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Monitoring Motility, Spreading, and Mortality of Adherent Insect Cells Using an Impedance Sensor

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An emerging sensor technology referred to as electric cell−substrate impedance sensing (ECIS) has been extended for monitoring the behavior of insect cells including attachment, motility, and mortality. In ECIS, adherent cells were cultured on an array of eight small gold electrodes deposited on the bottom of tissue culture wells and immersed in a culture medium. Upon the attachment and spreading of cells on the gold electrode, the impedance increased because the cells acted as insulating particles to restrict the current flow. Experimental data revealed that insect cells interacted differently with various proteins used to precoat the gold electrode with concanavalin A as the best promoter to accelerate the rate of cell attachment. After the cells were fully spread, the measured impedance continued to fluctuate to reflect the constant motion and metabolic activity of the cells. As the cell behavior was sensitive to external chemicals, the applicability of ECIS for inhibition assays was demonstrated with $HgCl_2$, trinitrotoluene, trinitrobenzene (TNB), and 2-amino-4,6-dinitrotoluene as model systems. Unlike conventional assays, the quantitative data obtained in this study are taken in real time and in a continuous fashion to depict cell motility and mortality.

In vitro, normal cells generally exhibit restricted growth requirements with a finite proliferation and they usually attach to a surface, a condition referred to as anchorage dependence. Prior to cell spreading, the cells secrete extracellular matrix proteins and proteoglycans which adhere to the substrate, normally with a slight net negative charge. The cells then bind to molecules in the extracellular matrix by specific cell surface receptors.[1] Interference reflection microscopy reveals that cells are capable of attaching to the substratum and there are spaces or channels (the order of nanometers) between the ventral side of the cell and the substratum.[2] Albeit several advances have been achieved in quantifying physiological and biochemical activities in tissue culture, it is still formidable and time-consuming to assess changes of cell morphology and motility. The microscope has been widely used to study cell attachment and spreading with the result described only in qualitative terms. For a continuous record of the event, cinematographic arrangements are necessary and data obtained are very difficult to quantify and usually require image processing with extensive data manipulation.[3]

Cell spreading, morphology, and micromotion, three important parameters in tissue culture, have been quantified using an emerging electrical method referred to as electric cell−substrate impedance sensing (ECIS).[4,5] The embodiment of this technique is a small electrode deposited on the bottom of tissue culture wells and immersed in a culture medium inoculated with a specific cell line. Inoculated cells have a tendency to drift downward and attach to the surface of the electrode. The size of the electrode will restrict the maximum cell population that can be observed by this technique. For instance, an electrode with a surface area of 0.001 cm^2 will restrict a maximum cell concentration to 50−100 cells.[6] Using culture medium as the electrolyte, a constant current source applies a small ac current of ∼1 μA at 4000−5000 Hz between the small detecting electrode and a larger counter electrode and the resulting voltage is monitored by a lock-in amplifier. The attached cells, acting as insulating particles because of their plasma membrane, will interfere with the free space immediately above the electrode for current flow. Owing to its smaller size compared to the counter electrode, the detecting electrode will dominate the overall impedance in the circuit, which can increase as much as 10-fold in a few hours.[7] The changing impedance can be continuously monitored and interpreted to reveal information about cell spreading and micromotion.[5−7] The impedance, a result of the coordination of many biochemical reactions, is very sensitive to operating conditions such as pH, temperature, and chemical compounds added to the culture medium. The broad response to changes in the environment allows this method to serve as a general tool for quantitation of cell spreading and motility as well as an alternative to animal testing for toxicology studies. Therefore, the applicability of ECIS for inhibition assays has been demonstrated using toxic or noxious agents such as cytochalasin D (cytoskeletal inhibitor),[7] prostaglandin E_2 (inflammatory mediator),[9] bacterial protease, and bacterial proteins that perturb

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extracellular matrix and cytoskeleton.[10] ECIS was also used to study the impedance changes upon removal of calcium from confluent Mardin-Darby canine kidney cell layers.[11] To date, a plethora of biosensors has been designed to be highly specific for a target analyte,[12] although there is an urgent need in designing sensors that can respond to a wide variety of chemicals in an integrated and continuous fashion. It should be noted that some related techniques have been reported for monitoring toxicity of compounds using whole cell approaches involving the use of a silicon microphysiometer (detection of pH changes)[13] or the measurement of the barrier function of a cell layer (transcellular electrical resistance)[14−16] upon exposure to toxicants. However, unlike ECIS, these methods require cell layers grown on membrane inserts which are not suitable for continuous and real-time analysis.

