STUDY OF A CELL-BASED ELECTROCHEMICAL BIOSENSOR FOR FUNGICIDE CYTOTOXICITY EVALUATION ON MAMMALIAN CELL LINES

Flampouri E[1,*], Mavrikou S[1], Kintzios S[1].

[1] Agricultural University of Athens, Department of Agricultural Biotechnology, Faculty of Plant Biology, Laboratory of Plant Physiology and Morphology, 75 Iera Odos Street, 118 55, Athens.

Motivations

The ultimate concern over pesticides in the environment is their toxic impact on nontarget organisms, including humans. For over the last two decades, the toxicological research has focused on pesticide-induced oxidative stress in terms of monitoring alterations in various biochemical and molecular compositions. A relative new group of agricultural fungicides, strobilurins, represent a major class of plant protection products and world's largest selling fungicides. They act as complex III Qo site inhibitors and thus disrupt electron transport in the respiratory chain, which generates superoxide and potentially results in oxidative stress.
Cell-based biosensors arise as powerful tools for the rapid detection of xenobiotics in food industries, agriculture and the environment. The present study was aimed at developing an electrochemical biosensor for cytotoxicity assays on mammalian cells, cultured on PEDOT electrodes. The cellular mono layers formed on top of the conductive material, support cell interactions, growth and metabolism as cells keep their original characteristics of morphology and functionality. In this framework, the action of kresoxim-methyl, of the stobilurins group, was examined on murine neuroblastoma cells (N2a). Square wave voltammetry (SWV) and a three-electrode configuration (W: PEDOT, C: Carbon, R: Silver) were used for recording the electrochemical changes after drug treatment. Moreover, standard viability/cytotoxicity protocols were conducted as references for assay comparisons. Due to PEDOTs transparent composition morphological observations and adhesion tests of the seeded cells were also made. Electrochemical responses against the fungicide provided evidence of the possible use of this assembly as a toxicity biosensor.

Materials and Methods

-Cell culture: cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS (Figure 1A), harvested for subculture using trypsin/EDTA digestion and grown in a humidified atmosphere containing 5% CO_2 and 95% air at 37℃.
-According to the EU Pesticide Database (Reg. (EC) No 396/2006) the Maximum Residue Limits (MRLs) of kresoxim-methyl for the majority of agricultural commodities range from 0.05 to 1 ppm, depending on the individual product. The selected concentrations were: 0, 0.05, 0.1 and 1 ppm.
-Assessment of cellular viability was conducted through formazan formation by the MTT reduction assay.
-Before experiment, the cells were pre-cultured on PEDOT electrodes (DropSens, Ref. P10), which consist of a 4mm PEDOT working electrode, a carbon counter electrode and a silver reference electrode (Fig. 1B). Polylysine was deposited on top of PEDOT electrodes prior to cell seeding. Cells were seeded on top of the electrodes with the use of a cut-off pipette tip which formed a 4mm culture well (Fig. 1C). The disposable electrode was connected to the potentiostat (Uniscan PG580) through a DropSens DSC interface (Fig. 1D).
-Cytoplasmic membrane depolarization of was measured using the membrane potential-sensitive probe, 3,3′ - dipropylthiacarbocyanine [diSC$_3$(5)].

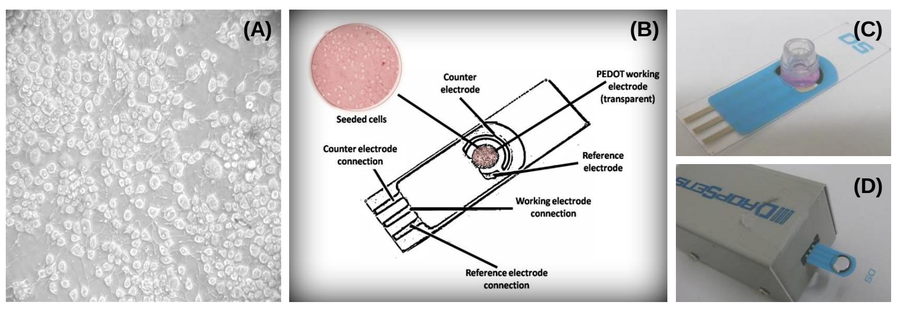

Figure 1. A, Phase-contrast microphotograph of neuroblastoma N2a cells; B, PEDOT/cells electrode configuration; C, Cell culture well on top of PEDOT electrode consisting of a cut-off pipette tip (polystyrene, diameter 4mm); D, DropSens DSC interface .

