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# Dynamic Monitoring of Cytotoxicity Using Electric Cell Substrate Impendence Sensing

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# Dynamic Monitoring of Cytotoxicity Using Electric Cell Substrate Impendence Sensing

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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## DEDICATION

to my wife Lydia and children Sandra and Ashley

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**Dynamic Monitoring of Cytotoxicity using Electric Cell Substrate Impendence Sensing**

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ABSTRACT

Electric cell-substrate impedance sensing (ECIS) pioneered by Giaever and Keese is suitable for continuous, automatic and real-time cell attachment analysis. ECIS is a novel electrical method to study, in real time, many of the activities of animal cells when grown in tissue culture. These include morphological changes, cell locomotion, and other behaviors directed by the cell's cytoskeleton. One of the most direct ECIS measurements is that of the attachment and spreading behaviors of cells. These measurements allow one to study and quantify the interaction of cultured cells with extracellular matrix (ECM) proteins and other macromolecules continuously and in real time. Traditionally, cell attachment and spreading measurements are labor intensive, requiring many manipulations of the cultures for microscopic evaluation of cell behavior. With ECIS, these same measurements can be made in an automated approach without opening the door of the incubator. The ECIS core technology is based

on a technique of measuring the change in impedance of a small electrode to AC current flow. The heart of the measurement is a specialized slide that has 8 individual wells for cell culturing. The base of the device has an array of gold film electrodes that connect to the ECIS electronics to each of the 8 wells.

In our work we used ECIS to study the attachment and spread of HUVEC and 3T3 cells. The curve of HUVEC showed higher resistances than that of 3T3 cells. This was due to the fact we used gelatin to aid in attachment of HUVECs which accounted for the high resistances. 3T3 cells attached easily without help of gelatin. We also studied the cytotoxicity of HUVEC and 3T3 cells. The drugs that we used were CB, H7 and $CdCl_2$. We found that the best drug was CB since it affected the cells even at low concentrations. H7 effects were mild while $CdCl_2$ only worked at high concentrations. HUVEC cells make loose contact on electrodes and are easily detached by drugs. 3T3 makes firm attachment to the electrodes and are not easily detached from the electrodes.

Electrical impedance measurements on multiple electrodes are highly attractive in this application because of the potential for direct computer control.

# CHAPTER 1

## INTRODUCTION AND BACKGROUND

We all would like to enjoy good health forever. But sadly from time to time our body's basic unit, the cell, is attacked by various pathogens found in the environment around us and within us. This make us get sick and eventually die. For us to enjoy good health at least for sometime, it is crucial to find out how cells react to various poisons. This will help in preparing drugs that can combat the pathogens that have similar ill effects as the poisons studied.

Cell – based assays refer to any experiments based on the use of live cells. This includes a variety of methods for measuring cell viability, cytotoxicity, proliferation, motility, and morphology (1-3). The cell viability assay includes the method measuring the number of live cells while cytotoxicity assay indicates the number of dead cells. These cell-based assays are frequently used for drug discovery using high-throughput screening (4), environmental assessment of chemical (5) and biosensors for monitoring cellular behavior(6). According to one report on drug discovery (2), 30% of the attrition of potentially new drug candidates are due to the failure of toxic verification and the

presence of toxic side effects. Therefore, cytotoxicity testing in various cell types has become one of the fundamental tools for drug discovery (1, 4). As an example, hepatotoxicity is a major concern for drug toxicity because most toxic effects are influenced by drug metabolism in hepatocytes which have been used in most drug discovery and development laboratories (7-10).

Cell attachment is often studied with a radiation label (11) technique but this cannot provide dynamic information. Microscopes have been widely used to study cell attachment with the result described only in qualitative terms. For a continuous record of cell adhesion, cinematographic arrangements are necessary and data obtained are very difficult to quantify and usually require image processing with extensive data manipulation.

Some biochemical methods such as the MTT assay (8, 10), the neutral red uptake assay (5, 7) and ATP measurement are widely used in hepatotoxicity screening assays. However, these traditional biochemical methods are time consuming and laborious and require complex steps with multiple reagents at every prescheduled time point. For in vivo experiments they do not give quantitative results without affecting target cells. In addition, it is difficulty to monitor continuous behavior against toxic effects and to analyze the real time change of cell viability. As a result many researchers have lately

focused on the development of an alternative method for continuous monitoring of toxic effects (12, 13).

Among these methods, an impedance measurement using the microfabricated electrode may provide the ideal method for detecting cellular behavior without multiple reagents (14). Electric cell- substrate impendence sensing method [ECIS] has emerged as a means for screening cells. This method was first described by Giaever and Keese (15), is suitable for continuous automatic and real time cell attachment analysis. This technique uses an array of small electrodes that are deposited on the bottom of tissue culture wells and immersed in a culture medium. Upon inoculation with a specific cell line, inoculated cells drift downward and attach to the electrode surface. The attaching cells will interfere with the free space immediately above the electrode for current flow. When an AC voltage is applied between the two electrodes through a resistance of 1 MΩ, a lock- in amplifier can monitor the voltage between the two electrodes. Owing to its significantly smaller size compared to the counter electrode, the detecting electrode will dominate the overall impedance of the circuit. The increased impedance, a coordination of many biological reactions within the cell, can be continuously monitored and interpreted to reveal information about cell spreading and micro motion.  ECIS has

been demonstrated as a useful tool for kinetics and quantitation of mammalian cell spreading and motility as well as an alternative to animal testing for toxicity studies.

Giaever and Keese in 1984, designed a new electrical method to monitor fibroblastic behavior in vitro (12). Mammalian fibroblasts were cultured on evaporated gold electrodes driven with an alternating electric field at 4000 Hz. The dish used consisted of one large ( 2 $cm^2$) and four small ( 3 X $10^{-4}$ $cm^2$) electrodes bathed in tissue culture medium. The applied electric field produced a voltage drop of a few $mv/cm^2$ at the boundary between the solution and the small electrode. The authors suggested that the small population of cells that had attached and spread on the small electrodes had a marked effect on the measured impedance and caused it to fluctuate with time. The amplitudes of these fluctuations was greatly reduced by cytochalasin B (10 μM), suggesting these fluctuations were a consequence of cell movement. Later in 1986 they studied the attachment and spreading of two cell types, one normal cell, WI-38, and one transformed cell, WI-38 VA13 (16).They studied the cells response to various protein-covered electrodes in which they found that fibronectin and gelatin proved to be good substrates for most cell types. By studying the fluctuations of the in-phase voltage they detected the motion of the cells. The transformed cells shifted their anchoring of the surface more

often than the normal cells. In 1991, Giaever and Keese studied micro motion of mammalian cells (17), where they showed that average motion of the cell layer of 1 nm could be inferred from the ECIS measurements. In 1997 Lo and Ferrier studied the impedance analysis of fibroblastic cell layers (18). Here they proposed an extended model of impedance analysis for cell layers where cells are considered rectangular. They also measured average cell-substrate separation of human gingival fibroblasts at different temperatures. McCoy and Wang (19) used electric cell-substrate impedance sensing (ECIS) to monitor the progression of cytopathic effect due to influenza A virus infection. A healthy monolayer of cells was insulted with influenza A virus infection and exhibited a characteristic rounded cell morphology and cell detachment. These effects resulted in reduced impedance, which was monitored with ECIS. Since data obtained through ECIS are both quantitative and in real-time, it was possible to monitor continuously cell behavior during infection. This, in turn, allowed for a more detailed and comprehensive data set to analyze. More importantly, through ammonium chloride treatment of cells, it was also shown that ECIS may be exploited to examine a treatment's effect on the reduction of resistance because of its antiviral activity. Thus, ECIS may be a powerful approach for screening antiviral compounds quantitatively in a real-time fashion.

In 2004, De Blasio et al (20) used modified electrical cell-substrate impedance sensor (ECIS) and were able to simultaneously record both growth and motility, thus enabling cell confluence on the electrodes to be systematically correlated to the impedance in regular time intervals of seconds and for extended periods of time. Furthermore, the technique provides an independent measure of monolayer cell densities that they compared to calculated values from a theoretical model. They followed the attachment and spreading behavior of epithelial Madin-Darby canine kidney strain I (MDCK-I) cell cultures on microelectrodes for up to 40 h. The studies reveal a high degree of correlation between the measured resistance at 4 kHz and the corresponding cell confluence in 4- to 6-h intervals with typical linear cross-correlation factors of r equaling approximately 0.9. In summary, the impedance measured with the ECIS technique provides a good quantitative measure of cell confluence. De Blasio et al (21), in 2004, found that Madin- Darby canine kidney (MDCK) cells elicit synchronous, multicellular Ca2+ oscillations in response to hormone stimulus. The periods of the Ca2+ oscillations and the electrical fluctuations are found to coincide. Further, blocking of gap junctions by 18a-glycyrrhetinic acid causes a loss of synchrony in Ca2+ signals and inhibition of impedance oscillations, emphasizing the importance of gap junctions in the signal transduction process.

They concluded that based on these observations the co-ordinated adhesive changes in MDCK cells are a direct consequence of synchronized Ca2+ oscillations. Calcium signalling represents an efficient way of organizing physiological responses in a tissue. A possible functional implication of the structural changes might be to modulate transportation of various substances across the cell sheet. However, no work on HUVEC cells response to drugs has been reported. In this study, we have used ECIS to monitor cell attachment and spreading on the electrodes and cytotoxic effects of 3T3 and HUVEC cells under varying concentrations of drugs H7, cytochalasin B and cadmium chloride ($CdCl_2$). The purpose of our work was to understand how the impedance change is related to the cell activity, and to analyze cells' responses to the drugs.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1 Cells and drugs

Two type of cells were used in this work. One type was Human Umbilical Vein Endothelial (HUVEC) cells that were taken from the inside of umbilical cord of babies. They are isolated from normal human umbilical vein. They are cryopreserved at the end of primary culture and can be cultured and propagated at least 16 population doublings. HUVEC are responsive to cytokine stimulation in the expression of cell adhesion molecules. These cell systems are commonly used for physiological and pharmacological investigations, such as macromolecule transport, blood coagulation, and fibrinolysis.

Endothelial cells may be derived from any part of the vascular tree. They may come from large and small veins and arteries, from capillaries, or from specialized vascular areas such as the umbilical vein of newborns, blood vessels in the brain or from vascularized solid tumors. A convenient source of endothelial cells has been the human umbilical vein.

The other cell used was 3T3 which is given in figure 2.0 below.

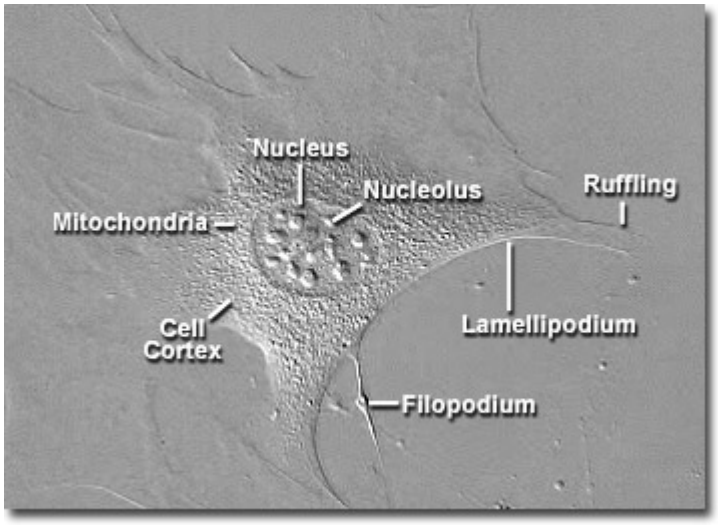



Figure 2.0 Albino Swiss Mouse Embryo Fibroblasts (3T3 Line)

The 3T3 cell line is an important fibroblast culture, widely utilized in laboratory research, which was established from disaggregated tissue of an albino Swiss mouse (***Mus musculus***) embryo. Most 3T3 variants have been demonstrated to be negative for mousepox and for the polymerase reverse transcriptase, an indication of the absence of integral retrovirus genomes. When the 3T3 line was established in the early 1960s by George Todaro and Howard Green, the cells were considered somewhat of an anomaly.

