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Assessment of Cytotoxicity Using Electric Cell−Substrate Impedance Sensing: Concentration and Time Response Function Approach

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This paper describes a simple and convenient method to measure the concentration and time response function *f* (*C*,*t*) of cells exposed to a toxicant by electric cell−substrate impedance sensing. Attachment and spreading of fibroblastic V79 cells cultured on small gold electrodes precoated with fibronectin were detected as electrical resistance changes. With this method, chemical cytotoxicity was easily screened by observing the response function of attached cells in the presence of inhibitor. The cytotoxicities of three test models, cadmium chloride, sodium arsenate, and benzalkonium chloride, were quantified by measuring the percentage inhibition as a function of the inhibitor concentration. The half-inhibition concentration, the required concentration to achieve 50% inhibition, derived from the response function agreed well with the results obtained using the standard neutral red assay.

Assays performed on cells in tissue culture have been considered as one of the established areas of in vitro assays that involve measurements of cytotoxicity, i.e., the ability of cytotoxic compounds to inflict damage and often kill cells. There is an essential need for determination of the cytotoxic potential of chemicals in environmental and medical research and in the food, cosmetic, and pharmaceutical industries. Cytotoxicity or the cell-killing property of a toxicant can be measured by following the cellular metabolic rate (e.g., tetrazolium salt cleavage),[1] the activity of a cytoplasmic enzyme (e.g., lactate dehydrogenase),[2] or the release of an artificial label, radioactive or nonradioactive.[3] Although useful and widespread, the two principal methodologies for testing cytotoxicity, the neutral red uptake assay (NR)[4] and the total cellular protein assay,[5] are not suitable for continuous, automation and real-time analysis. Cytotoxicity assays based on the loss of membrane integrity may suffer a serious shortcoming since the initial sites of damage of many, if not most cytotoxic agents are intracellular. Therefore, cells can be irreversibly damaged and commit to die whereas the plasma membrane is still intact.[6] Other cytotoxicity methods involve the detection of pH changes in the neighborhood of cultured cells by a silicon microphysiometer[7] and the measurement of the barrier function of a cell layer (transcellular resistance) upon exposure to test compounds.[8] Both of these noninvasive methods present quantitative data but require cell layers grown on membrane inserts.

There have been attempts to study cytotoxic effects by following intracellular motion of small vesicles or organelles in cultured cells using image analysis[9] and video-enhanced microscopy.[10] The procedures, however, require extensive data processing and only provide semiquantitative results. Of particular interest is the development of electric cell−substrate impedance sensing (ECIS) as a tool for in vitro toxicity testing.[11−14] This technique uses a small electrode that is deposited on the bottom of tissue culture wells and immersed in a culture medium. Inoculated cells drift downward and attach to the electrode surface to form a confluent layer. The attached and spread cells often act as insulating particles because of their plasma membrane to interfere with the free space immediately above the electrode for current flow.[11−14] Consequently, there will be a drastic change in the measured impedance due to the cell attachment and spreading.[11−16] Nevertheless, attached and spread neuronal cells were reported to be electrically transparent until tetrodotoxin, a highly specific sodium channel blocker, was added.[17] To date, ECIS has been

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able to provide quantitative information with respect to cell morphological changes, cell movements, and alteration in cellular function under various drug, chemical, and biochemical treatments.[18−22] As the response of a cell line is different from one toxicant to another, the ability to follow the time course of such cytotoxicity effects might reveal important information about the mechanism of cell death. Prevailing cytotoxicity assays such as NR[4] and the total cellular protein assay[5] only provide very limited information on the time course of cytotoxicity effects.

In this study, we describe a simple and convenient cell-based assay based on cell−substratum interactions as valuable predictors of in vivo response to sodium arsenate, cadmium chloride, and benzalkonium chloride (BAK) as three test models. The inhibition assays provide the inhibitor concentration required to achieve 50% or half-inhibition in real time, which agrees well with that of conventional inhibition assays. Here, we also attempt to quantify the response of cells induced by a chemical as a function of the cytotoxicant concentration and the exposure time.

EXPERIMENTAL SECTION

Materials. Cadmium chloride, sodium arsenate, Neutral Red, and benzalkonium chloride were purchased from Aldrich (Toronto, ON, Canada). Sodium chloride was a product of BDH (Toronto, ON, Canada), and fibronectin (purity over 99%) was obtained from Sigma (St. Louis, MO). Chinese hamster lung fibroblast V79 cells (93-CCL) were purchased from the American Type Culture Collection (ATCC, Rockville, MD).