Results

-Morphological observations: microphotographs were taken from cell suspensions and from live cells plated on PEDOT working electrodes, with an inverted phase contrast microscope. Attached cells and their cellular extensions (dendrites), were clearly visible in the PEDOT cultures (black arrows, Fig. 2A).

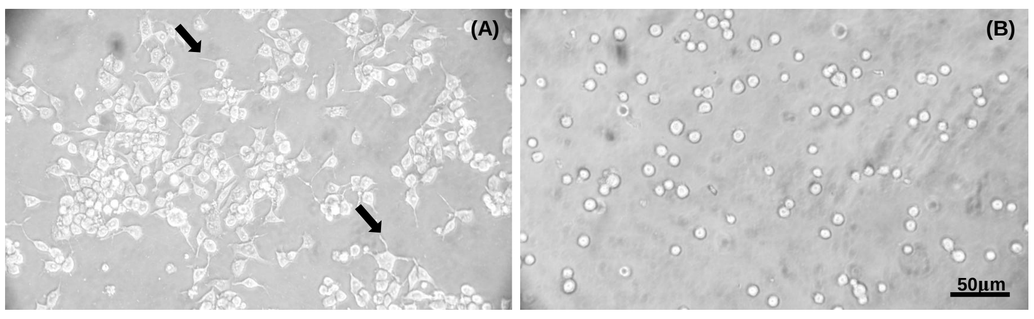

Figure 2. Microphotographs of neuroblastoma cell cultures and cell suspensions on Pedot electrodes; A, In cultures cells become adherent and develop cellular extensions; B, In suspensions cells are smaller, spherical and detached.

Cancer cells in monolayer cultures show a different morphology than in cell suspensions. While attached cells showed an extended neuron - shaped morphology , suspended ones were morphologically round with no axonal outgrowth.

-Attachment rates: the suitability of the electrode substrate for cell culture was investigated through the adhesion of cells on the coatings. Tissue culture plates commercially available for cell culture were used as control. Attachment rates in different time intervals are presented in Table 1. Four hours after cell seeding 93.82 (±2.91)% of cells have attached on the electrode. This rapid attachment shows that the modified surface is probably suitable for the growth of cells.
-Proliferation assay: The selected fungicide concentration appears to be non lethal for the N2a cells after 1 hour incubation time. Viability was assayed through the MTT proliferation assay. With all three concentrations cellular viability was higher than 90 %.

Table1. Attachment of N2a cells on the Pedot electrodes, 30min, 1 hour, and 4 hours after seeding of neuroblastoma cells.

Time after seeding (min)	30	60	240
Attachment rate (% control)	61.23	84.66	93.82
Standard deviation	4,25	3.94	2.91

Table 2. Viability of kresoxim-methyl on N2a cells using the MTT proliferation assay.(N=6)

Fungicide Concentration (ppm)	0	0.05	0.1	1
Viability (% control)	115,875	95,354	96,149	96,8101
±SEM	1,407	1,639	14,916	3,450

-Electrochemical measurements: Square wave voltammograms were recorded using a Uniscan PG 580 potentiostat. SWV measurements were performed applying a sweep potential between +0.3V and -0.3V at a pulse amplitude of 25 mV and frequency of 50Hz. Cells are considered non-conductors consisting of cell membranes and their binding on the electrodes reduces current as the cellular layer may partially obstruct the electron flow (Strehlitz et.al 2008),. However, gap junctions of cells act as channels through the cell membrane that allow electrical connections between neighboring cells. When cells are electrically stimulated gap junctions effectively connect the cells. As a result cells may act as a conductor for a sort time if stimulated to undergo some biochemical changes (Kotnik and Miklavcic, 2000).

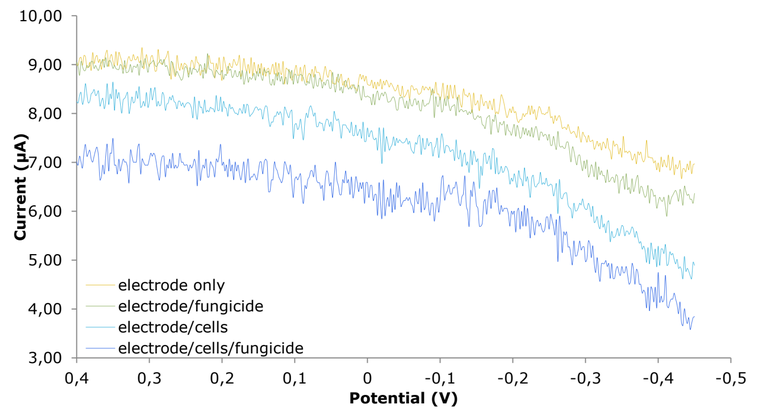

Figure 3. Change of electrical current under different electrode conditions. Values of SWV are different depending on the condition of the electrode. Cell presence causes lower current values along with a less gradual current decrease. This decrease was used for the fungicide evaluation(N=6).

