurement results are given in Figure 5. By analyzing the impedance response of the salt solutions, the corresponding electrical components, i.e., the double layer capacitance C_{DL} and the total resistance of the agarose scaffold and the solution R_A, can be determined. At the frequencies below 15 kHz, the phenomenon of C_{DL} is shown from the impedance responses of the culture medium and 1 M NaCl

solution. At the frequencies above 15 kHz, the amplitudes show constant in all solutions. That indicates the conduction of ions in the solution determines the signal. The R_A is defined by the stable impedance amplitude response at the frequency range from 15 to 100 kHz. The correlation between R_A and the NaCl concentration from 10^{-2} to 1 M is shown in Figure 6. The result is in agreement with the previous works [27, 28], which indicate the impedance response was dominated by the conductivity of the solution in this frequency range. Considering the culture medium, impedance above 15 kHz was dominated by R_A, but impedance below 15 kHz was contributed by C_{DL} than R_A.

3.2 Impedance characterization of cell number in 3D cell culture construct

In order to conduct a precise and physiologically-meaningful cellular assay, micro-scale perfusion 3D cell culture was proposed to provide a well-defined and biologically-relevant culture environment [9, 10]. The miniaturized culture chamber can eliminate the chemical gradients existing in such a 3D culture construct. Moreover, the perfusion setting provides continuous nutrient supply and waste removal. That can enable a steadier extracellular environment for a precise cellular assay compared with the conventional static culture. However, to study the cellular responses, e.g., cell number and cell viability, quantifying the cellular components such as DNA is widely used. Sacrifice of cultured cells is necessary and thus hampers the subsequent cellular assays. Therefore, impedance measurement of the culture construct is proposed to determine the cell number in the 3D construct. The on-chip vertical electrodes are utilized for the measuring electrodes. Uniform electric field can be generated to penetrate through the cells/agarose construct. The total impedance change of the cells/agarose construct can be measured and represents the cell number in the construct.

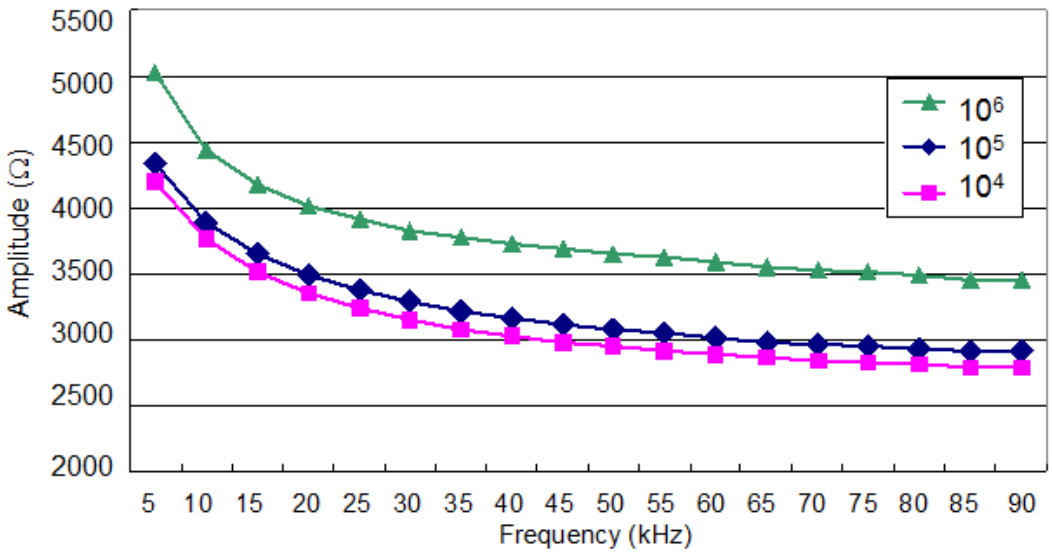

Figure 7. Impedimetric spectroscopy of breast cancer cells/agarose construct. The measurement was conducted with the cells in different cell numbers of 10^4, 10^5, and 10^6 cells/ml.

