Assessment of Organophosphate and Carbamate Pesticide Residues in Cigarette Tobacco with a Novel Cell Biosensor

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Abstract: The conventional analysis of pesticide residues in analytical commodities, such as tobacco and tobacco products is a labor intensive procedure, since it is necessary to cover a wide range of different chemicals, using a single procedure. Standard analysis methods include extensive sample pretreatment (with solvent extraction and partitioning phases) and determination by GC and HPLC to achieve the necessary selectivity and sensitivity for the different classes of compounds under detection. As a consequence, current methods of analysis provide a limited sample capacity. In the present study, we report on the development of a novel cell biosensor for detecting organophosphate and carbamate pesticide residues in tobacco. The sensor is based on neuroblastoma N2a cells and the measurement of changes of the cell membrane potential, according to the working principle of the Bioelectric Recognition Assay (BERA). The presence of pesticide residues is detected by the degree of inhibition of acetylcholine esterase (AChE). The sensor instantly responded to both the organophosphate pesticide chlorpyriphos and the carbamate carbaryl in a concentration-dependent pattern, being able to detect one part per billion (1 ppb). Additionally, tobacco leaf samples (in blended dry form) were analyzed with both the novel biosensor and conventional methods, according to a double-blind protocol. Pesticide
residues in tobacco samples caused a considerable cell membrane hyperpolarization to neuroblastoma cells immobilized in the sensor, as indicated by the increase of the negative sensor potential, which was clearly distinguishable from the sensor’s response against pesticide-free control samples. The observed response was quite reproducible, with an average variation of $\pm 5.6\%$. Fluorescence microscopy observations showed that treatment of the cells with either chlorpyrifos or carbaryl was associated with increased $[\text{Ca}^{2+}]_{\text{cyt}}$. The novel biosensor offers fresh perspectives for ultra-rapid, sensitive and low-cost monitoring of pesticide residues in tobacco as well as other food and agricultural commodities.

**Keywords:** Bioelectric Recognition Assay (BERA), carbamates, neuroblastoma, organophosphates, tobacco.

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1. **Introduction**

More than 130 different pesticides are used worldwide for the production of tobacco, with approximately 25 compounds used on a regular basis. They belong to different chemical groups, such as organophosphates, carbamates, organochlorine and heterocyclic pesticides, nitro compounds, pyrethroids and amides. Many of these compounds can cause moderate to severe respiratory and neurological damage or act as genotoxic and carcinogenic agents, therefore increasing the health risks associated with smoking. Organophosphate (such as chlorpyrifos) and carbamate pesticides (such as carbaryl) in particular, affect the nervous system by inhibiting acetylcholine esterase (AChE), an enzyme that is important for the transmission of nerve impulses [1, 2]. Furthermore, compared to other crops, tobacco leaves have more surface to weight ratio [3] and with excessive use of pesticides there is always a chance for accumulation of pesticide residues in the leaf tissue.

The conventional analysis of pesticide residues in agricultural commodities is a labor-intensive procedure, since it is necessary to cover a wide range of different chemicals, using a single procedure [4]. Standard analysis methods include extensive sample pretreatment (with solvent extraction and partitioning phases) and determination by gas chromatography (GC), high-pressure liquid chromatography (HPLC) and mass spectrometry (MS) to achieve the necessary selectivity and sensitivity for the different classes of compounds under detection [5]. As a consequence, current methods of analysis provide a limited sample analysis capacity, on a day/instrument basis. Therefore, rapid pesticide residue testing is necessary. In a region-specific pattern, this results to a general lack of resources for implementation and enforcement of environmental, pesticide and labour regulations in tobacco-producing countries.

A biosensor is a device that detects, transmits and records information regarding a physiological or biochemical change. In recent years there has been a rapid increase in the number of diagnostic applications based on biosensors, including live, intact cells and -in some cases- tissues, organs or whole organisms. A cell-based sensor design employs the physiological responses of whole living cells as the sensing component, namely oxygen consumption, surface chemical or electrical potential, mobility or genetic activity [6].
A number of biological methods and protocols for toxicity measurement (including pesticide residues) have been developed, based on microorganisms such as bacteria and yeast. The advantages of toxicity bioassays include their low cost, easy detection protocol, reproducibility and rapid response. In addition, bioassays offer direct toxicity information on tested chemicals or samples that analytical methods cannot provide. Cairns and Mount [7] stated that chemical concentrations can be measured with an instrument, but living organisms can measure toxicity only. The number of toxic compounds that can be assayed by cell-based sensors is virtually endless. Current cell-based sensors for environmental monitoring can detect heavy metals or their salts, organic compounds, various carcinogens and pesticides such as diuron, atrazine, organotin compounds, organophosphates, glyphosate and various antibiotics (for a review see [8, 9]). However, it must be mentioned that cell sensors commonly are non-selective, not being able to discriminate between individual bioactive compounds. Also, cell-based sensors must provide for an environment maintaining sufficient cell viability (e.g. CO₂-enriched culture atmosphere).