In this study, the question of whether the ECIS approach could be extended for studying the spreading and micromotion of insect cells has been addressed. First, it is not understood whether insect cells must be attached to a surface prior to their spreading and replication. Second, rates of mammalian cell attachment and spreading are well known to be dependent upon the type of protein coating the substratum; however, this phenomenon is not well studied for insect cells. Insect cell−substratum interactions were then proven as valuable predictors of in vivo response to $HgCl_2$, trinitrotoluene (TNT), and two of its derivatives. To our knowledge, this is the first demonstration using the ECIS system to study the spreading, micromotion, and inhibition of toxins with respect to insect cells. The insect cell−baculovirus host vector system has been increasingly utilized for the expression of heterologous proteins, including many high-value biologicals such as growth factors, hormones, and vaccines for both human beings and animals.[17,18]

EXPERIMENTAL SECTION

Cell Line and Culture Conditions. *Spodoptera frugiperda Sf* 9 cells were maintained in 125-mL disposable Erlenmeyer flasks with a working volume of 20 mL in serum-free SF-900 II medium (Gibco BRL, Canadian Life Technologies, Burlington, ON, Canada). Cells were cultured weekly at 0.4×10^6 cells/mL at 27 °C, pH 6.2, with agitation at 110 rpm. The cell counts were determined using a hemacytometer and Coulter Counter Multisizer II (Coulter Electronics, Hialeah, FL), and the cell viability was performed via the trypan blue exclusion method. *Sf* 9 cells, inoculated at an initial cell density of 0.4×10^6 cells/mL, were grown to the midexponential phase ($(3-3.5) \times 10^6$ cells/mL) and the resulting cells were aseptically centrifuged at 1000 rpm for 10 min. The supernatant, referred to as the spent medium, was collected and stored at 4 °C until needed. Pellets were thereafter suspended at a cell concentration of 1×10^6 cells/mL in a medium containing 50% of the fresh culture medium and 50% of the spent medium.

The ECIS Biosensor System. The ECIS biosensor system (model 100, Applied Biophysics, Troy, NY) has the capability of simultaneous measurements of from 1 to 16 individual cultures. In this system, a 1-V ac signal is applied to the electrodes through a 1-MΩ series resistor at 4000 Hz and the in- and out-of-phase voltages across the electrodes are measured using a phase-sensitive lock-in amplifier/preamplifier.[4] Both data acquisition and processing were performed using the software supplied by Applied Biophysics. Each ECIS disposable electrode array consists of eight gold film electrodes (0.5×10^{-3} cm^2) and delineated with insulating films with a much larger common counter electrode (0.2 cm^2) located at the base of 10-mm square wells (volume of ∼0.5 mL). For the attachment studies in this work, the voltage data were used to calculate a value for the resistance of the system, treating it as a series RC circuit (a resistor and a capacitor in series). Detailed information on this treatment and the ECIS system can be found elsewhere.[4,19,20]

To follow the cell growth using the ECIS biosensor system, cells were taken from the midexponential growth phase and inoculated in the ECIS biosensor at 150−200 cells/gold film electrode. Cells were then allowed to attach and spread for a few minutes before measurement. Unless otherwise indicated, all culturings were at 27 °C with high humidity. Each well was normally used with 0.4 mL of medium, ∼80% of the well volume, and the sampling time was 5 min.

Monitoring of Protein Binding to Gold Surface. The binding of concanavalin A (Con A) and fibronectin to the bare gold surface was performed using a surface plasmon resonance (SPR) biosensor (BIAcore 2000, Biacore AB, Uppsala, Sweden). The system was equilibrated using a PBS buffer (50 mM, pH 7.4) at a flow rate of 5 μL/min. Four chambers on the chip were used for Con A with concentrations of 0.1, 0.25, 0.5, and 0.75 mg/mL, and three chambers of another chip were used for fibronectin at concentrations of 0.05, 0.1, and 0.5 mg/mL. After the sample injection (350 μL), the buffer was introduced to the chamber and sensorgrams were recorded. By nonlinear regression analysis, K_s was obtained from the sensorgram as $R = R_{max}(1 - e^{-K_s t})$ for each protein concentration, where R is the optical response of the BIAcore biosensor system, R_{max} is the maximum response, and $K_s = k_{assn}\, C + k_{dis}$. From the K_s vs C (concentration of Con A or fibronectin) plot, k_{assn} (association constant) and k_{dis} (dissociation constant) were obtained as slope and intercept of the linear regression line and $K_d = k_{dis}/k_{assn}$. It should be noted that $1/K_d$ is known as the binding constant.