-Membrane depolarization: Cytoplasmic membrane depolarization was measured using a membrane potential-sensitive probe, 3,3′ - dipropylthiacarbocyanine [diSC$_3$(5)]. Fluorescence was measured before and after the addition of kresoxim-methyl and their difference was expressed as Relative Fluorescence, (Fig. 4). The increase of fluorescence after the fungicide addition suggests depolarization of the membranes and for concentrations below 1 ppm, it follows a dose dependent pattern.

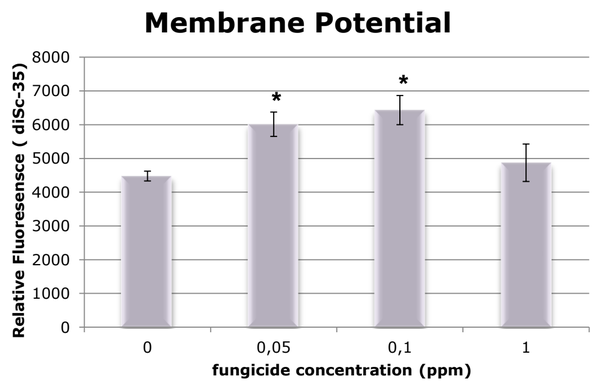

Figure 4. Depolarization of the neuroblastoma cell membranes by kresoxim-methyl. The release of DiSC3(5) dye from untreated and treated prelabeled DiSC3(5)-N2a was measured with a spectrofluorometer at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. The release follows a concentration-dependent pattern from to ppm. (N=6) .The asterisks (*) indicate values that are significantly different from the control (P<0.05 by one way ANOVA).

-Drug dose response: The selected fungicide concentrations were applied to the biosensor with the experimental conditions applied to Fig.2. A dose-response curve was constructed using the decreases in current for every condition, (Fig.5).

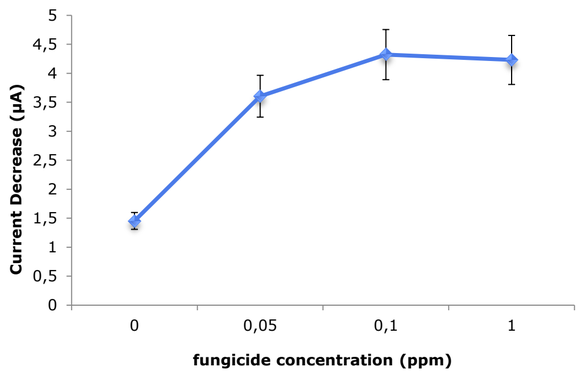

Figure 5. Dose response graph of current decreases after fungicide treatments. Electrochemical responses show a significant correlation to the doses applied and to the to the fluorescent data. (N=6) ±SEM.

Conclusions

-The PEDOT electrode surface showed satisfactory affinity with cellular monolayer allowing cell adhesion and relatively high viability. These results suggest that these modified electrodes could be a promising electroactive material for application in the field of biosensors, especially in view of the physiological (bioactivity) information provided by the cellular responsive elements.
-Results suggested that mammalian cell lines can act as conductors when plated on conducting materials.
-Results show that exposing the cells to small, non–lethal doses of the fungicide can produce rapid biological changes as recorded by the biosensor.

Future Work

Future experiments could focus on the investigation of the possible analyte-specific pattern of biosensor response, as well as the limit of detection of a given analyte. In addition, various operational parameters should be elaborated, including for example, the density of attached cells, assay temperature, etc

Acknowledgments:

«This research has been co-financed by the European Union (European Social Fund-ESF) and Greek national funds through the Operational Program "Education and Lifelong Learning" of the National Strategic Reference Framework (NSRF) - Research Funding Program: Heracleitus II. Investing in knowledge society through the European Social Fund».

*corresponding author, email: flampouri_kel@aua.gr