They were different than most other cell lines in regard to the fact they did not induce tumors to develop when injected into murine species. However it was quickly realized that they were not normal cells either,

since they are capable of growing indefinitely. In fact, the unusual behavior of the line enabled researchers to make a clear distinction for the first time between immortal cells and cells that have the ability to form tumors; previous to studies of the 3T3 line, it was believed that these characteristics were necessarily synonymous with one another. However, due to examination of 3T3 cells and subsequent research it has become widely accepted that for immortalization of cells to take place, telomere shortening, which can instigate chromosomal rearrangements, must be overcome, a process that is not necessarily related to a cell's ability to undergo oncogenic transformation.

These cells are known to double every 24 hours. They form confluent monolayers, with contact inhibited cell motility and can be serum starved and activated by specific growth factors. These cells can be readily transfected: using calcium/phosphate, lipofectamine, adenovirus.

Three drugs were used in this research. The first one was Cytochalasin B (CB) whose structure is given below. Its formula is $C_{29}H_{37}NO_5$

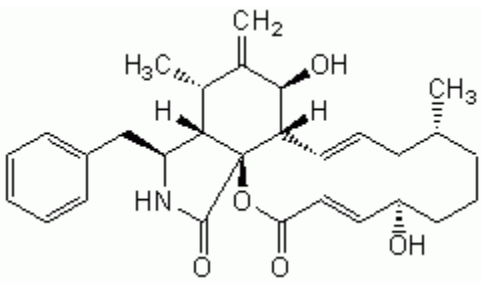



Figure 2.1. Structure of cytochalasin B

Cytochalasin B is a cell permeable fungal toxin that disrupts contractile microfilaments by inhibiting actin polymerization. This, in turn, induces DNA fragmentation, inhibits cell division and disrupts many cellular processes. It also inhibits glucose transport. It is an alkaloid isolated from a fungus, Helminthosporium dermatioideum, and its Mw is 479.6.
The second drug was H-7. E. coli O157:H7 is one of hundreds of strains of the bacterium Escherichia coli. Although most strains are harmless and live in the intestines of healthy humans and animals, this strain produces a powerful toxin and can cause severe illness.
It was first recognized as a cause of illness in 1982 during an outbreak of severe bloody diarrhea; the outbreak was traced to contaminated hamburgers. Since then, most infections have come from eating undercooked ground beef.
The combination of letters and numbers in the name of the bacterium refers to the specific markers found on its surface and distinguishes it from other types of E. coli.
E.coli *O157:H7* infection often causes severe bloody diarrhea and abdominal cramps; sometimes the infection causes non bloody diarrhea or no symptoms. Usually little or no fever is present, and the illness resolves in 5 to 10 days. In some persons, particularly children under 5 years of age and the elderly, the infection can also cause a complication called hemolytic uremic syndrome, in which the red blood

cells are destroyed and the kidneys fail. About 2%-7% of infections lead to this complication. In the United States, hemolytic uremic syndrome is the principal cause of acute kidney failure in children, and most cases of hemolytic uremic syndrome are caused by E. coli O157:H7.Most persons recover without antibiotics or other specific treatment in 5-10 days. There is no evidence that antibiotics improve the course of disease, and it is thought that treatment with some antibiotics may precipitate kidney complications.

Hemolytic uremic syndrome is a life-threatening condition usually treated in an intensive care unit. Blood transfusions and kidney dialysis are often required. With intensive care, the death rate for hemolytic uremic syndrome is 3%-5%.

The third drug used was cadmium chloride. Cadmium is one of the most toxic transition metal pollutants and is associated with air and water pollution as well as cigarette smoking and its potential harm has increased with increasing industrial usage of the element (22).It is shown to have a wide physiological function and it activates the expression of several mammalian genes. Cadmium has been shown to have toxic effects on human neuroblastoma cells, porcine and rat kidney cells, and human prostate epithelial cells. Correlation of cadmium with estrogen receptors in breast cancer has been found suggestive. Cadmium has been shown to attribute to carcinogenicity

by enhancing DNA mutation rates and to stimulate mitogenic signaling pathways and expression of oncoproteins that control cellular proliferation.

Different routes of cadmium uptake, such as via airborne particles, smoking, drinking water, and food have been identified. Cadmium uptake into cells occurs by simple diffusion and cadmium also utilizes the transport pathways for calcium, zinc, and copper. Cadmium uptake also requires interaction with membrane sulfhydryl groups.

### 2.2 Instrumentation

The ECIS core technology is based on a technique of measuring the change in impedance of a small electrode to AC current flow. The heart of the measurement is a specialized slide that has 8 individual wells for cell culturing. The base of the device has an array of gold film electrodes that connects the ECIS electronics to each of the 8 wells. The current flows between a 250 μm diameter electrode and a larger counter electrode using normal culture medium as the electrolyte. The leads from one of the small electrodes and large electrodes were connected to a SR830 lock-in amplifier from Stanford Research Systems. To study cell morphological changes, usually a 1V 4000 Hz AC signal through a 1 MΩ resistor was used to provide an approximately constant current of 1 μA through the system. All

connections were done by coaxial cable to minimize any background noise. The setup is shown in Fig.2.2 below. As the cells attach and spread on the electrode, it changes the impedance in such a way that morphological information about the attached cells can be inferred.

### 2.3 Experimental Setup

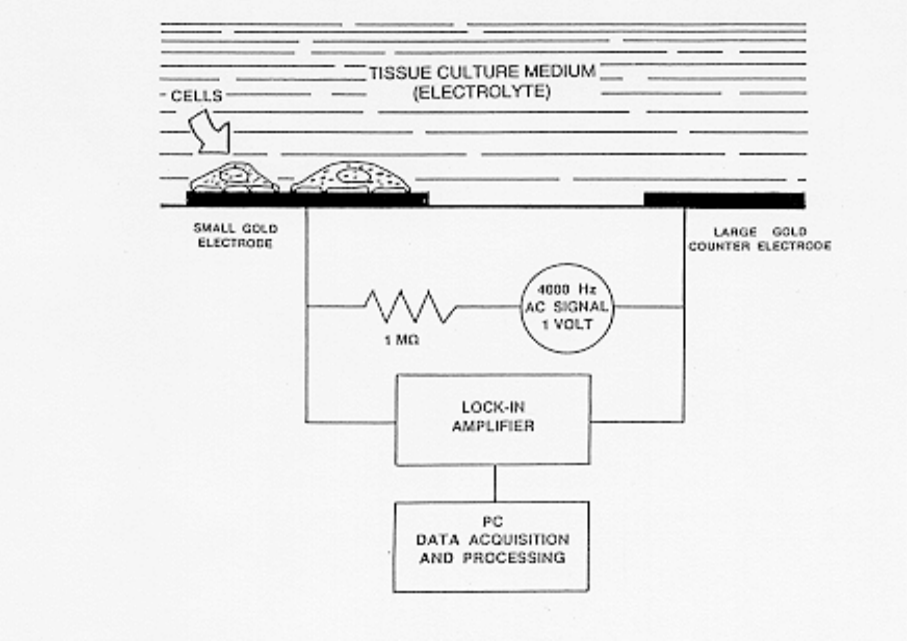



Figure.2.2 Set up for Impedance measurement

In addition to the impedance measurements the data were also converted to resistance and capacitance, considering the cell electrode system as a simple resistor and capacitor in series. When the cells attach and spread upon the electrode, they block the current. This compels the current to flow beneath and between the cells and result in large increase in impedance. Since the microampere currents and

the resulting voltage drop of a few millivolts have no measurable effect on the cells, the monitoring of the cell behavior is noninvasive (3).

### 2.4 Electrode Array

Each electrode well used for the ECIS measurements is well explained by Keese et.al. All eight electrode contains small working electrode (area= $5\times10^{-4}$ $cm^2$) and a large counter electrode (area=0.15 $cm^2$). Because of the difference in surface area, the impedance of the small electrode determines the total impedance of the system. The active electrode is delineated by circular openings (diameter = 250 μm) in a photoresist overlayer that insulates the rest of the deposited gold film from bulk electrolyte. As cells in the culture attach to the electrode, we can relate the cell behavior by the impedance measured by this device. We selected the size of the electrode to be this small as cell related signals are difficult to measure with large electrodes. This is because solution resistance in the culture dish is much larger than the electrodes impedance. The impedance of the electrode electrolyte interface (Faradaic impedance) is proportional to the inverse of the electrode area, but constriction resistance (spreading resistance) for the circular disk electrode in a conducting medium of infinite extent varies as ρ/2d, where ρ is the resistivity of the medium and d is the diameter of the electrode. So the electrode impedance can always be

made to dominate the constriction resistance by using sufficiently small electrodes. When the electrode area is reduced to $10^{-4}$ $cm^2$, the Faradaic resistance of the electrode electrolyte interface at 4000 Hz is many times larger than the constriction resistance, so the motility of the cells can be easily studied.

The image of the electrode we used in our lab is given in Fig.2.3 A. while that HUVEC is given in Fig 2.3 B

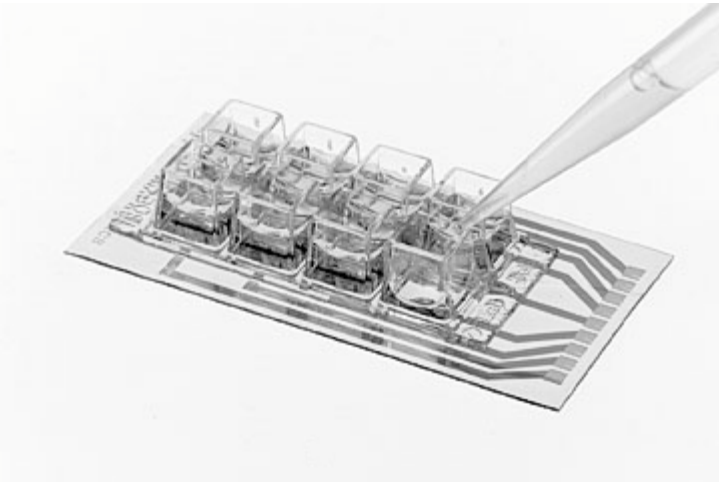


Figure 2.3a. Eight well electrode

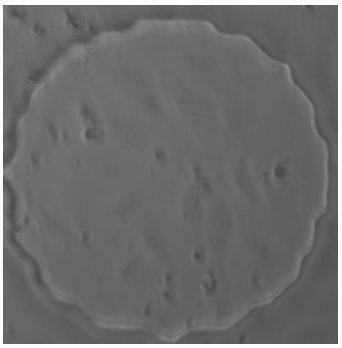


Figure 2.3b. Microscopic view of HUVEC on the electrode

### 2.5 Cell Culture Procedures

The Human Umbilical Vein Endothelial Cells (HUVECs) were obtained from Clonetics Corp. and were grown in endothelial cell growth which is added with following: 10 ng/ml human recombinant Epidermal Growth Factor, 1 µg/ml hydrocortisone, 50 µg/ml getamicin, 50 ng/ml amphotericin B, 12 µg/ml bovine brain extract and 2% fetal bovine serum (amounts indicate final concentration). For the ECIS studies the cells were taken from slightly subconfluent culture usually 48 hrs after passage. The culture medium was changed twice a week. Cell suspension was prepared using standard tissue culture technique with 0.05% or 0.25% of trypsin/EDTA. The cells were then kept in the incubator to equilibrate before adding to each wells of the electrode for about 3 minutes.