Commercial environmental cell biosensors utilize luminescence from natural or genetically modified microbial species, whereas specificity of the response against an analyte is based on the fusion of reporter genes, with a metabolic operon induced by its respective analyte. Bioluminescence-based systems have been extensively used to monitor cellular stress responses, either by inducing the response at low analyte concentrations or by inhibiting the luminescence at higher concentrations [5, 10-15].

Fluorescence-based systems are not always suitable for quantitative determinations. They are also far more expensive than amperometric techniques [16]. Fluorescent dyes may also have considerable side-effects on cellular physiology, such as uncoupling or respiratory inhibition and membrane permeabilization [17, 18] and may interfere with the cell parameter that they assay [19].

Approached from different angles, electrically active cells can be interfaced with microelectrodes which allow the capture of extracellular spikes or impedance changes associated with cellular or tissue response. An example is the Bioelectric Recognition Assay (BERA) which has been originally developed for the detection of viruses on the basis of their specific interaction with appropriately immobilized, mammalian cells and the measurement of the change of the electric potential that is caused by the aforementioned interaction [9, 20, 21].

In the present study, we developed a cellular biosensor based on the BERA working principle for detecting organophosphate and carbamate pesticide residues in tobacco. The sensor is based on neuroblastoma N2a cells. The presence of pesticidal compounds is detected by the degree of inhibition of AChE, which is directly associated with changes of the immobilized cell membrane potential after administration of acetylcholine (ACh).

2. Experimental Section

2.1. Materials

Mouse neuroblastoma (N2a) cell cultures were originally provided from LGC Promochem (UK). Acetylcholine iodide (ACh) was purchased from Sigma Chem. Co. (USA). Fresh stock solutions of 14 mM S-acetylthiocholine-iodide (ATCi; Alfa Aesar Gmbh & Co KG) were made weekly in distilled
water and kept at 4°C in the dark. Dithiobisnitrobenzoic acid (DTNB (Ellman's reagent; Aldrich) was used as an indicator for ATCi hydrolysis. Fresh stock solutions were made monthly in P-buffer (0.1 M KH$_2$PO$_4$/K$_2$HPO$_4$; pH = 7.4) and kept at 4°C in the dark. Clorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate; CAS [2921-88-2]; Mw = 350.59; DowAgroSciences, USA) was used as a standard organophosphate insecticide and carbaryl (1-naphthyl methylcarbamate; CAS [63-25-2]; Mw = 201.221 Bayer Cropscience, France) as a standard carbamate insecticide. Pesticide mixes which contained 10 µM of each pesticide, were prepared daily in acetone solution. All other reagents were purchased from Fluka (Switzerland).

2.2. Sensor fabrication from N2a cells

Cells were cultured in Dulbecco’s medium with 10% heat-inactivated foetal calf serum (FCS), 10% antibiotics (streptomycin) and 10% l-glutamine. After cell detachment from the culture vessel by adding trypsin/EDTA for 10 min at 37°C and cell concentration by centrifugation (6 min, 1200 rpm, 25°C), 1 ml of cells (at a density of 2.5x10$^6$/ml) were mixed with 2ml of 4% (w/v) sodium alginate solution and then the mixture was added drop wise, by means of a 22G syringe, in 0.8M CaCl$_2$. Each of the resulting calcium alginate beads had an approximate diameter of 2mm and contained approximately 50x10$^3$ cells.

2.3. Sample preparation

Tobacco leaves (of the oriental cultivar Basmas) were kindly provided by the local tobacco processing company SEKE S.A. in blended dry form. Leaves were derived from plants grown at thirteen different locations in Northern Greece.

Control samples contained strictly organically-grown tobacco, which was free of pesticide residues.

Standard solutions of either chlorpyriphos or carbaryl were prepared by spiking control (pesticide-free) tobacco samples with the respective pesticide at concentrations of 0.0001, 0.001, 0.01, 0.1, 1 and 2 ppm. This was done in order to avoid the so-called “matrix effect”, i.e. the interference of sample-specific components on the sensor performance. In this way, the baseline (control) response of the sensor would reflect the effect of plant constituents on the sensor.