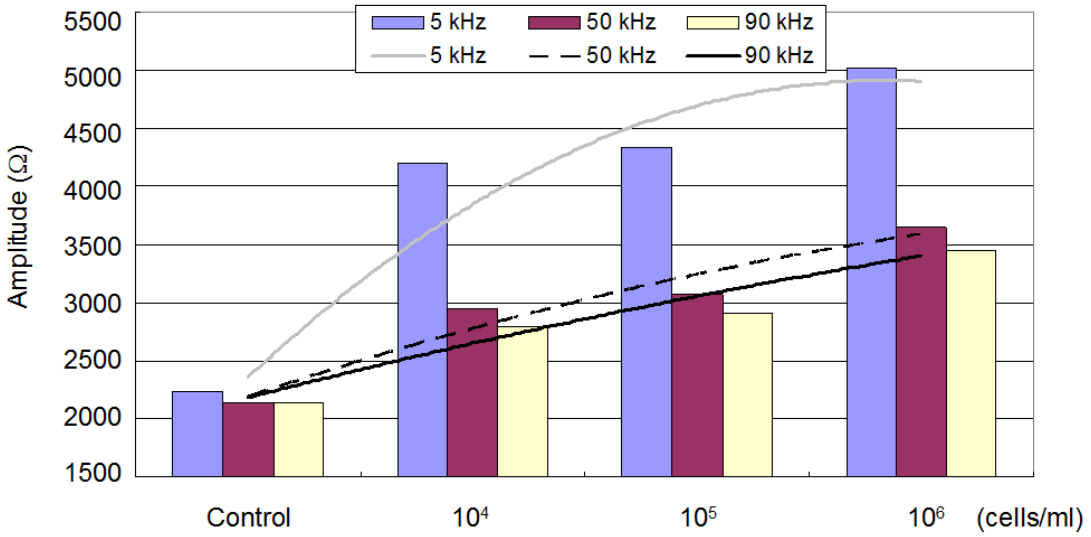

Figure 8. Impedance amplitudes of breast cancer cells/agarose construct under the frequencies of 5, 50, and 90 kHz. The trend lines of cell number in the construct are respectively shown by grey line, dash line, and solid line. The measurement was conducted with the cells in different cell numbers of 10^4, 10^5, and 10^6 cells/ml. The control (without cells encapsulated in the agarose construct) is also shown in the figure.

In this study, breast cancer cells in different cell numbers of 10^4, 10^5, and 10^6 cells/ml, were encapsulated in the agarose gel and agarose gel without cells encapsulated was defined as control. The agarose suspensions were respectively loaded into the culture chamber based on the above described procedure in Section 2.2. Then, culture medium was applied to the culture chamber. The impedance data of the cells/agarose construct was acquired by the impedance analyzer with potential of 0.1 V and frequency ranging from 5 kHz to 100 kHz across the vertical electrodes. Impedimetric spectroscopy of the cells/agarose construct in cell number of 10^4, 10^5, and 10^6 cells/ml, is shown in Figure 7. At frequency above 50 kHz, the responses show resistive effect, i.e., the amplitudes show relatively constant across the frequencies. That indicates the response is determined by the combination of the total resistance of the agarose scaffold and the solution R_A, the resistance of the cell membrane R_{BLM}, and the resistance of the breast cancer cell R_C. At frequency below 50 kHz, a combination of the resistances and capacitances from agarose scaffold and cells was measured. In 3D measurement, a pair of vertical electrodes was used for the detection of cells suspended in the agarose scaffold. Cells were sitting in the agarose scaffold and treated as dopant from the viewpoint of electric characteristic. Therefore, the electric circuit model is interpreted as having two parallel passageways, i.e., impedance from agarose scaffold and impedance from cells. At high frequency, capacitances, i.e., C_{DL} and C_{BLM}, were minimal and the total impedance was dominated by the resistances, i.e., R_A, R_{BLM}, and R_C. Since resistance value was not changed by the measuring frequency, the total impedance measured in high frequency, i.e., >50 kHz, is suggested to be suitable for the estimation of the cell number. Moreover, impedance amplitudes of the cells/agarose constructs and the control measurement at the frequencies of 5, 50, and 90 kHz were plotted in Figure 8. Generally, the impedance amplitude of the construct was proportional to the cell number in the construct. At frequency of 5 kHz, the total impedance of the

construct was the combination of the resistances and capacitances from agarose scaffold and cells. And capacitances, i.e., C_{DL} and C_{BLM}, were relatively significant in this frequency range. The trend line, i.e., grey line in the figure, does not show linear response to the cell number in the construct. At frequencies of 50 and 90 kHz, the trend lines, i.e., dash line and solid line in the figure, show linear response to the cell number. That indicates the impedance amplitude was dominated by the resistances, i.e., R_A, R_{BLM}, and R_C because the capacitances were minimized at these frequencies. It is obvious that the resistance of the construct was proportional to the cell number in the construct. However, the cell number below 10^3 cells/ml cannot be detected because of insufficient number of cells for the impedance measurement.

4. CONCLUSIONS

A measurement platform has been developed for the impedimetric detection of cell number in the 3D cell culture construct. The platform mainly consists of a culture chamber embedded with a pair of vertical electrodes located at its opposite sidewalls. The total impedance of the cell culture construct can be measured via the vertical electrodes and the cell number in the construct can be determined. The impedance amplitude of the construct was proportional to the cell number in the construct. By analyzing the electrical model of the construct, it is suggested that the measurement is suitable in the frequency above 50 kHz. The proposed technique provides a fast and easy measurement compared with the conventional cell analysis techniques. These preliminary results indicate that the impedimetric detection of cell number in the 3D construct has a high potential for on-chip cellular analysis during 3D cell culture process.