3T3 fibroblasts were obtained from America Type Culture Collection (Manassas, VA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % newborn calf serum, 4 Mm glutamine, 4 Nm insulin, 10 mM Hepes and with 25µg of sodium ascorbate, 100 IU of penicillin, 100 µg of streptomycin, and 0.25 µg of amphotericin B per millileter at 37ºC in water saturated atmosphere at 5% $CO_2$ in air, in a Heraeus incubator (BBI6).

### 2.6 Cell Attachments and Spreading

The suspension of HUVECs in the wells was prepared from the confluent cell layers. The cells were first washed with Hanks' Balanced Salt solution of nearly 5 ml. The cells were then kept in the incubator with 0.05% of trypsin (2.5 ml) in it. Adding nearly 4 ml of the complete culture medium terminated the trypsinization. The cells were then spun down at 1500 rpm for 5 min. These made the cells to settle down. The media with trypsin in the cell was separated. Fresh culture media was then added and density of the cells was known by using standard hemacytometer. The impregnate was adjusted to give a final cell number of $9 \times 10^4$ cells and final cell density in each well of $10^5$ cell/$cm^2$. Before suspending the cells the electrode was coated with gelatin and kept in the incubator for 15 min. A solution of 200 µg/ml gelatin in 0.15 M NaCl was used. Gelatin settled down and protein solution was then aspirated. The electrode-containing wells were rinsed twice with the culture medium. Prior to inoculation, ECIS electrodes were rinsed with small amount of serum free culture medium. The Fig.2.4 B. shows the attachment of the HUVEC cells to the electrode. The image was taken 24 after inoculation of $10^5$ cells/$cm^2$ HUVEC cells into the well.

3T3 fibroblast cells were grown on standard 10 cm diameter polystyrene dishes and maintained in Dulbecco's Modified Eagle

medium supplemented with calf serum (10%), 500 unit/ml penicillin, 500 μg/ml streptomycin, 2 Mm L- Glutamine, and Mm HEPES buffer, at 37 $^{o}$ C, and 5 % $CO_2$ in air. The same medium was used to sustain cells during the impedance monitoring experiments.

### 2.7 Drug Invasion Assay

We also used ECIS to monitor the effects of cytochalasin B, H-7 and cadmium- chloride on HUVEC and 3T3 cells. The cells confluence was verified both microscopically and by ECIS measurements. The HUVECs were generally incubated for 1-2 days before the drugs were added. Impedance measurements were used to test if confluence was achieved, maintained and that the cells exhibited normal levels of impedance fluctuations indicative of healthy cell layers (23). The detachment data were then run for 90 min with just the HUVEC cells to establish a baseline. Before the challenge of drugs, they were allowed to grow in the culture dish for 2 days to reach confluence. The medium with the trypsin was then aspirated and the drug medium was added. We then counted the cells with hemacytometer (added more media to make the total cell density as $10^{5}$ cells/$cm^{2}$). 3T3 were similarly prepared.

# CHAPTER 3

# THEORY

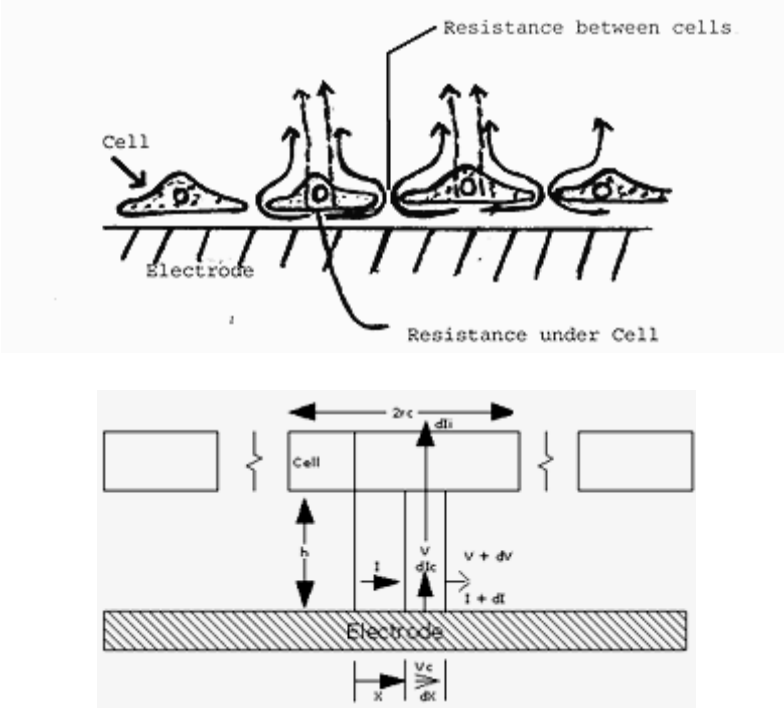



Figure 3.1 Model of the cells in tissue culture emphasizing space between the cell and substratum. Calculated resistance is due to the current flow under the cells and an additional resistance because the current must flow out between the cells. Broken line represents the capacitive current flow through the membranes. The cells are regarded as disk shaped and viewed from top. ρ is the resistivity of solution.

## 3.1 ECIS Theoretical Model

Fig.3.1 above shows a schematic lay out of the electrodes and the basic electrical setup used in these measurements. The model derivation for the ECIS has been well explained by Giaever and Keese

in 1991. In this two-probe electronic instrument, the resistance of the bulk tissue culture medium is in series with the impedance of the electrodes and will govern the measurement except when an electrode is small. The solution resistance will then be shown itself as a spreading or constriction resistance that depends on the size of the electrode. Take an example, for a circular disk electrode in a conducting medium of large radius, the constriction resistance varies as ρ/2d, where ρ is the resistivity of the medium and d is the diameter of the electrode (24).As the impedance measured with the electrode-electrolyte interface should be inversely proportional to the area of the electrode, $4/\prod d^2$, it can always govern the constriction resistance by making the diameter sufficiently small. For an electrode of $10^{-3}$ $cm^2$, the real part of the impedance of the electrode (the faradaic resistance) is several times larger than the constriction resistance at 4 kHz. Under these conditions, the activities of attached cells are clearly studied. If two large electrodes had been used, the solution resistance would have concealed the measurement and the presence of cells would be barely noticed. The model used to calculate the specific impedance for a unit area of a cell-covered electrode as a function of the frequency $\nu$, is shown in Fig. 3.1. It depends on the measured specific impedance $Z_n(\nu)$, of a cell-free electrode, the specific impedance $Z_m(\nu)$, through the cell layer which is mainly the capacitance of the upper and lower

cell membranes in series and the resistivity p, of the tissue culture medium. The cells have been considered as circular disks of radius $r_c$. We considered that the current flows radially in the space formed between the ventral surface of the cell and the substratum and that the current density under the cells does not change in the z direction. The equations can be stated as

$$\frac{-dV}{dr} = \frac{\rho}{2h\pi r} I$$

$$V_c - V = \frac{Z_n}{2\pi r dr} dl_c$$

$$V = \frac{Z_m}{2\pi r dr} dl_i$$

$$dl = dl_c - dl_i$$

These equations can be combined to yield

$$\frac{d^2V}{dr^2} + \frac{IdV}{rdr} - \gamma^2 V + \beta = 0$$

here

$$\gamma^2 = \frac{\rho}{h}(\frac{1}{Z_n} + \frac{1}{Z_m}$$

and

$$\beta = \frac{\rho V_c}{hZ_n}$$

where $V_c$ is the potential of the electrode, and h is the height of the space between the ventral surface of the cell and the substratum. The

solution of above equation is a sum of modified Bessel functions of first and second kind (25). Using proper boundary conditions, the specific impedance for a cell-covered electrode is obtained as follows:

$$\frac{1}{Z_c} = \frac{1}{Z_n}\left[\frac{Z_n}{Z_n + Z_m} + \frac{\frac{Z_m}{Z_n + Z_m}}{\frac{\gamma r_c}{2}\frac{I_0(\gamma r_c)}{I_1(\gamma r_c)} + R_b\left(\frac{1}{Z_n} + \frac{1}{Z_m}\right)}\right]$$

where $I_0$ and $I_1$ are modified Bessel functions of the first kind with order 0 and 1. Here the solution depends on two parameters i.e. $R_b$, the resistance between the cells for a unit area, and $\alpha$ which can be given as:

$$\gamma r_c = r_c\sqrt{\frac{\rho}{h}}\left(\frac{1}{Z_n} + \frac{1}{Z_m}\right) = \alpha\sqrt{\left(\frac{1}{Z_n} + \frac{1}{Z_m}\right)}$$

Since $Z_n(\nu)$ is measured and $Z_m(\nu)$is the impedance of two cell membranes in series. Now, $\alpha$ and $R_b$ are the only adjustable parameters in the equation stated above. The expression does not depend on frequency directly but is contained in the impedances $Z_n(\nu)$ and $Z_m(\nu)$.

The value of the resistance used 4 kΩ will ensure that the amplitude of current in the system is in μA scale, and the amplitude of voltage between the two electrodes in mV scale. This is very important

because if current or voltage were too high, cells attached on the electrodes would die.

We would also calculate the variance for different time periods to analyze the electrical fluctuations resulting from cell motion. The data set of 7168 points is first split in 1792 equal sets with 4 points each, and the variance is calculated and averaged for all the sets. The process is then repeated with 8, 16, 32, etc. points in each set, and the average variance is plotted versus the number of points (sampling period) in a log-log plot. The variance is equivalent to power, and the inverse of the number of points is similar to a frequency.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Inoculation of HUVECS

Figure 4.1 given below shows the initial attachment of the HUVECs. The resistance value is obtained with respect to the change in time. Seven individual electrodes were used to show the impedance measurement from the time zero when the cells were inoculated to 20 hours later. The data were taken at frequency of 4 kHz. Initially when the cells are not attached, their resistance is nearly 2000 Ω. On the inoculation of HUVECs, the cell attached to electrodes in each well. Now the insulating plasma membrane present in the cell, block the flow of the current so the current flows beneath and between the cells. This path of the current causes more change in the impedance value. This takes place at the small and the counter electrode but the impedance of the small electrode is too large compared to the larger electrode and thus can be ignored. The initial rise in the resistance is due to the cell attachment and spreading. Spreading is completed in nearly 2.5 hours. Here from the Fig.4.1, we notice that the resistive portion of the impedance increased much higher than cell free

electrode. The fluctuation observed in the increased impedance is because the cells are alive and they change their morphology, which affect the impedance. The important thing to understand is that the ac current used for this measurement (1μA) and relative voltage drop across the cells (few millivolts) has nearly no effects so the measurements taken are noninvasive.