Impedance Measurement with ECIS. On an ECIS sensing chip (8W1E, Applied Biophysics, Troy, NY), there are eight integrated culture wells, and the volume of each well is ~9 × 9 × 10 mm^3. On the bottom of each well there is a detecting gold electrode (circular, 0.057 mm^2) and a counter gold electrode (rectangular, 2 × 9 mm^2). There are two gold−liquid interfaces in the well when cell culture medium is added to the well. The impedance (Z_s) of the well is equal to the sum of the impedance on the two interfaces and the resistance of liquid between the two electrodes. Because the surface area ratio between the detecting electrode and the counter electrode is so small (~1/300), the impedance of the detecting electrode interface dominates Z_s. The impedance of an equivalent circuit with a resistance (R_s) and a capacitor (C_s) in series can be used to represent the impedance of the well.

In the ECIS system, an alternating potential (ac) is applied to the two electrodes through a 1-MΩ resistor. The two electrodes of the well are connected to a lock-in amplifier in the ECIS by a 120-cm cable (Figure 1). Our previous work[23] confirmed that the 120-cm cable contributed an appreciable parasitic impedance (Z_p: 1 kΩ, 0.19 nF), connected in parallel with Z_s. Because of the existence of Z_p, there is a significant difference between sample impedance and the impedance indicated by the commercial ECIS. A balance impedance (Z_b: 2.21 kΩ, 4.7 nF) was used to modify the ECIS, so that the modified ECIS can measure sample impedance precisely between 1 and 10 kHz. Detailed information on this subject including modified ECIS circuits and the impedance measurement can be found elsewhere.[23] Because the larger changes occur in the resistance, we have chosen to concentrate on those changes in the present study.

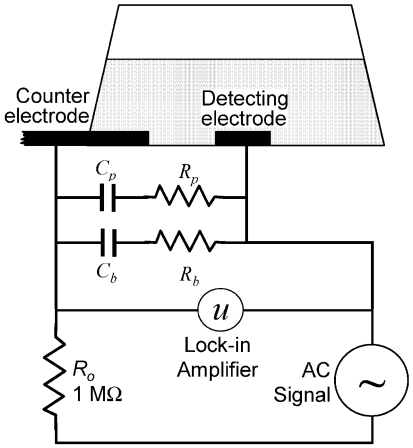

Figure 1. ECIS circuit and the structure of a well on an 8W1E sensing chip. The parasitic impedance (R_p = 1 kΩ, C_p = 0.19 nF) is contributed by a 120-cm cable connecting the well; the balance impedance (R_b = 2.21 kΩ, C_b = 4.7 nF) is used to calibrate the ECIS.

Cell Culture and Neutral Red Cytotoxicity Assay. Fibroblastic V79 cells (93-CCL) were grown at 37 °C in a humidified incubator containing 5% CO_2 for pH and humidity control. Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY) for the cell culture was supplemented with 5% fetal bovine serum (FBS, Sigma). Cell suspensions were prepared using 0.05% (v/v) trypsin, and cell viability was assessed using the trypan blue exclusion technique.[24] The NR assay was carried out as described by Borenfreund and Puerner[4] with slight modifications. The NR stock solution (20 mg/mL) was prepared by dissolving Neutral Red (Sigma, MW. 288.78, λ_{max} = 540 nm) in dimethyl sulfoxide. The NR working solution (50 μg/mL) was prepared by dilution of the stock solution in 10 mM HEPES-buffered DMEM, pH 7.2. BAK was used as a reference toxicant in the NR assay.

Adding a Toxicant to the Cell Suspension. Cadmium chloride, BAK, and sodium arsenate were dissolved in 0.85% NaCl to a concentration of 10 mM and used as the stock solutions. In a doubling dilution series, 0.5 mL of the stock solution was used to obtain a variety of diluted toxicant solutions. The diluted solutions (C_n) with volume V (10 μL ≤ V ≤ 20 μL) were added to a microtube consisting of 1000 μL of cell suspension (2.5 × 10^5 cells/mL). After thorough but gentle mixing, 0.2 mL of the resulting solution was introduced to each tissue culture well. The final toxicant concentration in the culture well is estimated as $C_nV/(V + 1000)$.

