Environ Chem Lett (2003) 1:2–7
DOI 10.1007/s10311-002-0001-8

ORIGINAL PAPER

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Insect cell-based impedance biosensors: a novel technique to monitor the toxicity of environmental pollutants

Accepted: 12 August 2002 / Published online: 23 January 2003

Abstract Cell biosensors are currently emerging as novel, sensitive techniques to monitor the toxicity of environmental pollutants. Here, we have developed electric cell-substrate impedance sensing (ECIS) for on-line monitoring of the behavior of insect cells. Cells were cultured on a microarray of eight small gold electrodes, deposited on the bottom of tissue culture wells. Upon inoculation, cells showed a tendency to drift downward and attached to the gold surface precoated with the protein Concanavalin A to accelerate the cell attachment. The impedance increased because the cells acted as insulating particles to restrict the current flow. The resulting impedance, a coordination of many biological reactions within the cell, was continuously monitored in real-time to reveal information about cell spreading and micromotion. As the cell behavior was sensitive to external chemicals, the applicability of ECIS for inhibition assays was demonstrated with $HgCl_2$, 2,4,6-trinitrotoluene (TNT), 2-amino 4,6-dinitrotoluene (2-ADNT) and 1,3,5-trinitrobenzene (TNB).

Keywords Biosensor · Impedance spectroscopy · Insect cells · Cell attachment · Cell mobility · Cell mortality · Inhibition assays, nitroaromatics · Hg · TNT

Introduction

A biosensor is a device incorporating a biological sensing element either intimately connected to or integrated within a transducer. This powerful device combines the specificity and sensitivity of biological systems with the measuring power of electronics (Turner et al. 1987). To date, a plethora of biosensors has been designed to be highly specific for a target analyte (Luong et al. 1988). However, there is an urgent need to design sensors that can respond to a wide variety of chemicals in an integrated and continuous fashion, particularly the behavior of cells and cytotoxicity. 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 (Giaever and Keese 1992, 1993). Although several advances have been achieved in quantifying physiological and biochemical activities in tissue culture, it is still time-consuming to assess changes of cell viability, morphology, and motility (Giaever and Keese 1993). The microscope has been widely used to study cell attachment and spreading with the result described only in qualitative terms (Nobel and Levine 1986). Another elegant approach is to follow the tracks left by moving cells as they transverse surfaces coated with colloid gold particles (Albrecht-Buehler 1977). However, 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.

In vitro, cells normally secrete extracellular matrix (ECM) proteins and proteoglycans which adhere to the substrate, usually with a slight net negative charge. The cells then bind to molecules in the extracellular matrix by specific cell surface receptors (Freshney 1994). Interference reflection microscopy revealed a nanogap or nanochannel between the ventral side of the cell and the substratum (Heaysman and Pergum 1982). Cell spreading, morphology, and micromotion have been quantified using electric cell-substrate impedance sensing (ECIS). This emerging technique uses a small electrode which is deposited at the bottom of tissue culture wells and immersed in a culture medium (Giaever and Keese 1993). Inoculated cells normally drift downwards and attach to the surface of the electrode. The attached cells act as insulating particles because of their plasma membrane to

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interfere with the free space immediately above the electrode for current flow (Mitra et al. 1991). A constant current source applies a small AC current (1 μA, 4–5 kHz) between the small detecting electrode and a larger counterelectrode, i.e., the detecting electrode will dominate the overall impedance in the circuit (Keese et al. 1998; Kowolenko et al. 1990). Besides pH and temperature, the changing impedance is very sensitive to chemical compounds added to the culture medium as a result of the coordination of many biochemical reactions within the cell (Lo et al. 1993, 1995; Smith et al. 1994; Ko et al. 1998). Such behavior allows this method to serve as a useful tool for quantitation of cell spreading and motility as well as an alternative to animal testing for toxicology studies. Although some related techniques have been reported for monitoring toxicity of compounds using whole cells, they are not suitable for continuous and real-time analysis (Giaever and Keese 1993; Pasternak and Miller 1996).