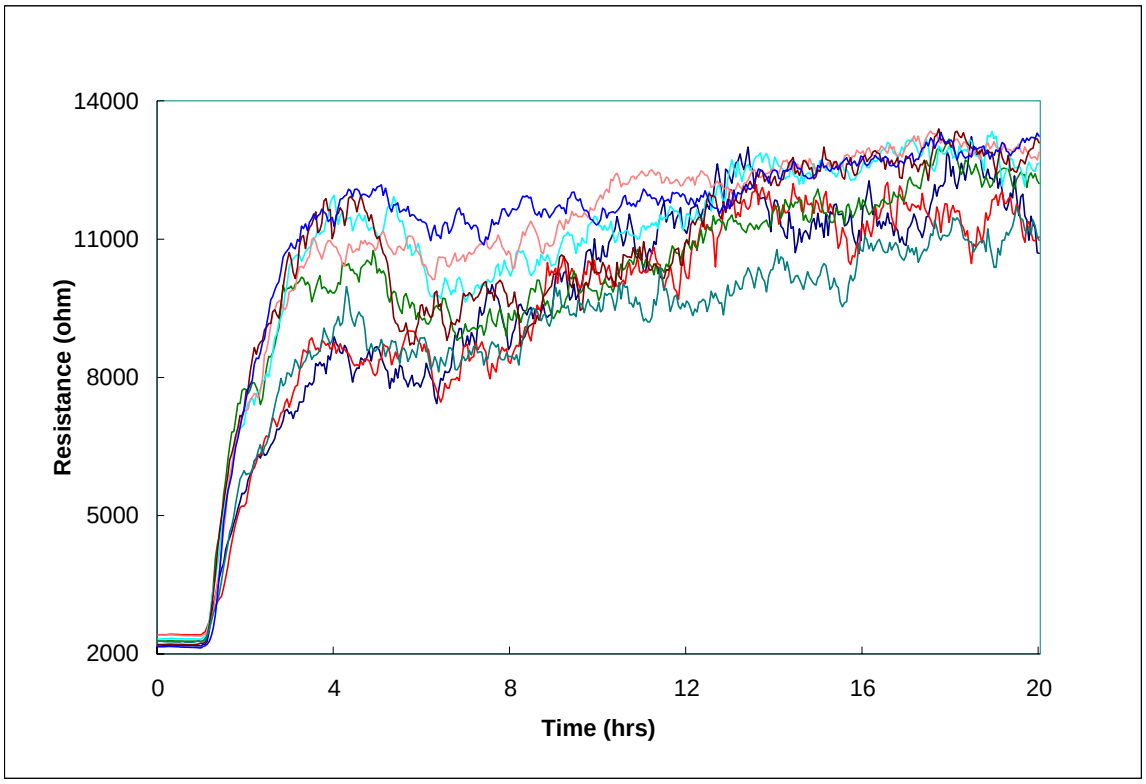



Figure 4.1 ECIS measurement of HUVEC inoculations. Measurements made in eight individual wells were at 4 kHz, and the resistive portion of the impedance is presented. All electrodes were precoated with 0.2 mg/mL gelatin for 15 minutes.

### 4.2 Inoculation of 3T3 Cells

The attaching and spreading of 3T3 cells is shown in figure 4.2 given below. Before attachment the resistance of the electrodes was about

1700 Ω. This value is less than the one of HUVECS where gelatin was used to facilitate the attachment of cells. 3T3 cells attached well on the electrodes without help of gelatin. As cells attached, resistance shot up to about 3400 Ω. This cells' attachment on the electrode blocked the passage of current and so current was forced to flow between cells and between cells and the electrodes. After about 5 hours the attachment and spreading of the cells on the electrodes was complete and resistance settled down to about 2500 Ω. Since this final resistance is lower than the peak value, perhaps the cells loosen contact on the electrodes allowing more current to pass through.

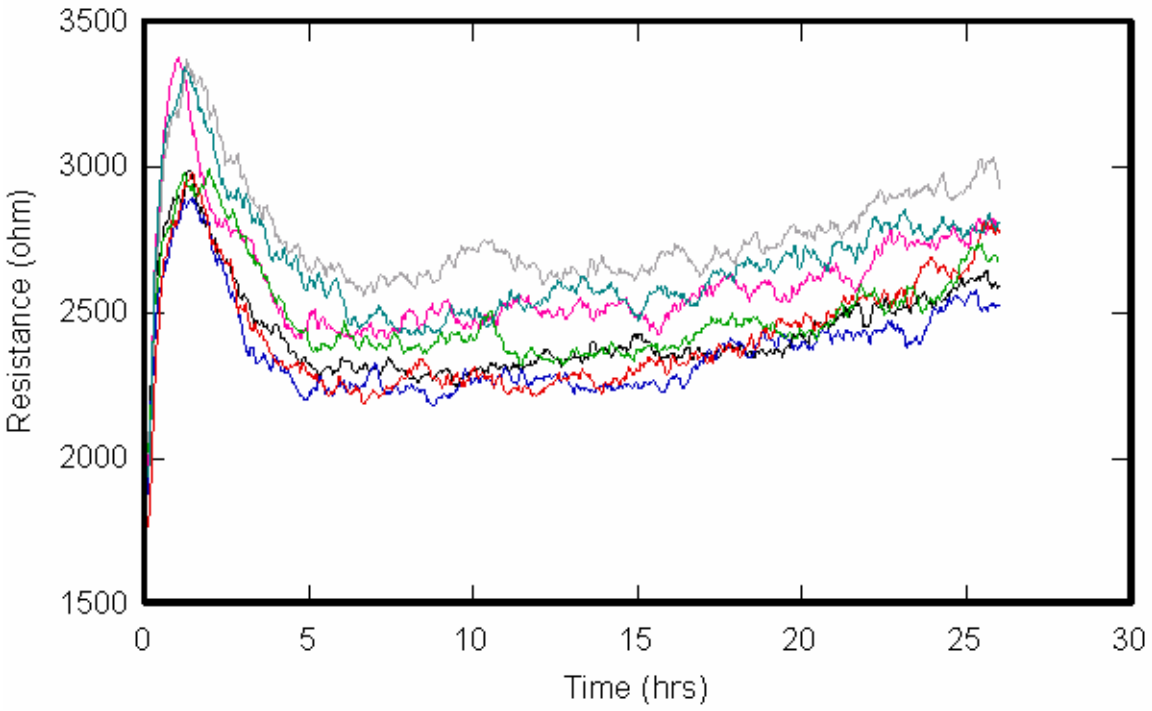



Figure 4.2 Attachment and spreading of 3T3 cells.

### 4.3 CYTOTOXICITY RESPONSE of 3T3 to CB, H7 and $CdCl_2$

(a) Cytochalasin B effects on impedance sensor

Resistance measurements were performed to study the cell response to Cytochalasin B. Resistance response to concentration of the drug was monitored for 20 hours. The results are shown in figure 4.3a given below.

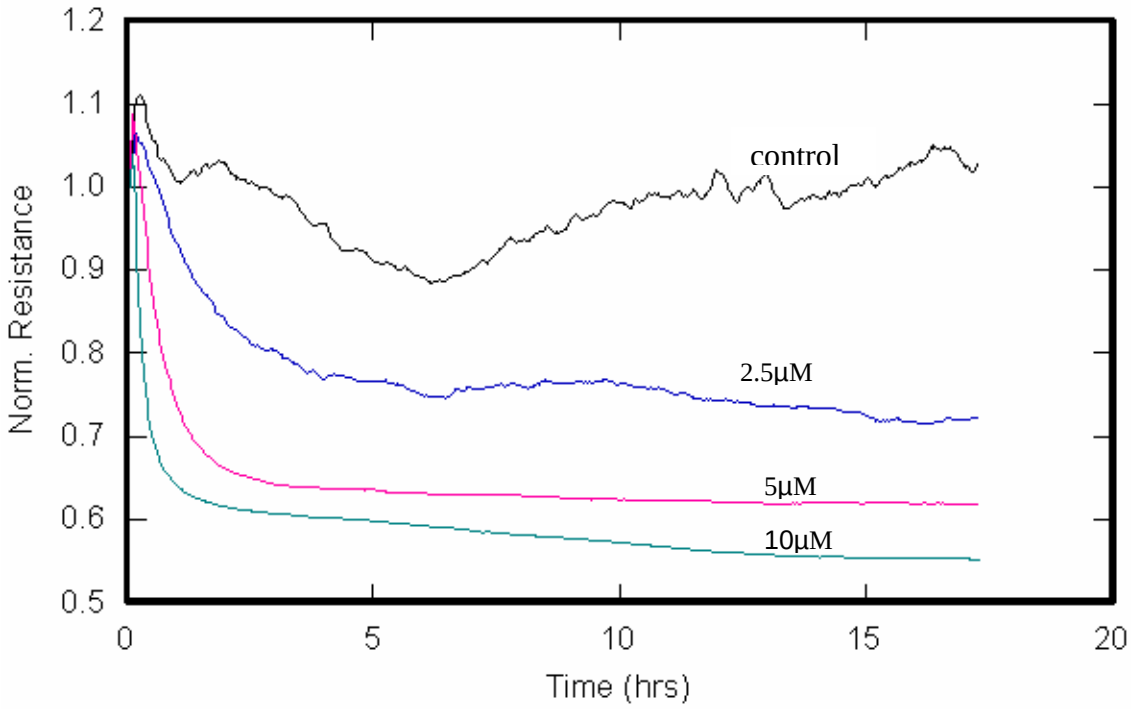
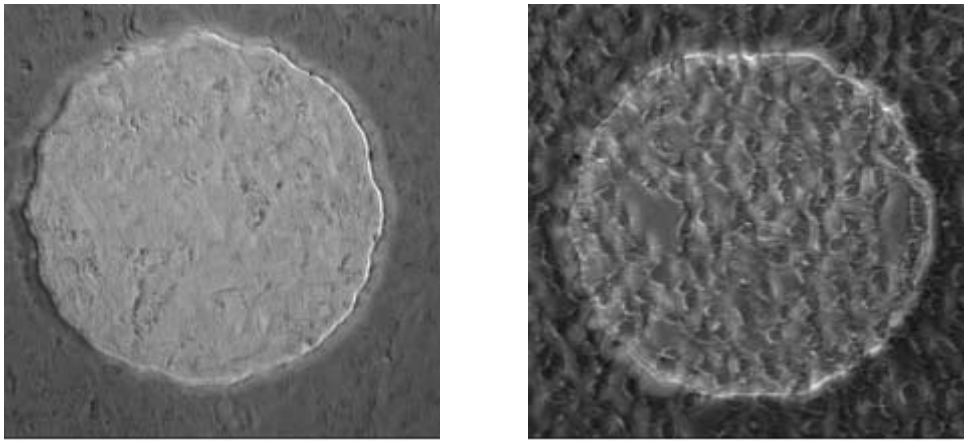



Figure 4.3a. Variation of 3T3 cells normalized resistance with time at different concentration of CB

The resistance dropped with increase in concentration. In less than 3 hours the resistance became more or less constant for all concentrations ,save the control, with time showing that either the cells were dead or the contact on the electrode had been loosened (19) allowing more current to pass through the gap between the cell

and the electrode. The resistance of cells in the control well changed with time since they all alive, and the morphology changing all the time. This effect of the drug can also be seen from figure 4.3b of the image of the cells taken 20 hours after introduction of the drug.