RESULTS AND DISCUSSION

Optimized Conditions for Cell Growth and Adhesion. It is well known that the electrode can be represented as a series

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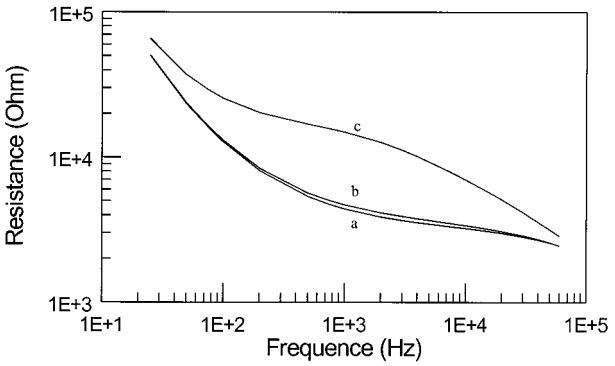

Figure 1. Relationship between resistance and frequency for a cell-free electrode (curve a), electrode coated with Con A (curve b), and Con A-coated electrode inoculated with 3.2×10^6 cells/mL (curve c).

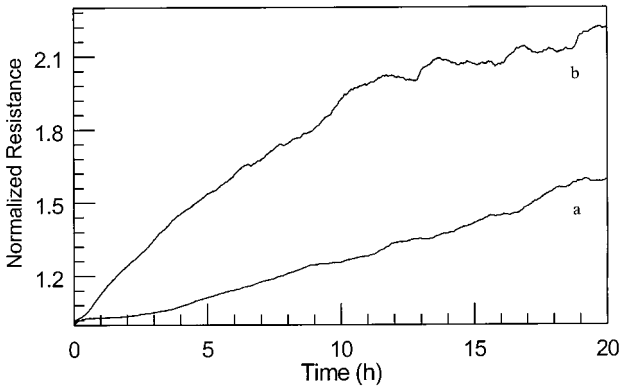

Figure 2. Effect of cell resuspension medium on the cell adhesion with ECIS data presented as normalized resistance at time zero. The bare electrodes were inoculated from a cell stage of 3.2×10^6 cells/mL: (a) fresh medium; (b) 50% spent medium.

RC circuit[21] and both the resistance and the capacitance of a conductor electrolyte vary as $f^{-\kappa}$, where $0 < \kappa < 1$ and f is the frequency. The numerical value of R_{sol} or the constriction resistance is simply equal to the asymptotic value at high frequency of the measured resistance for bare electrode in the absence of the cells (Figure 1, curve a). The result was almost identical for a cell-free electrode coated with Con A (Figure 1, curve b) whereas the resistance of the Con A-coated electrode was significantly higher in the presence of the insect cells at frequencies below 10 000 Hz (Figure 1, curve c). The R_{sol} value of the Con A-coated electrode before inoculation was determined to be 3100 Ω, and strictly speaking, this value should be subtracted from the measured impedance before the calculations are done and then added back for comparison with the experimental results. It has been recognized that the impedance of the electrode will decrease inversely with the electrode area whereas the solution resistance above the open electrode will decrease inversely with the square root of the electrode area.[7] As the detecting electrode is significantly smaller in area compared to the counter electrode (~400-fold), the impedance of the counter electrode and the tissue culture solution can be mostly neglected; i.e., the impedance of the detecting electrode dominates the measurement. Notice that the cells influence the impedance measurement but are not adversely affected by the ac current and the weak electric fields. The frequency used in this study is still low so that the cells can be regarded as insulating materials. At much higher frequency than 4000 Hz, the current will start to flow through the cells because of the high capacitance of the cell membranes.[22] At +1 V and 4000 Hz, the current should be ~1 μA with a resulting voltage drop of a few millivolts across the cell layer. This level was proven not to have any detectable effect for fibroblast and endothelial cell lines.[23] Based on such information, the measurement at this frequency was anticipated to be also noninvasive for the insect cells used in this study.