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Electrode Coating and Cell Inoculation. Fibronectin was dissolved in 0.85% NaCl to a concentration of 0.1 mg/mL, and then 0.1 mL of the resulting solution was injected into each well to coat the detecting gold electrodes. The chips were placed into an incubator for 60 min to ensure complete protein adsorption. After washing the wells with deonized water, 0.2 mL of culture medium was injected into each empty well and the impedance baseline of each well was monitored. The wells were then emptied, washed with water, and 0.2 mL of cell suspension (2.5×10^5 cells/mL) admixed with a toxicant was injected into each well. In the cytotoxicity assays, the toxicant was admixed with the cell suspension just before cell inoculation. The toxicant level in the 16 wells ranged from zero to the lethal concentration, which was predetermined by ABS_{559} as described later. One hour after inoculation, the chips were taken out from the CO_2 incubator and placed on an inverted microscope enhanced with video (Wilovert S AFL, Hund, Germany) for counting the number of attached cells (N_o). The chips were then brought back to the incubator and the impedance measurement was carried out for another 24 h.

Cytotoxicity Screening by ABS_{559}. DMEM consists of phenol red, a dye that serves as an indicator of the pH change during cell culturing. Due to the pH change, cells grown on DMEM affect the color of DMEM from red (pH 8.2) to yellow (pH 6.8). If the cell growth is inhibited by a toxicant, the DMEM color remains unchanged or changes very little compared to the negative control. In brief, 1 mL of cell suspension (2.5×10^5 cell/mL) admixed with a toxicant was injected into each well of a 24-well cell culture cluster (Corning, Corning, NY). The cluster was placed in the incubator for 56 h and then ABS_{559} (absorbency at 559 nm) of the medium in each well was measured by a spectrophotometer (model DU 640, Beckman, Fullerton, CA).

Real-Time Measurements of Cell Responses to Toxicants. After a well is inoculated with cells exposed to a toxicant, the resistance change (ΔR_s) of the well is dependent on the number (N_o) of initial cells attached on the detecting electrode, the toxicant concentration (C), and the exposure time (t). The resistance change normalized by N_o is defined as the cells response to the toxicant measured by ECIS.

$$f(C, t) = \Delta R_s / N_o \quad (1)$$

As a control, no toxicant is added in the culture medium; i.e., C is equal to zero and $f(0, t)$ will increase with time due to the attachment, spreading, and mitosis of cells on the electrode. When cells are exposed to some acute toxicants with high concentration, $f(C, t)$ will decrease and even approach zero, indicating total cell death. The inhibitor concentration required to achieve 50% inhibition measured by the ECIS response is defined as the half-inhibition concentration ($ECIS_{50}$).

$$f(ECIS_{50}, t)/f(0, t) = 50\% \quad (2)$$

Safety. Cadmium chloride, sodium arsenate, and BAK must be handled with extreme care. $CdCl_2$ is a carcinogen, teratogen, and tumorigen. Sodium arsenate is a clear, colorless crystalline material, and animal tests show that this substance possibly causes fetal malformations. BAK is an antimicrobial germicide[25] that acts by disrupting the cell wall of disease-causing bacteria and other microorganisms. To avoid inhalation or any skin contact with these chemicals, protective gloves and clothing were worn. Eye protection using safety glasses is included in the recommended respiratory protection. In addition, any preparation and handling of these materials must be performed in an exhaust hood equipped with ventilation. It is necessary to contain and dispose of these chemicals as hazardous waste.

RESULTS AND DISCUSSION

Responses of V79 to Chemical Compounds. Our previous work[23] showed that the gold electrode could be completely covered by fibronectin, a protein that strongly binds to cell surface protein integrins. Since integrin molecules connect with cytoskeleton inside cells, fibroblast cells can firmly attach to the fibronectin-coated gold electrode. Therefore, each electrode was precoated with an adsorbed fibronectin layer before exposing it to the cell medium containing cells. Cell−substratum interactions including spreading, morphology, and cell motility require a complex series of events to occur in a regulated and integrated fashion.[12] This includes the regulation of metabolic energy supplies, the formation/contraction or breakdown of actin microfilaments, and the formation and breakdown of attachments to the substratum. The resistance of the well filled with 0.2 mL of culture medium (DMEM containing 5% FBS) without cells was ~2 kΩ. When 0.2 mL of cell suspension without toxicant was injected in the well, all the cells descended to the well bottom within 30 min as observed by a video-enhanced microscope. After contacting the well bottom including the sensing area of the electrode, cells changed from round shapes (diameter ~9 μm) to flattened forms with much larger dimensions.