Control 5 μM

Figure 4.3b. Effects of CB on 3T3 cells

The image for control cell is smooth but the image of the cell with 5μM concentration of drug is rough and some portion of it has been destroyed and lifted of the cell. Philips et al (26) point out from similar studies of CB that there are a number explanations for this decrease of resistance with increasing drug concentration, including: inhibition of actin gel formation in crude cytoplasmic extracts; a direct effect on actin, resulting in inhibition of actin filament network formation; and reduction in the rate of actin polymerization. However, they say that

there is now agreement from a number of laboratories that CB inhibits actin polymerization in vitro and that, in the erythrocyte membrane, at least the polymerization site is both membranes associated and sensitive to CB. Painter et al (27) also agree with this effect on CB on cells. They say that CBs are known to have at least two possible in vitro effects on F-actin. First, the drug inhibits actin polymerization by blocking monomer addition at the barbed end of growing filaments. Blocking one end of the filament would also inhibit tread milling of actin. Second, the drug appears to affect bulk gel strength by blocking direct filament interaction and /or by reducing the length of F-actin filaments. Both effects would be expected to drastically alter the overall bulk strength of the cytoplasmic gel. This explanation seems to agree with our work as resistance decreased with concentration of CB. The drug CB inhibits actin polymerization in vitro by blocking monomer addition at the end of growing filaments. The drug also appears to block direct filament interaction and / or by reducing the length of actin filaments. Therefore, the higher the concentration of the drug, the more the damage on the cells, and the resistance goes down. Figure 4.3c given below shows log variance of the cells versus time.

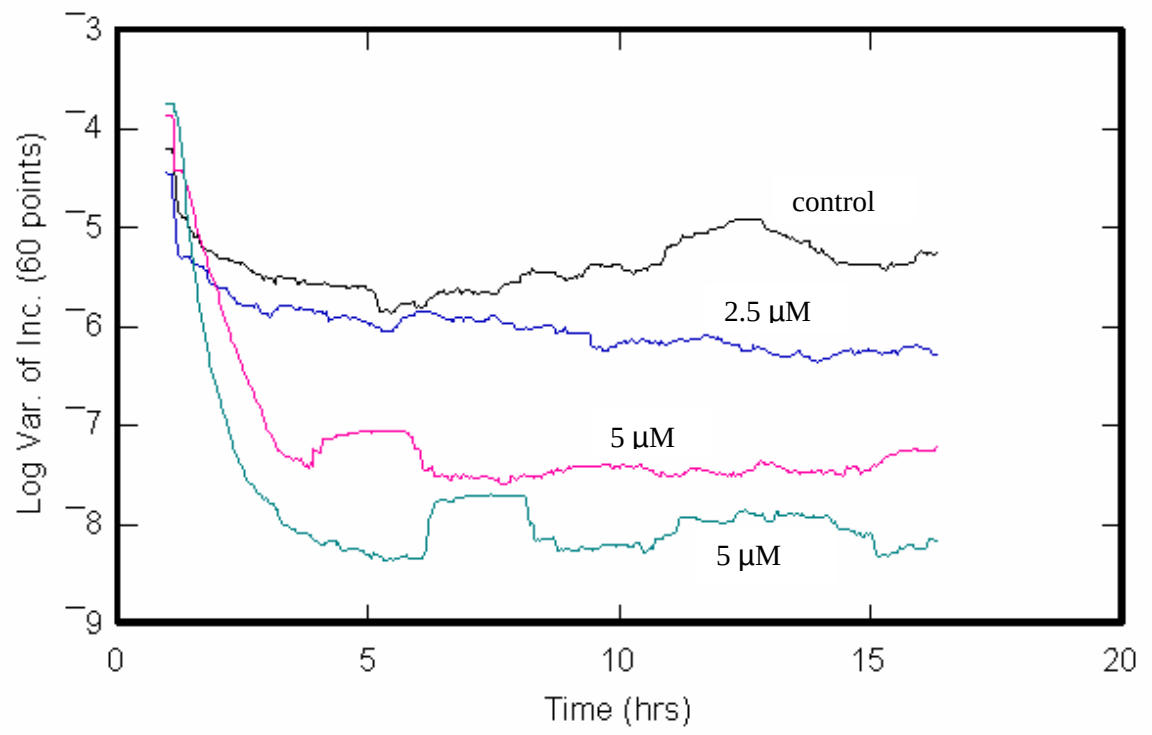



Figure 4.3c. Log variance of 3T3 cells with CB drug versus time

The variance fluctuates a lot at low concentrations since the cells are still alive and are changing their shapes all time, but at high concentrations there is less fluctuation because some cells are dead and others are under attack by the drug. Variance decreases with concentration (b) Effects of drug H7 on impedance sensor. The cell response to the drug H7, a protein kinase inhibitor, was studied in culture dishes by monitoring resistance for 20 hours, figure 4.4a.

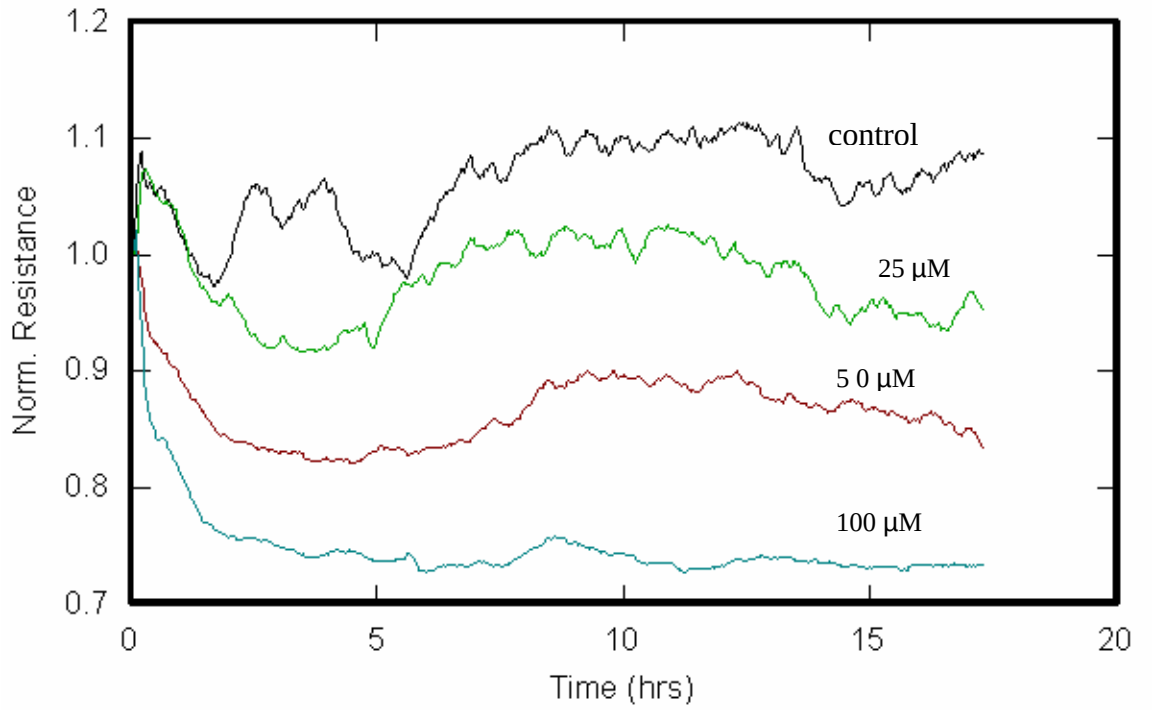
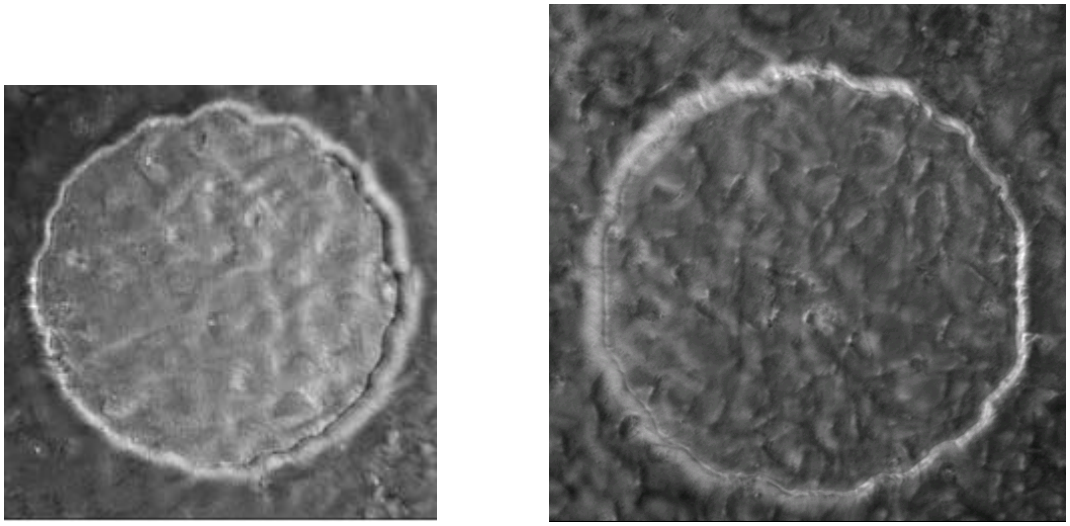



Figure 4.4a. Variation of 3T3 cells normalized resistancewith time at different concentration of H7.

The higher the concentration, the more the resistance fell. As can be seen from the figure H7 is not as toxic as cytochalasin B. CB starts affecting cells at a lower concentration that H7. The pictures also show how concentrations affect the cells in figure 4.4b



Control 50 μM

Figure 4.4b. Effects of H7 on 3T3 cells

The control cell is smooth while the other is rough showing the effect of the drug on the cell after 20 hours.

The more concentration, the rougher the surface showing that more cells are dying. Huang et al (28) made this observation clear through optical observation. The stained 3T3 cells were visualized using epifluorescence microscopy. They found that H-7 treatment of the cells reduced the abundance of large fibers over the course of 4 hours. The loss of the fibers resulted in less cell-substrate contacts without dramatic change of cell morphology and hence the reduction of resistance with concentration. Similar results were found through impedance study. They found that the drug lifted cells of the electrodes giving more room for the current to flow through and hence

resistance dropped. Figure 4.4c given below shows log variance of the cells versus time.