In this study, the electrical cell-substrate sensing (ECIS) approach was extended for studying the spreading and micromotion of 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 and micromotion of insect cells and its applicability for inhibition assays.

Experimental section

Cell line and culture conditions

Spodoptera frugiperda Sf 9 cells (Luong et al. 2001) were maintained in serum-free SF-900 II medium (Gibco BRL, Canadian Life Tech., Burlington, ON, Canada). Cells were cultured weekly at 0.4×10^6 cells/mL at 27 °C, pH 6.2, and 110 rpm. The cell counts were determined using a hemacytometer and Coulter Counter Multisizer II (Coulter Electronics, Hialeah, FL). The cell viability was performed via the trypan blue exclusion method. *Sf* 9 cells, inoculated at an initial cell density of $0.4\text{x}10^6$ cells/mL, were grown to the mid-exponential phase ($3–3.5\times10^6$ cells/mL) and the resulting cells were aseptically centrifuged at 1,000 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 electrical cell-substrate sensing (ECIS) biosensor system

The ECIS biosensor system (model 100, Applied Biophysics, Troy, NY) has the capability of measuring 1 to 16 individual cultures simultaneously. In this system

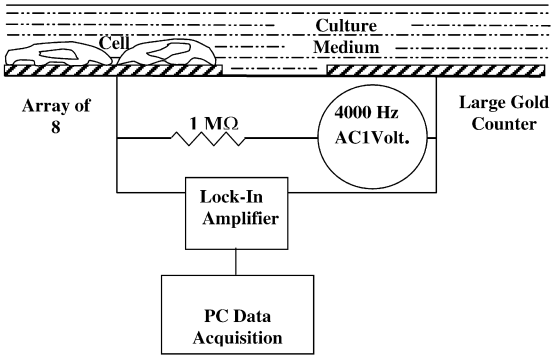

Fig. 1 Schematic diagram of electric cell-substrate impedance sensing (ECIS) for monitoring cell attachment and motility

(Fig. 1), a 1-volt AC signal is applied to the electrodes through a 1-MΩ series resistor at 4,000 Hz and the in- and out-of-phase voltages across the electrodes are measured using a phase-sensitive lock-in amplifier/preamplifier (Giaever and Keese 1993). Each ECIS-disposable electrode array consists of eight gold film electrodes (0.5×10^{-3} cm^2) and delineated with insulating films with much larger counter electrodes (0.2 cm^2) located at the base of 10-mm square wells (volume of about 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 can be found elsewhere (Giaever and Keese 1991, 1993; Ghosh et al. 1994; Xiao et al. 2002). To follow the cell growth, cells were taken from the mid-exponential growth phase and inoculated in the ECIS biosensor at 150–200 cells per gold film electrode. Cells were then allowed to attach and spread for a few minutes before measurement. Unless otherwise stated, all culturings were at 27 °C with high humidity. Each well was normally used with 0.4 mL of medium, about 80% of the well volume and the sampling time was 5 min.

Monitoring of protein binding to gold surface

Fibronectin has been known as a glue protein for several animal cells since the fibronectin receptor of mammals, which is part of the integrin family, has a site that recognizes and binds fibronectin at the cell surface (Xiao et al. 2002). The most notable domain, arginine-glycine-aspartate (RGD), is recognized by integrins and mediates cell adhesion. Concanavalin A (Con A), isolated from jack beans (*Concanavalin ensiformis*), is one of the best-known sugar-binding proteins or lectins that agglutinate cells and/or precipitate glycoconjugates (Scott and Eagleson 1988). Lectins bind erythrocytes, leukemia cells, yeast, and several types of bacteria. As the binding is saccharide-specific, lectins will not agglutinate cells that do not carry the appropriate surface saccharides.