As fibroblast cells attached and spread on the protein-coated electrode surface, they altered the effective area available for current flow, causing a significant increase in the resistance of the well. The changing resistance showed a characteristic multiphasic pattern. Initially, as the cells attached to the detecting electrode, the resistance of the well increased very rapidly and then approached steady state, normally within 1 h in the experiments. For the inoculated 5×10^4 cells within 0.2 mL of culture medium, the number of cells attached on the detecting electrode of a well ranged between 10 and 70. One hour after cell inoculation, the resistance change contributed by each cell attached on the detecting electrode was estimated as ~43 Ω. About 6 h later, the resistance of the well increased significantly (Figure 2, control); therefore, we limited our cell culture time to 24 h during the toxicity assay.

When a toxicant at the lethal concentration is mixed with the cell suspension and used for inoculating the tissue culture well, the inhibitory effect on attachment, spreading, mitosis, and cytolysis could be very complex and time dependent. With an acute toxicant, a majority of cells die instantly and there will be no increase in the measured resistance. If cytotoxicity is not very acute, effector cells might still be able to attach and spread on the detecting electrode, leading to an increase in the response function. However, over the lengthy course of toxicant exposure, the attached and infected cells will eventually die or cannot bind

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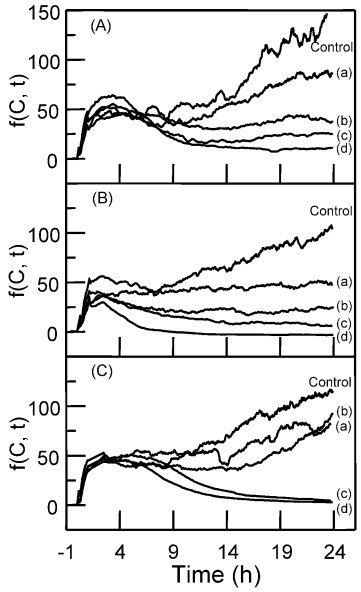

Figure 2. Responses (Ω per cell) of fibroblastic V79 cells to three cytotoxic chemicals (μM): cadmium chloride, (a) 2.9, (b) 4.6, (c) 6.2, and (d) 8.1; benzalkonium chloride, (a) 15.2, (b) 18.3, (c) 21.3, and (d) 30.4; sodium arsenate, (a) 45, (b) 60, (c) 140, and (d) 200.

firmly to the gold electrode, corresponding to a gradual decrease in $f(C, t)$. In an extreme case, the value of the response function $f(C, t)$ approaches zero, an indication of total cell death.

As shown in Figure 2, the cytotoxicity effect was concentration dependent for all three compounds tested. Although the lethal concentration of $CdCl_2$ to V79 is 8.1 μM, the value of $f(8.1, t)$ was bigger than $f(0, t)$ in the first 6 h of exposure of V79 cells to $CdCl_2$ (Figure 2A), and then $f(8.1, t)$ continuously decreased to near zero. With $CdCl_2$ at concentrations less than the lethal concentration, some cells might die, and some cells could survive. The surviving cells usually covered a broader area on the detecting electrode than the control cells. One might reason that when exposing V79 to $CdCl_2$, effector cells began with an impairment of the cell's ability to maintain ion homeostasis.[6] Consequently, there was an influx of water, extracellular ions, and cytotoxic chemicals from the culture medium to the cytoplasm.[6] The cytoskeleton of the V79 cells could also fall apart when the cells were subjected to $CdCl_2$, so the effector V79 cells were spread and occupied a broader surface area on the gold electrode. The mechanism of cell apoptosis and proliferation is a subject for further study and beyond the scope of this study. It should be noted that cadmium has been widely used for electroplating and galvanization processes, in the production of pigments, in rechargeable batteries, as a chemical reagent, and in miscellaneous industrial processes.[26] Cadmium is therefore naturally present in air, water, soil, and foodstuffs. It has been estimated that 98% of ingested cadmium comes from terrestrial foods, whereas only 1% comes from aquatic foods such as fish and shellfish, and 1% arises from cadmium in drinking water.[27] Cadmiun was chosen as one of the three test models in this study as a wide variety of adverse health effects, including kidney damage, kidney stones, heart diseases, and depressed immune functions have been attributed to chronic cadmium exposure, mainly from drinking water.[28] Cadmium may also increase cancer risk, for the lungs and prostate.[28] In higher doses, it can cause bone and joint aches and eventually lead to deformities or to more easily broken bones, known as the "itai-itai" disease, a fatal disease in many cases.[29]