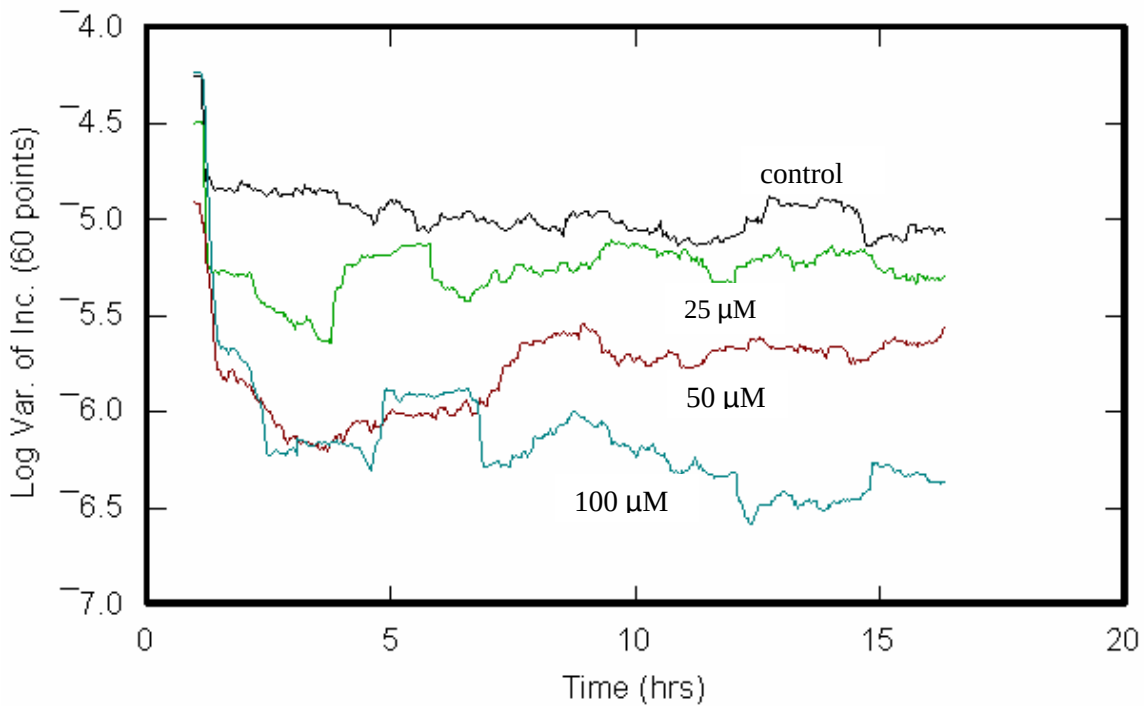



Figure 4.4c Log variance of 3T3 cells with H7 drug versus time

The variance fluctuates a lot at low concentrations since the cells are still alive and are changing their shapes all time, but at high concentrations there is less fluctuation because some cells are dead and others are under attack by the drug. The variance decreases with concentration (c) Effects of drug $CdCl_2$ on impedance sensor.

The drug effect on resistance of 3T3 cells with time at different concentrations is shown in figure 4.5a given below.

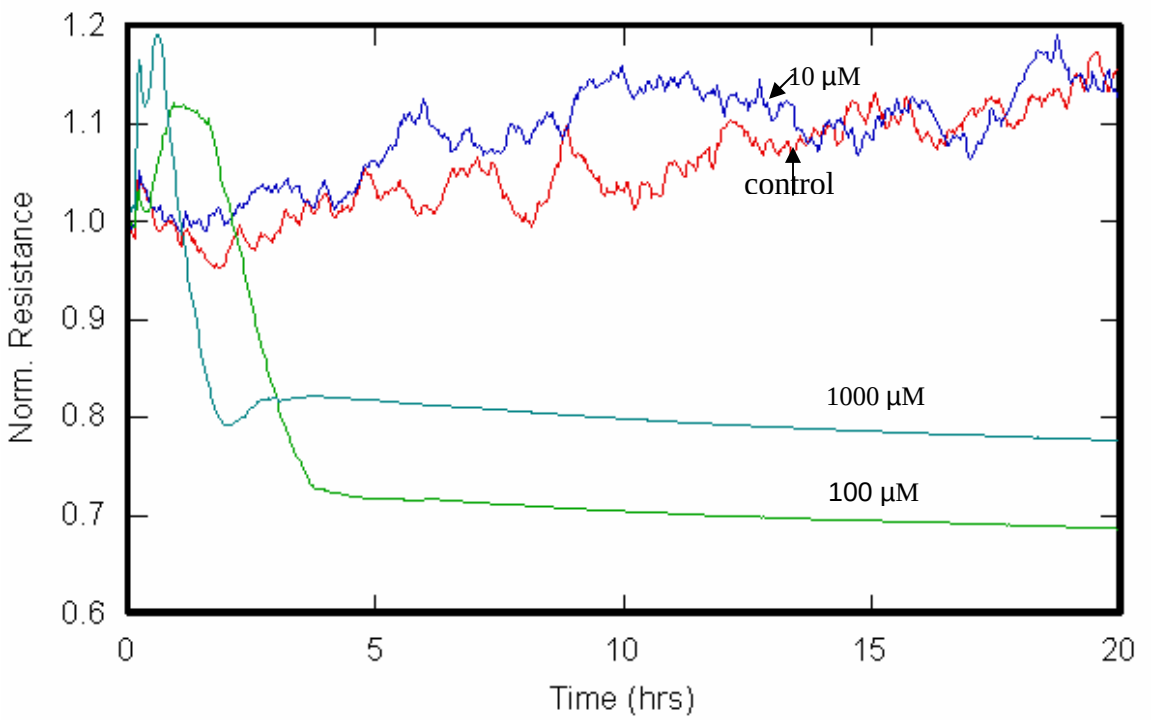
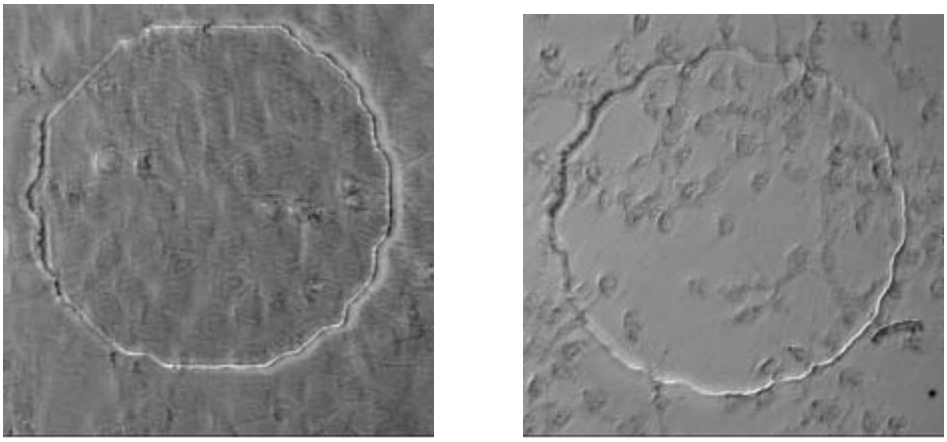



Figure 4.5a. Variation of 3T3 cells normalized resistance with time at different concentration of $CdCl_2$

The resistance dropped with increase in concentration of the drug. Small concentration of the drug did not affect the cells at all. At high concentrations the cells were attacked severely and after about 2 and 4 hours for 1000 µM and 100 µM respectively the cells were either killed or severely destroyed, since there was hardly any micromotion. The effect of the drug can also be seen on the image of cells shown figure 4.6b given below. The control cell is smooth. The cell at concentration of 100 µM has changed much showing that the drug only works at high concentrations.



Control 100 μM

Figure 4.5b. Effects of $CdCl_2$ on 3T3 cells

The effect on the drug on the cells can be explained as follows: toxic doses of transition metals are capable of disturbing the natural oxidation/reduction balance in cells through various mechanisms stemming from their own complex redox reactions with endogenous oxidants and effects on cellular antioxidant systems, which deranges the cellular signaling and gene expression systems (22) Ultimately, there is a variety of toxic effects, including apoptosis and carcinogenesis. Cadmium-induced apoptosis occurs through major mitogen-activated protein kinases, which have been shown to regulate apoptosis.

The effect of the drug is further seen from log variance with time as given in figure 4.5c given below.

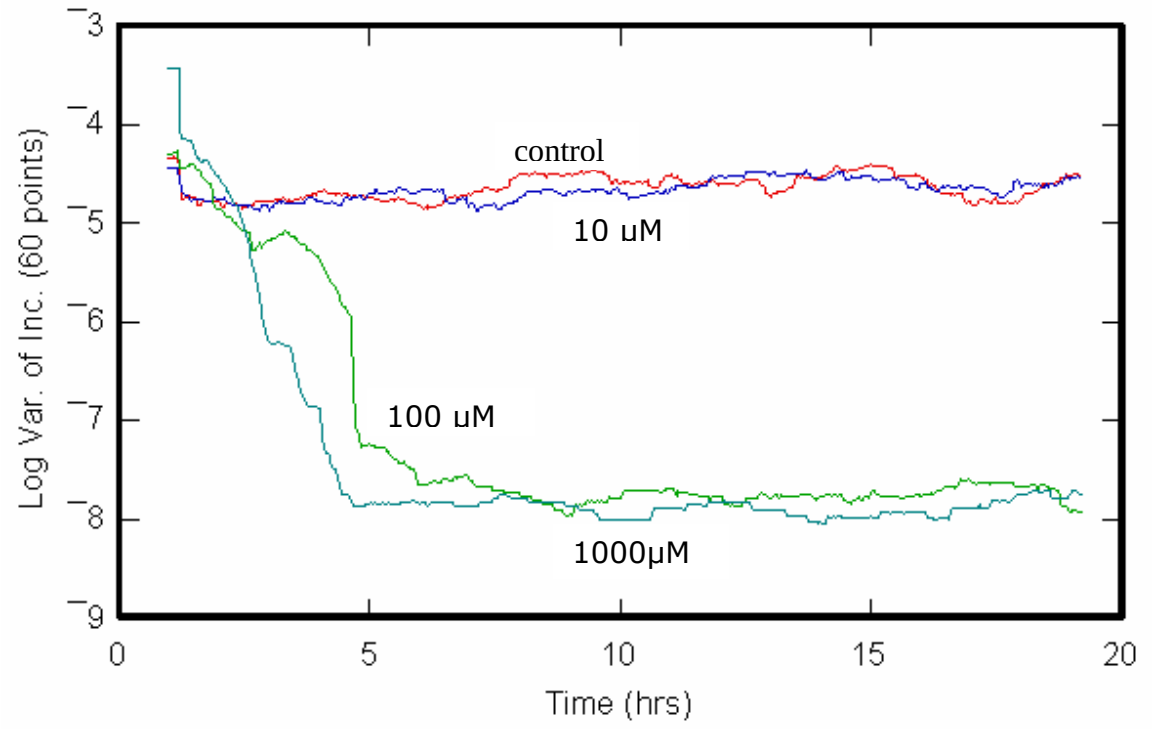



Figure 4.5c Log variance of 3T3 cells under $CdCl_2$ drug versus time

The variance fluctuates a lot at low concentrations since the cells are still alive and are changing their shapes all time, but at high concentrations there is less fluctuation because some cells are dead and others are under attack by the drugs. From the figure above, variance decreased with concentration.

A summary of the effect of the drugs on 3T3 cells is given in the table below. The table shows normalized resistances at different concentrations of the three drugs used.

| | CB | H7 | $CdCl_2$ |
|---|---|---|---|
| Control/ μM | 0.94 | 1.02 | 1.04 |
| 2.5 | 0.76 | | |
| 5 | 0.64 | | |
| 10 | 0.60 | | 1.08 |
| 25 | | 0.97 | |
| 50 | | 0.85 | |
| 100 | | 0.74 | 0.72 |
| 1000 | | | 0.82 |

Table 4.1. Effects of CB, H7 and $CdCl_2$ on 3T3 cells

Of the three drugs used on 3T3, CB affected 3T3 cells even at low concentration. The drug H7 also did affect the cell at low concentrations but rather mildly. $CdCl_2$ has little effect on the cells at low concentrations; however at high concentrations it kills the cells very effectively.