The binding of Con A and fibronectin to the bare gold surface was performed using a surface plasmon reso-

nance (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 a concentration of 0.1, 0.25, 0.5, and 0.75 mg/mL and three chambers of another chip were used for fibronectin at a concentration 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 $R=R_{max}(1-e^{-Ks t})$ for each protein concentration where R is the optical response of the BIAcore biosensor system, and R_{max} is the maximum response and K_s k_{ass} $C+k_{dis}$. From the K_s vs. C plot, k_{ass} and k_{dis} were obtained as slope and intercept of the linear regression line and $K_d=k_{dis}/k_{ass}$.

Results and discussion

Relationship between resistance and frequency

The electrode-electrolyte interface can be represented as a series RC circuit (Lo et al. 1995) 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 (about 3,100 Ω) at high frequency of the measured resistance for a cell free electrode (Fig. 2).

As the detecting electrode is significantly smaller in area compared to the counterelectrode (about 400-fold), the impedance of the counterelectrode and the tissue culture solution can be mostly neglected. It should be noted 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 4,000 Hz, the current will start to flow through the cells because of the high capacitance of the cell membranes (Giaever and Keese 1992). At +1 V and 4,000 Hz, the current should be about 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 on fibroblastic and endothelial cell lines (Keese and Giaever 1994). Based on such information, the measurement at this frequency was also considered as noninvasive for the insect cells.

Effect of cell resuspension medium on the cell adhesion

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 (Fig. 3, curve a). Microscope 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.

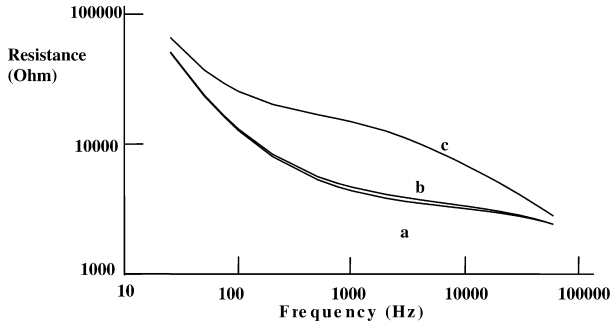

Fig. 2 Relationship between resistance and frequency. Bare electrode (*curve a*); electrode coated with Concanavalin A (*curve b*), and Con A-coated electrode inoculated with 3.2×10^6 cells/mL (*curve c*)

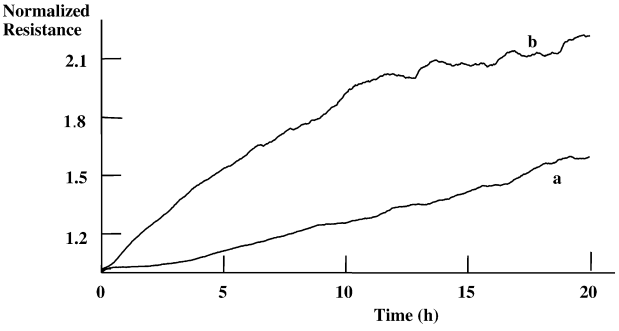

Fig. 3 Effect of cell resuspension medium on the cell adhesion with electric cell-substrate impedance sensing (ECIS) data presented as normalized resistance at time zero. The bare electrodes were inoculated from a cell stage of 3.2×10^6 cells/mL: *a* fresh medium; *b* 50% spent medium

Nonadherent cells, however, caused practically no change in the impedance and the increase in impedance or resistance was not exactly proportional to the fraction of the covered electrode area. Similar to mammalian cells, insect cells were anticipated to attach to substrata by means of small adhesion plaques leaving much of the lower surface of the cell above the substratum. In order to get the cells to thrive and replicate more rapidly, it was necessary to augment the culture medium with 50% of the spent medium (Fig. 3, curve b). The spent medium was expected to contain 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 on the initial cell concentration used for inoculation. The best result was obtained with an inoculum size consisting of about 3.2×10^6 cells/mL harvested in the mid-exponential phase (3.6–4.6 h of incubation in the shake flask). Microscope examination revealed that the number of cells attached to the surface of the gold electrode was about 150–200 cells with an aver-

age diameter of 13 μm. In principle, the detecting electrode with a surface area of 0.0005 cm^2 should accommodate up to 300 cells.