An experiment was first conducted to study the growth and adhesion of insect cells using a bare gold electrode. Although the insect cells were capable of drifting downward and then attaching to the bare gold electrode, this phenomenon only became noticeable after 10 h of incubation with a corresponding increase in the normalized resistance of about 1.4−1.5-fold (Figure 2, curve a). Microscopic examination revealed the spherical cells attached to the electrode and other parts of the well, and there was an increase in the cell population during the course of the experiment. Nonadherent cells, however, caused practically no change in the impedance, and the impedance or resistance increase was not exactly proportional to the fraction of covered electrode area. To get the cells to thrive and replicate more rapidly, it was necessary to augment the culture medium with 50% of the spent medium (Figure 2, curve b). The spent medium was expected to contain several unknown and complex compounds or growth factors required by the cells for their survival and proliferation. Growth factors are generally secreted as diffusional proteins and transduce proliferation and differentiation signals by binding to specific receptors on the target cell membrane or specific extracellular matrix proteins.

The final normalized resistance and the response time were dependent upon the size of the inoculum, i.e., the initial cell concentration used for inoculation. The best result was obtained with an inoculum size consisting of ~3.2×10^6 cells/mL harvested in the midexponential phase (3.6−4.6 h of incubation in the shake flask). Microscopic examination revealed that the number of cells attached to the surface of the gold electrode was about 150−200 cells with an average diameter of 13 μm. The value of cell loading was not completely unexpected since the detecting electrode with a surface area of 0.0005 cm^2 should be able to accommodate up to 300 cells.

In all cases, after the steady-state normalized resistance had been attained, the measured resistance continued to fluctuate as a result of the constant motion of the cells altering the current flow in subtle ways. Although motions responsible for the observed impedance fluctuations are the end result of a chain of complex reactions in the cells, one might anticipate that as cells change shape within one place, there will be a change in the average size of spaces existing between the ventral surface of cells in culture and their substratum.[8] Therefore, the impedance fluctuations reflected the vertical motion of cells in confluent layers[4,8] and changes in the transcellular resistance of the layer[3] in contrast to the horizontal cell locomotion, a movement across a substratum. This behavior was referred to as micromotion[8,11] since the size of the vertical motion is of the order of nanometers, significantly below the resolution of optical microscopy. The

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fluctuations were observed to cease immediately when 20 μg/mL $HgCl_2$ was added to the well, which killed the cells almost instantaneously (figure not shown). There was a significant drop in the normalized resistance value (~30%) and the resulting signal became very flat, confirming that electronic noise of the ECIS system was considerably below the level of the measurement. The response to $HgCl_2$ of the ECIS system using the insect cells thus illustrated a potential application of the technique for inhibition/toxicity measurements of heavy metals.

Rate of Cell Attachment Using Protein-Coated Electrodes. Although insect cells were capable of attaching directly to bare gold films, the elapsed time was very lengthy and generally not useful for sensor applications. Since the rapid anchoring behavior of most animal cells is crucial for the ECIS system, a series of experiments was conducted to evaluate the anchoring-dependent property of the insect cells using gold electrodes precoated with monolayers of different proteins. Cell attachment studies reported in the literature have demonstrated certain proteins are involved in the process of cell surface binding in tissue culture as the cells possess receptors specific for fibronectin and for the Fc portion of IgG molecules.[23−26] This was the main reason proteins such as fibronectin, bovine serum albumin, and gelatin were used to coat gold electrodes to study the spreading and motility of mammalian cells.[22−24] In view of this, fibronectin, concanavalin A, gelatin, and *Mytilus* adhesive protein (MAP) were used to coat the detecting electrodes in this study. All chemicals were obtained from Sigma (St. Louis, MO). Such results were achieved by applying 5 μL of protein (100−500 μg/mL) directly over the detecting gold electrodes. Following 30-min incubation to ensure adsorption of a complete monolayer of protein molecules, the unadsorbed protein was rinsed away and the protein-coated electrodes were inoculated with cell suspension. The final average resistance provides a convenient measure when the cells have fully spread and reached confluence. The insect cells showed clear preferences among the various proteins. There was hardly any appreciable increase in resistance with gelatin and the MAP-coated electrodes. Con A, a lectin purified from *Concanavalis ensiformis*, promoted the best adhesion behavior for the insect cells. For the Con A-precoated electrode, confluence was reached within a few hours with a resistance increase of ~2-fold from that of a bare electrode. Interestingly, Con A was superior to fibronectin, a well-known cellular "glue" for various mammalian cells. Surface plasma resonance experiments also confirmed that Con A adsorbed more strongly to a gold surface in comparison to fibronectin as reflected by a significantly smaller K_d value, 0.89 nM (the binding constant, $1/K_d = 1.12$ nM^{-1}) for Con A in comparison to 63 nM for fibronectin ($1/K_d = 0.015$ nM^{-1}).