The lethal concentration of BAK to V79 is 30.4 μM (Figure 2B). The value of response functions $f(C, t)$ was lower than its control immediately after cell inoculation. Microscopic examination revealed that there were many vesicles in V79 cells exposed to BAK. The microscopic observation thus validated the resistance data obtained for BAK since ECIS measurements were sensitive to any change in the current path, and in this case, there was a significant decrease in the transcellular resistance of the cell layer, a result of membrane damage. As widely used in many household products as a germicide, BAK has been known to act upon microorganisms by altering cell membrane permeability and lysing cytoplasmic contents.[25,30] In 1992, the FDA proposed a ban on the use of BAK to treat insect bites and stings and in astringent drugs because it has not been shown to be safe and effective for stated claims in over-the-counter products.

With sodium arsenate, the inhibitory effect only began from 6 h into the experiment (Figure 2C) and this compound was less toxic than both $CdCl_2$ and BAK as reflected by the lethal concentration of 200 μM. The amplitude of the noise in the response functions with toxicants was smaller than that of the control. Notice that the fluctuation in the measured resistance ceased when the cells were exposed to high toxicant levels (Figure 2). Such killed cell data supported the biological nature of the fluctuation, a unique feature of viable and attached cells as detected by ECIS. Inorganic arsenic is considered as the most toxic form of the element and is found in groundwater and surface water, as well as in many foods.[31] A wide variety of adverse health effects, including skin and internal cancers and cardiovascular and neurological effects, have been attributed to chronic arsenic exposure, mainly from drinking water.[31]

Half-Inhibition Concentrations $ECIS_{50}$. ECIS can simultaneously measure the response functions $f(C, t)$ of 16 wells integrated on two 8W1E sensing chips. At a given time t_o, the relationship between $f(C, t_o)$ and C can be used to construct an inhibition curve at time t_o. Based on eq 5, the half-inhibition concentration ($ECIS_{50}$) of a toxicant measured by ECIS could be estimated from the inhibition curve. Figure 3 showed the inhibition curves of cadmium chloride, BAK, and sodium arsenate at t_o = 16 h. Their $ECIS_{50}$ was then determined to be 4.0, 14.0, and 50.0 μM, respectively. Notice that, on the BAK inhibition curve, there were four points with small negative values ($-5 < f(C, t) < 0$). The response functions of other acute toxicants such as $HgCl_2$ at lethal concentration also had small negative values when V79 cells

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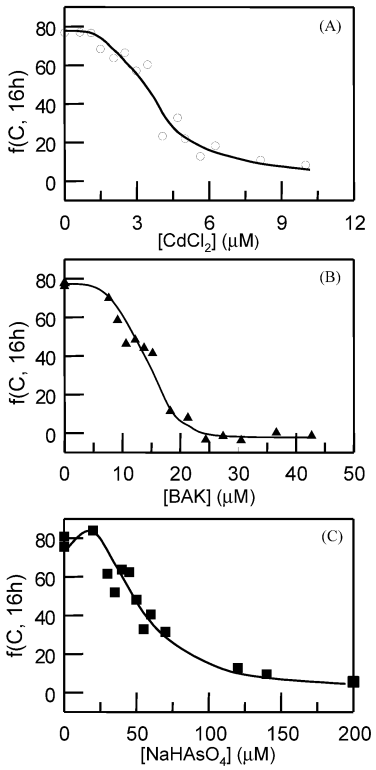

Figure 3. Inhibition curves derived from Figure 2 at $t_o = 16$ h: (A) $CdCl_2$, (B) BAK, and (C) $NaHAsO_4$. Half-inhibition concentrations were 4.0, 14.0, and 50.1 μM, respectively.