Impedance fluctuations: the vertical motion of cells

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 in 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 (Lo et al. 1993). Therefore, the impedance fluctuations reflected the vertical motion of cells in confluent layers (Giaever and Keese 1993; Lo et al. 1993) and changes in the transcellular resistance of the layer (Nobel and Levine 1986) in contrast to the horizontal cell locomotion, a movement across a subtratum. This behavior was referred to as micromotion (Lo et al. 1995; Ko et al. 1998) since the size of the vertical motion is of the order of nanometers, significantly below the resolution of optical microscopy. The 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 (about 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. Mercury is one of the most toxic substances known to humanity, particularly when it is combined with other atoms and molecules such as the methyl or chloride forms.

Rate of cell attachment using various protein-coated electrodes

Although insect cells were capable of attaching directly to bare gold films, the elapsed time was very lengthy and not useful for sensor applications. Therefore, 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 that 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 (Abercrombie 1982; Molnar et al. 1987; Keese and Giaever 1994). This was the main reason why proteins such as fibronectin, bovine serum albumin, and gelatin were used to coat gold electrodes to study the spreading and motility of mammalian

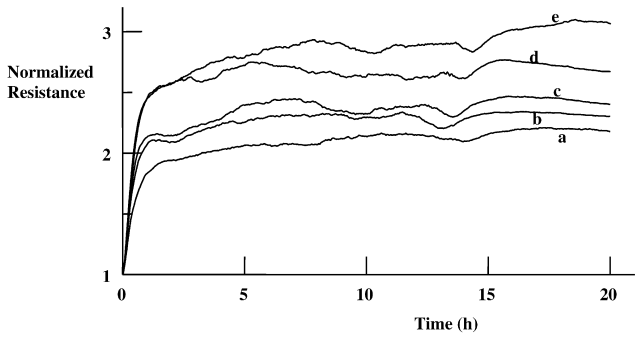

Fig. 4 Effect of precoating the gold electrode with various concentrations of Concanavalin A with respect to cell adhesion as monitored by electric cell-substrate impedance sensing (ECIS) data presented as normalized resistance at time zero. The cell stage was 3.2×10^6 cells/mL. *a* 50, *b* 100, *c* 200, *d* 500, and *e* 800 μg/ml

cells (Abercrombie 1982; Giaever and Keese 1992; Keese and Giaever 1994; Xiao et al. 2002). Thus, fibronectin, Con 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 20 μL of protein (100 μg/mL) directly over the detecting gold electrodes. Following a 30-min incubation to assure 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. Concanavalin A or 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 about twofold 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 (SPR) 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 for Con A in comparison to 63 nM for fibronectin (Luong et al. 2001).

Optimization of the concanavalin A concentration

An experiment was then performed to study the effect of Con A concentration on the response time and the final normalized resistance of the electric biosensor 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 (Fig. 4). In contrast to the resistance change, the capacitance decreased when the cells at-

tached and spread on the gold electrodes (figure not shown). 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. 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 could be an interesting subject for further studies in cell biology.

The inhibitory effect of explosives

As mentioned earlier, the electric cell-substrate sensing (ECIS) approach can be useful to detect and determine the inhibitory effect of chemicals on culture cells. Trinitrotoluene (TNT) 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, 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 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitrobenzene (TNB), and 2-ADNT on the insect cells was investigated by continuously monitoring the impedance using an array of eight electrodes. Identification and determination of such explosives together with their cytotoxicity are of importance in environmental analysis and remediation technology because there are growing concerns over the environmental fate and toxicity of such pollutants with the closures of several military bases throughout the world (Luong and Guo 1998). 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. It was reasoned 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 (figure not shown). The ID_{50} value determined for 2-ADNT, TNT, and TNB was 530, 31.7, and 21.4 ppm, respectively.

Conclusion

In brief, the electrical cell-substrate sensing (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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