An experiment was then performed to study the effect of Con A concentration on the response time and the final normalized resistance of the ECIS system. As expected, the final value of the normalized resistance was dependent upon the amount of Con A used to precoat the detecting electrode with an optimum at about 500−800 μg/mL (Figure 3). In contrast to the resistance change, the capacitance decreased when the cells attached and spread

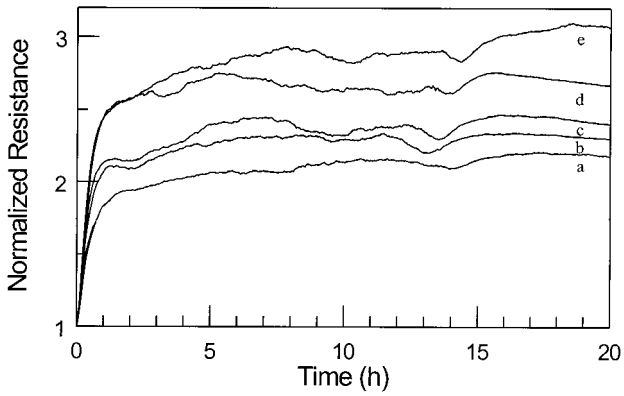

Figure 3. Effect of precoating the gold electrode with various concentrations of Con A with respect to cell adhesion as monitored by ECIS data presented as normalized resistance at time zero. The cell stage was 3.2×10^6 cells/mL: (a) 50, (b) 100, (c) 200, (d) 500, and (e) 800 μg/mL.

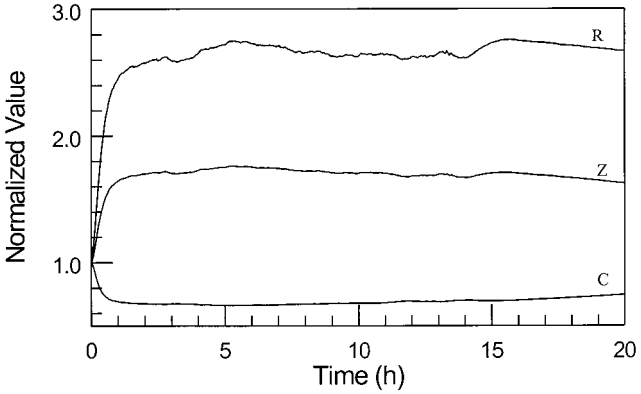

Figure 4. Gold electrode coated with 500 μg/mL Con A and inoculated from a cell concentration of 3.2×10^6 cells/mL monitored by ECIS data presented as normalized impedance (Z), resistance (R), and capacitance (C). The impedance Z is expressed as $R + 1/i2\pi fC$; i.e., $Z^2 = R^2 + 1/(2\pi fC)^2$, where f is the frequency and i is the imaginary number.

on the gold electrodes (Figure 4). However, the changing capacitance was less pronounced than the changing resistance, and only the results related to the normalized resistance were presented and discussed here. The phase difference between the voltage and the current, estimated as $-\arctan(1/2\pi fRC)$, increased from −67.5° at time zero to about −51.5° when the ECIS system approached steady state. Reproducibility of the ECIS system was judged to be satisfactory since the general behavior of the cell line in four identical wells was very similar in overall form. The fluctuations, which related to cell micromotion and viability, were random in nature, and there was no correlation between one well and its counterparts (Figure 5). The rationale behind the adhesion promotion of Con A for the insect cells was not understood; however, one could reason that the insect cells may possess specific receptors for Con A, presumably glycosylated proteins, a situation that is somewhat similar to the possession of receptors specific for fibronectin by mammalian cells. This is an interesting subject for further studies in cell biology.