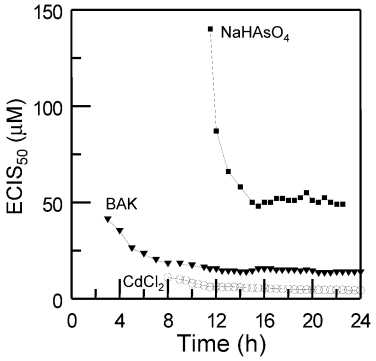

Figure 4. Relationship between the half-inhibition concentration and time during cell culture for the three toxicants: ■, $NaHAsO_4$; ▼, BAK; ○, $CdCl_2$.

completely died and floated in culture medium. Various intracellular substances released by lysed cells might significantly alter the conductivity of the culture medium. Under this circumstance, the resistance measured by ECIS is diminishing throughout the course of measurement as soon as cytotoxicity comes into play and the value of $f(C, t)$ would fall slightly below zero.

From the response functions shown in Figure 2, inhibition curves at other times during cell culture could also be obtained in the same way as at $t_o = 16$ h, and the relationship between $ECIS_{50}$ and time during the course of cytotoxicity assays can be found (Figure 4). This is a unique feature of ECIS that can monitor the half-inhibition concentration changing with time. Although the effect of cadmium chloride could be seen immediately, its inhibition could be detected 7 h later. Between the seventh to tenth hour, $ECIS_{50}$ quickly decreased from 10 to ~5 μM, and in the later period, it slowly decreased and leveled off at 4 μM. The inhibition of BAK to V79 could be seen from the measurements of $ECIS_{50}$ immediately after cell inoculation. In the first 10 h, $ECIS_{50}$ drastically decreased from 41 to 18 μM and then slowly approached ~15 μM. The toxicity of sodium arsenate to V79 cells dramatically decreased 10 h after exposure to the toxicant. $ECIS_{50}$ quickly decreased from 140 to ~50 μM.

Table 1. Half-Inhibition Concentrations (μM) Obtained by ECIS and Other Procedures

	methods			
toxicant	ECIS (±SD)	NR (±SD)	ABS_{559} (±SD)	literature data
$CdCl_2$	3.9 (±0.4)	3.0 (±0.4)	3.9 (±0.2)	4[a]−20,[b] 1,[c] 10[d]
Na_2HAsO_4	51.0 (±6.7)	52.2 (±7.7)	58.0 (±2.0)	110[e]
BAK	13.8 (±0.5)	15.3 (±0.9)	15.2 (±2.3)	10[f]

[a] 3T3 cells with 1% FBS (fetal bovine serum), 24-h exposure.[32] [b] 3T3 cells with 10% FBS, 24-h exposure.[32] [c] V79 cells with 10% FBS, 4-h exposure.[35] [d] Sp2/0 cells with $Cd(O_2CCH_3)_2$.[33] [e] 3T3 cells with 10% FBS, 24-h exposure.[34] [f] 3T3 cells with 10% FBS, 24-h exposure.[34]

Table 1 compares the half-inhibition concentrations of the three toxicants measured by the three different methods. All data in Table 1 came from the average of three repeated experiments. The average $ECIS_{50}$ was measured between 14 and 24 h after V79 cells were exposed to the three toxicants; the average of NR_{50} and ABS_{559} were measured at 24 and 56 h, respectively, after cells were exposed to toxicants. The results obtained from ECIS agreed well with that of the Neutral Red and ABS_{559} assays. It is, however, somewhat difficult to compare the results obtained with the literature data since the half-inhibition concentration depends on the cell line, exposure time, and compositions of culture media. Cytotoxicity substances might react with medium components producing adduct modifications that alter the ability of the cells to maintain normal metabolism. For instance, the cytotoxicity of cadmium was reported to depend on the concentration of fetal bovine serum in the culture medium as this metal binds to serum proteins (Table 1).[32−34] In general, our results agreed reasonably well with some selected half-inhibition concentration values reported in the literature given the differences in the cell line, exposure time, culture medium, and the assay procedure.

CONCLUSION

In vitro cultures systems used for cytotoxicity testing are a unique combination of living cells, culture medium with appropriate nutrients, and solid supports including extracellular matrix components. In this context, ECIS is designed to provide a test system in which the biological component can be maintained in a defined environment that affects the attachment, spreading, and micromotion of living cells. Concentration and time response functions of cells exposed to toxicants can be directly measured by ECIS. The response time of living cells to different toxicants

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is noticeably different. In this context, the response functions can be used for a real-time cytotoxicity assay. ECIS is presented in a format that is convenient to manipulate toward the development of higher density microwell formats with virtually no modifications for rapid and parallel screening. This is a precise, fast, and simple alternative to quantitate cytotoxicity/cytosis for many in vitro cell systems based on the measurement of electrical resistance changes.

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