### 4.4 Cytoxicity Response of HUVECS to CB, H7 and $CdCl_2$

(a) Cytochalasin B effects on Impedance Sensor

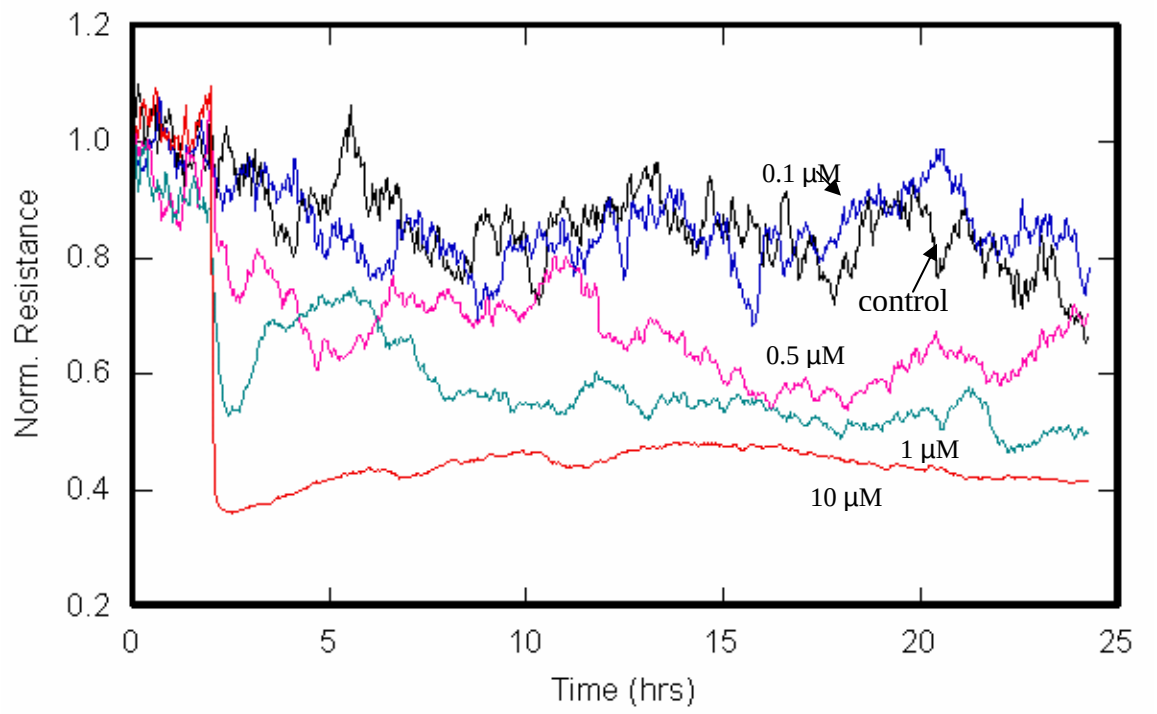



Figure 4.6a. Variation of HUVEC cells normalized resistance with time at different concentration of CB

Resistance measurement was performed to study HUVEC cell response to Cytochalasin B. Resistance response to concentration was monitored for 20 hours. The results are shown in figure 4.7a given above. As the concentration of CB increased, resistance dropped. At concentration of 10 µM after five hours, CB reduced the normalized resistance of HUVECs to 0.4 while that of 3T3 was reduced to 0.6. That means that HUVECs are easily detached from the electrode showing they make a weak contact on the electrode than 3T3 do. HUVECs are normal cells while 3T3 are abnormal in the sense that their cell division never

ceases. Such cells usually make a much firmer contact than normal cells on the electrodes.

The dependence of resistance on concentrations of CB can also been from figure 4.6b that show log variance of drug effect on HUVEC s against time. Variance is a measure of how spread out a distribution is. Log variance is large at high concentrations but small at low concentrations

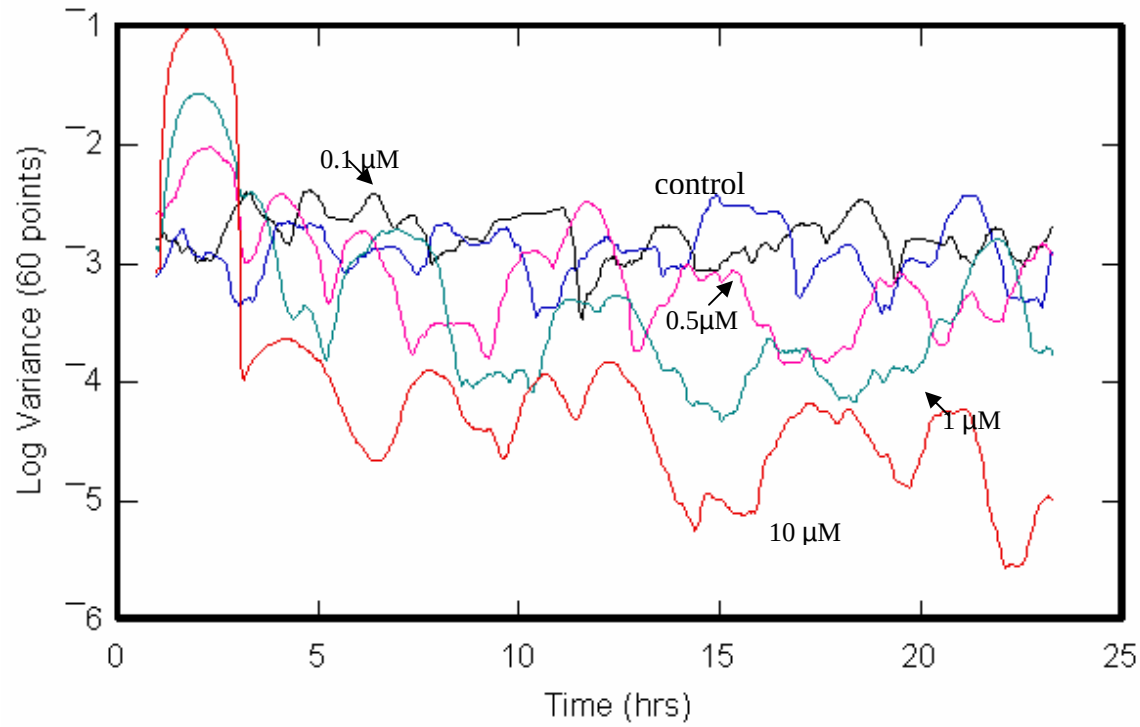



Figure 4.6b. Log variance of effects of CB on HUVECS verse time.

(b) H7 effects on Impedance Sensor

Figure 4.7a given below shows the variation of normalized resistance of HUVEC cells with time and various concentrations of H7.

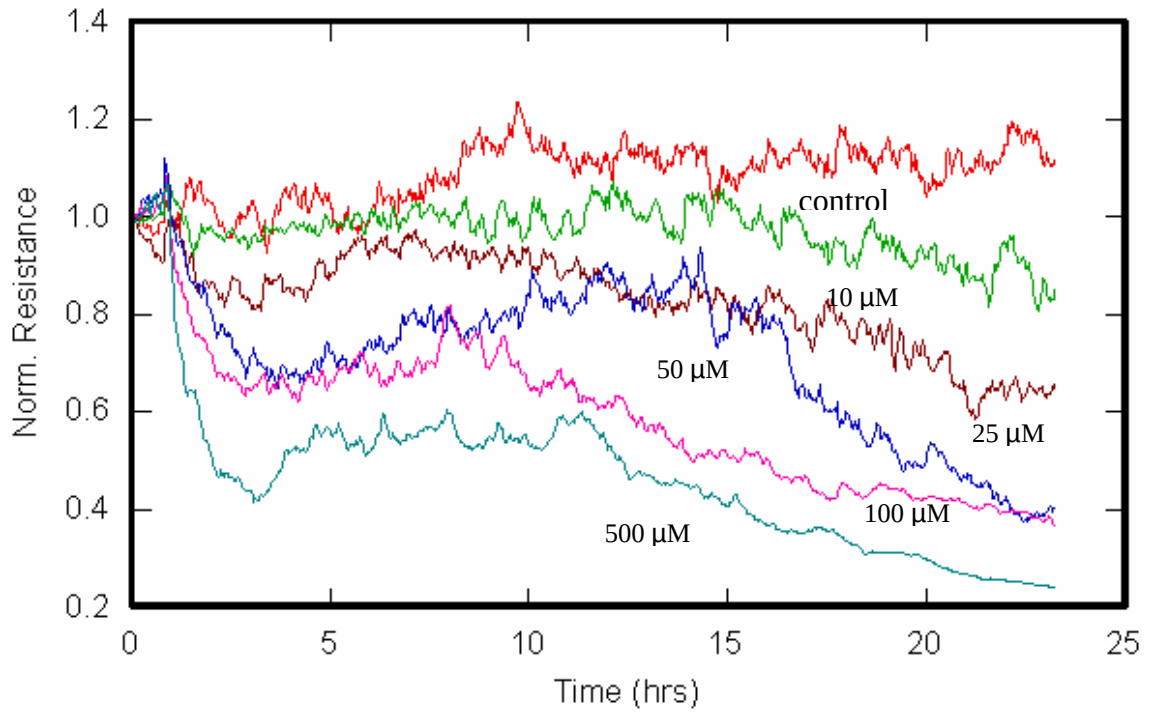



Figure 4.7a. Variation of HUVECs normalized resistance with time at different concentration of H7

Resistance dropped with increase in concentration of the drug. CB affects cells more than H7 does. For example, after 5 hours of exposure cells to drugs, at 10 µM, CB reduced HUVECs normalized resistance to 0.4 while at 500 µM of H7 reduced HUVEC resistance to only 0.6. This shows that CB is stronger drug that H7.The effects that H7 has on HUVEC and 3T3 cells is more or else the same as shown in table 4.1 below. The calculations were done after the cells had been exposed to drugs for 5 hours.

| Conc./ µM | HUVEC | 3T3 |
|---|---|---|
| 25 ± 1 | 0.88 ± 0.01 | 0.91 ± 0.01 |
| 50 | 0.70 | 0.85 |
| 100 | 0.55 | 0.74 |

Table 4.2. Effects of H7 on both HUVEC and 3T3 cells

Figure 4.7b given below shows the mildness of the drug on the cells.

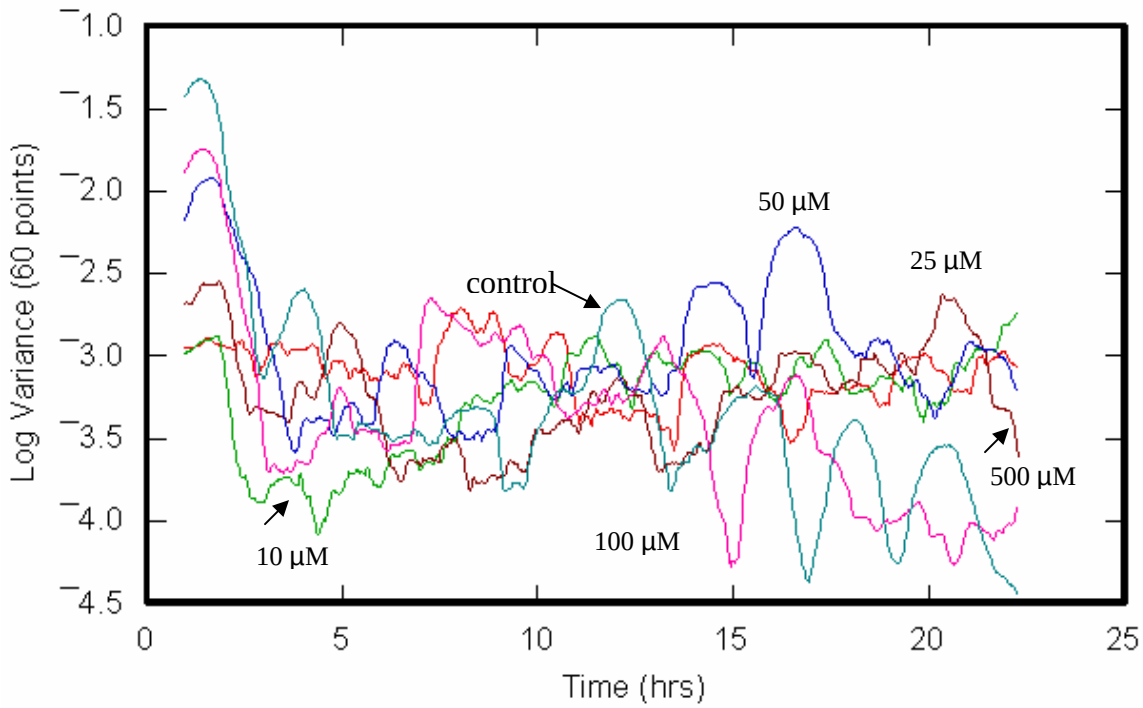



Figure 4.7b. Log variance of effects of H7 on HUVECS verse time.