The Inhibitory Effect of Explosives. In addition to the application for monitoring the activities and conditions of cells in tissue culture, the ECIS approach can be useful to detect and determine the inhibitory effect of chemicals on culture cells. TNT

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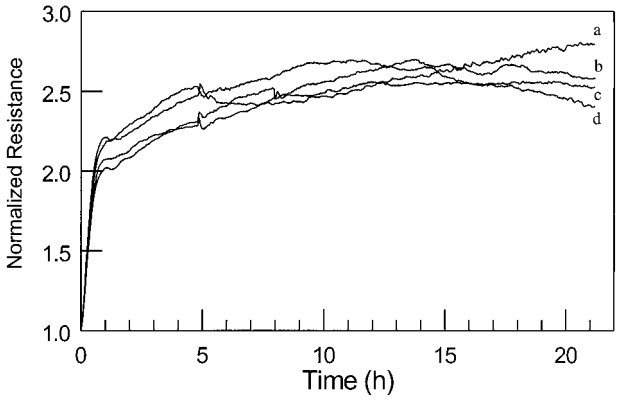

Figure 5. Reproducibility of four different electrode-containing wells inoculated with the insect cell. The gold electrodes were precoated with 500 μg/mL Con A and inoculated from a cell stage of 3.2×10^6 cells/mL. The ECIS data were presented as normalized resistance at time zero.

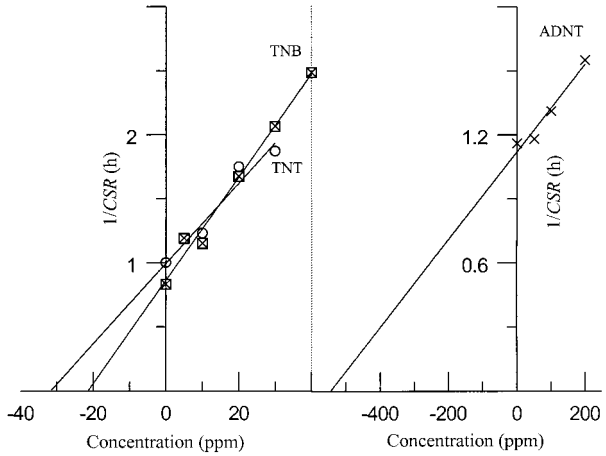

Figure 6. Inhibitory effect of explosives on the adhesion of insect cells to gold electrodes precoated with 500 μg/mL Con A and inoculated from a cell stage of 3.2×10^6 cells/mL.

and other explosives and their metabolites could pose a great health and ecological concern since such mutagenic, toxic, and persistent toxicants can leach from contaminated soil to accumulate in the food chain. Besides animal testing, the use of tissue culture is an alternative but requires more refined in vitro measurements. It should be noted that the spreading, replication, and motility require a complex series of events to occur in an integrated and regulated manner. Locomotion, for instance, has been known to be involved in the regulation of metabolic energy supplies and formation, contraction, and breakdown of actin filaments. When cells are exposed to a toxic chemical, the compound may affect the structural components of the cells and/or act at any stage of the cell metabolic cascade to alter the cell−substratum interaction.

The inhibitory effect of TNT, trinitrobenzene (TNB), and 2-amino-4,6-dinitrotoluene (2-ADNT) on the insect cells was investigated by continuously monitoring the resistance using an array of eight electrodes. Explosive-containing medium or medium alone as control was added to each well, and the resulting changes in electrode normalized resistance were followed. These data provided a quick overview of morphological changes and inhibitory effects induced by exposure to the explosives. At high concentrations of the explosives, there was a decline in the final normalized resistance value, corresponding to a significant increase in the elapsed time for the ECIS system to approach a steady-state value. Such a result implied that the cells were not alive or incapable of attaching to the electrode to block the current flow. One could reason that the explosives might bind to the receptors on the surface of the cell that may prevent or reduce cell−substratum interactions. The reciprocal value of the slope of the normalized resistance vs time curve, reflecting the cell spreading rate (CSR), was calculated and plotted against the explosive concentration to allow for estimating ID_{50}, the concentration at which the rate of cell spreading and growth is 50% of its maximal value. The ID_{50} values determined for 2-ADNT, TNT, and TNB were 530, 31.7, and 21.4 ppm, respectively (Figure 6).

In brief, ECIS, described in this study, has been extended to study the spreading and motility of insect cells in tissue culture. The concept of using the insect cells together with ECIS for monitoring the inhibitory effect of three key explosives was also demonstrated. It is anticipated that this noninvasive measurement in combination with an appropriate cell line will be a simple and reliable tool for screening potentially toxic compounds, drugs, and other compounds.

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