ACKNOWLEDGMENTS
The authors would like to thank the financial support from the National Science Council in Taiwan (NSC101-2221-E-182-003-MY3). Also, we would like to thank Prof. Tung-Ming Pan at the Department of Electronics Engineering, Chang Gung University for the instrumentation support.

References

1. M.H. Wu, J.P.G. Urban, Z.F. Cui, Z. Cui and X. Xu, *Biotech. Progress*, 23 (2007) 430-434.
2. M.H. Wu, Y.H. Chang, Y.T. Liu, Y.M. Chen, S.S. Wang, H.Y. Wang, C.S. Lai and T.M. Pan, *Sens. Actuators B*, 155 (2011) 397-407.
3. Z.F. Cui, X. Xu, N. Trainor, J.T. Triffitt, J.P. Urban and U.K. Tirlapur, *Toxicol In Vitro*, 21 (2007) 1318-1324.
4. J.M. Anderson, *Annu. Rev. Mater. Res.*, 31 (2001) 81–110.
5. M.H. Wu, S.B. Huang and G.B. Lee, *Lab Chip*, 10 (2010) 939-956.
6. A. Abbot, *Nature*, 424 (2003) 870-872.
7. U. Marx and V. Sandig, *Drug testing in vitro*, Wiley, Weinheim, 2007, 4, 54.
8. E. Cukierman, R. Pankov, D.R. Stevens and K.M. Yamada, *Science*, 294 (2001) 1708-1712.
9. M.H. Wu and C.Y. Kuo, *Biomed. Microdevices*, 13 (2011) 131-141.
10. M.H. Wu, J.P.G. Urban, Z. Cui and Z.F. Cui, *Biomed. Microdevices*, 8 (2006) 331-340.
11. P. Silley and S. Forsythe, *J. App. Bacteriology*, 80 (1996) 233-243.

12. A.G.E. Saum, R.H. Cumming and F.J. Rowell, *Biosens. Bioelectron.*, 13 (1998) 511-518.
13. S. Grant, F. Davis, K.A. Law, A.C. Barton, S.D. Collyer, S.P.J. Higson and T.D. Gibson, *Anal. Chem. Acta.*, 537 (2005) 163-168.
14. K.F. Lei, *Micro & Nano Letters*, 6(3) (2011) 157-160.
15. K.F. Lei, *Meas. Sci. Tech.*, 22 (2011) 105802.
16. K.S. Ma, H. Zhou, J. Zoval and M. Madou, *Sens. Actuators B*, 114 (2006) 58-64.
17. N.N. Mishra, S. Retterer, T.J. Zieziulewicz, M. Isaacson, D. Szarowski, D.E. Mousseau, D.A. Lawrence and J.N. Turner, *Biosens. Bioelectron.*, 21 (2005) 696-704.
18. E.E. Krommenhoek, J.G.E. Gardeniers, J.G. Bomer, A. Van den Berg, X. Li, M. Ottens, L.A.M. van der Wielen, G.W.K. van Dedem, M. van Leeuwen, W.M. wan Gulik and J.J. Heijnen, *Sens. Actuators B*, 115 (2006) 384-389.
19. K.F. Lei and P.H.M. Leung, *Microelectron. Eng.*, 91 (2012) 174-177.
20. S.M. Radke and E.C. Alocilja, *IEEE Sens. J.*, 4(4) (2004) 434-440.
21. S.M. Radke and E.C. Alocilja, *Biosens. Bioelectron.*, 20 (2005) 1662-1667.
22. M. Varshney and Y. Li, *Talanta*, 74 (2008) 518-525.
23. D.A. Boehm, P.A. Gottlieb and S.Z. Hua, *Sens. Actuators B*, 126 (2007) 508-514.
24. R. Ehret, W. Baumann, M. Brischwein, A. Schwinde, K. Stegbauer and B. Wolf, *Biosens. Bioelectron.*, 12(1) (1997) 29-41.
25. R. Ehret, W. Baumann, M. Brischwein, A. Schwinde and B. Wolf, *Med. Biol. Eng. Comput.*, 36 (1998) 365-370.
26. A.J. Bard and L.R. Faulkner, *Electrochemical Methods: Fundamentals and Applications*, Wiley, New York, 2001.
27. S. Hardeman, T. Nelson, D. Beirne, M. DeSilva, P.J. Hesketh, G.J. Maclay and S.M. Gendel, *Sens. Actuators B*, 24-25 (1995) 98-102.
28. Z. Zou, J. Kai, M.J. Rust, J. Han and C.H. Ahn, *Sens. Actuators A*, 136 (2007) 518-526.

© 2012 by ESG (www.electrochemsci.org)