There is no big difference in the variance at different concentrations showing that H7 drug does not affect the cells very much even at high concentrations.

(c) $CdCl_2$ effects on Impedance Sensor

Figure 4.8a given below shows the variation of normalized resistance of HUVECs with time at various concentrations of $CdCl_2$

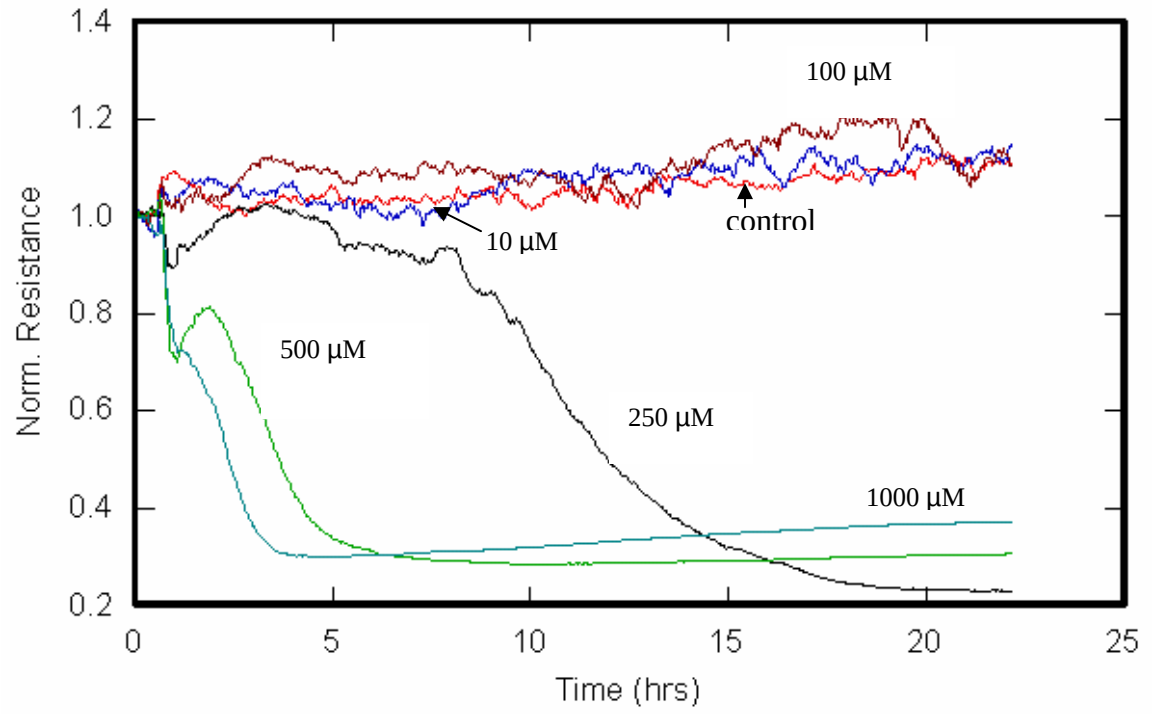



Figure 4.8a. Variation of HUVECs normalized resistance with time at different concentration of $CdCl_2$

The resistance varied inversely with concentration. Drug did not have effect on the cell at low concentration but high concentration it killed the cells or damaged them very severely since there was barely any micromotion of the cells. This effect of the drug is seen also from figure 4.8 B given below where variance is quite large at large concentrations but not well defined at low concentrations.

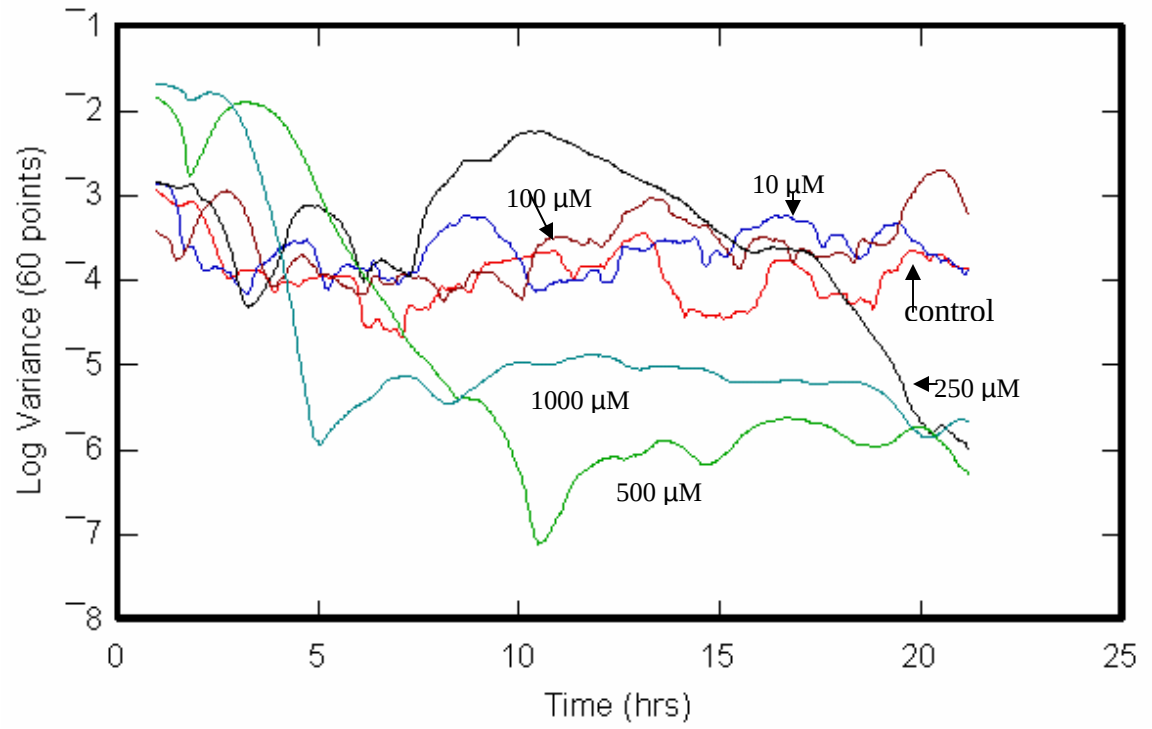



Figure 4.8b. Log variance of effects of $CdCl_2$ on HUVECS verse time.

The effects of the 3 drugs on HUVEC cells are summarized in the table 4.2 given below showing normalized resistance at various concentrations. The calculations were done after 5 hours of exposure of cells to the drugs.

| Conc/ µM | CB | H7 | $CdCl_2$ |
|---|---|---|---|
| control | 0.91 ± 0.01 | 0.98 ± 0.01 | 1.10 ± 0.01 |
| 0.1 | 0.84 | | |
| 0.5 | 0.71 | | |
| 1 | 0.63 | | |
| 10 | 0.43 | 0.97 | 1.05 |
| 25 | | 0.88 | |
| 50 | | 0.70 | |
| 100 | | 0.65 | 1.02 |
| 250 | | | 0.73 |
| 500 | | 0.55 | 0.31 |
| 1000 | | | 0.29 |

Table 4.3. Effects of CB, H7 and $CdCl_2$ on HUVECs

From the table above, we find that is CB drug is the best since it affects the cells at very low concentration. H7 is mild while $CdCl_2$ does affect a little cells at low concentration but very effective at high concentrations.

All the three drugs affect HUVECs more than 3T3 cells. This result was expected since 3T3 cells make a firm contact on the electrodes.

# CHAPTER 5

# CONCLUSION AND FUTURE PROSPECTS

## 5.1 Conclusion and Future Prospects

The impedance measurement technique is considered a very useful candidate because it can be applied to many quantitative, automated, and HTS experiments. We used it to study cytotoxicity tests of cells exposed to various drugs. The results described in this work reveal that the cell chip system would provide an easy and real-time monitoring method for cytotoxicity tests, especially for in vitro drug tests. Furthermore, this system could replace many conventional experiments dealing with not only animal cells but also bacterial cells. Two cells were used for cytotoxicity response namely 3T3 and HUVEC. We used the polystyrene dishes with culture medium to grow 3T3 cells but the HUVECs did not attach very well to the culture dish so gelatin medium was used to culture them. Cells were then added to the dish/flask as suspension and kept in the incubator at constant temperature of $37^0$C and 5% $CO_2$ to adjust pH of the mediums buffering system. The cells are usually spherical on the top of the dish and gets flattened on reaching the substrate as the begin attaching.

This process takes 2-3 hours for most commonly cultures cells. The individual cells then grow and divide. The daughter cell again spreads out, divide and continue the cycle till the dish is full confluent. Usually depending on the number of suspended cells, the culture dish gets confluent. Once the culture dish is confluent, the cells are then sub cultured after being trypsinized. We usually used HUVECs till passage 6 and 3T3 cells till passage 12.The cells were suspended on the evaporated gold electrode. The behavior of the cells is reflected in the measured impedance by using a lock-in amplifier. Voltage and phase data are stored and processed with a personal computer that controls the output of the amplifier and switches the measurement to different electrodes at various time during the measurement. This method thus provides the quantitative information of cell substrate interaction in real time. Moreover the current and voltage applied to the cell layer is sufficiently low so it does not affect the cell and can hopefully lead to many applications in future. A theoretical cell electrode model is developed in chapter 3, where the cells are regarded as spherical. The attachment and spreading data obtained were excellent. Through the ECIS measurements, we found the resistance of the cells as they were exposed to varying concentrations of different drugs. Thus we can conclude that ECIS can be very useful to study the cell morphology of different cell type.

From our results we found that both 3T3 and HUVEC cells made a similar firm contact on the electrodes. For the 3 drugs used, cytochalasin B, H7 and $CdCl_2$, cytochalasin B proved to be the best drug of all since it reacted with cells and reduced their resistances even at low concentrations. H7 reacted only mildly while cadmium chloride is only good at high concentrations. CB affected HUVECs more than 3T3 cells.

We envision the degree of decrease of resistance may be useful as a signature of the cell type and characterize the cell metabolism.

### 5.2 Future Aspect

To better understanding how ECIS can be used to test cytotoxicity of cells, further work will be done. More different types of cells, both normal and cancerous, would be investigated under different drug environments, especially anticancer drug environments. This may elucidate how ECIS can be used in identification of cell type and also to characterize cell metabolism.

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