In all samples the solvent used for extraction was water:acetone = 1:3 (v/v). A portion of ground tobacco (4 g) was placed in a 60 mL conical vial after adding 40 ml of the extraction solvent. The extract was placed under 37°C in a hot bath for 1 hour in order to allow the acetone evaporation. The supernatant was then collected and transferred to 1.5 mL eppendorfs.

Each sample solution consisted of 50 µl tobacco extract and 10µl acetylcholine iodide. Acetylcholine iodide (10 mM) was dissolved in a 50mM Tris-aminomethane buffer (Tris) of pH 8.

2.4. Assay principle

BERA sensors based on N2a cells were used for assaying the inhibition of cellular AChE to hydrolyze Ach to acetate and choline according to the following equation:

\[ \text{AChE} + \text{Ach} \rightarrow \text{Choline} + \text{acetate} \]
According to the working principle of the method, the presence of organophosphate or carbamate compounds is detected by the degree of inhibition of cellular AChE, which is inversely associated with ACh concentration. ACh is an excitatory neurotransmitter [22], the activity of which is regulated by AChE. Therefore, inhibition of AChE can lead to increased excitatory ACh transmission, which can be measured by the depolarization of the cell membrane. In other words, inhibition of AChE by pesticide residues in the sample will result in excessive stimulation of N2a cells by Ach, which will further lead to membrane depolarization above a pre-determined threshold. Membrane depolarization events and associated electrolyte influx/efflux will reflect themselves on the sensor’s response as a change of the sensor’s potential, due to changes in the concentration of electrolytes in the immediate vicinity of the working electrode.

2.5. Assay procedure.

Each cell-bearing bead (cell sensor) was connected to a working electrode made from pure silver, electrochemically coated with an Ag/AgCl layer and having a diameter of 0.75 mm. Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for recording the signal and data processing was InstaCal (Measurement Computing) (Fig. 1).

**Figure 1.** Schematic representation of the immobilized cell biosensor. The reference electrode is inserted directly in the liquid sample, while the measuring electrode is inserted in the immobilized cell-gel bead, which has an approximate diameter of two mm. Measurement and reference electrodes are connected via wiring to the PMD 1608-FS data converter. The displayed sensor response refers to a sample response containing Ach (blue) and a sample containing Ach + chlorpyrifos as the AChE inhibitor (red).

For each assay, the sensor system, comprising of the bead attached to the working electrode and a reference electrode, was immersed into each sample solution (200 µl). The response of each sensor was estimated by recording the average change of the sensor potential for a period of 180 sec after sample application.
2.6. Conventional sample analysis

Tobacco samples were also analyzed by GC and LC-MS/MS according to the International Standard ISO 4389 [23] and by following the protocols of Djordjevic et al. [24] and Haib et al. [4].

2.7. Acetylcholinesterase inhibition assay.

In order to demonstrate that (a) AChE is functionally expressed in N2a cells and (b) organophosphates and carbamates inhibit N2a-located AChE, we carried out AChE inhibition assays. AChE activity was determined spectrophotometrically with the Ellman AChE assay [25], using acetylthiocholine as the substrate. To prepare the cells for the AChE assay, 1 ml of P-buffer (0.1M sodium phosphate, 1% Triton X-100, pH 7.4) was added to a 15 ml sterile Cellstar® tube containing the cell pellet (approximately 7.5 × 10^6 cells) and the mixture was slowly trituated until the pellet was resuspended. All experiments were performed in P-buffer. S-acetyltiocholine-iodide (ATCi) was used as a substrate to determine specific cholinesterase activity. ATCi hydrolysis was measured as an increase in OD_{405} (50 s time intervals) caused by the reaction of thiocholine with DTNB to produce the yellow 5-thio-2-nitro-benzoic acid anion [25]. Specific activity is reported as nmol substrate hydrolyzed/min/mg protein. The protein content was determined by the Bradford method using bovine serum albumin as the standard [26]. The spectrophotometric assays were carried out with a spectrophotometer (UV-160A SHIMADZU) at a wavelength of 595nm.

2.8. Assay of pesticide-cell interaction

Changes in cytoplasmic Ca^{2+} concentration in N2a cells before and after the addition of an organophosphate (chlorpyrifos) or a carbamate (carbaryl), each at two different concentrations (0.03 or 0.3 µM) were monitored by the uptake of the acetomethyl ester of Fluo3 [27]. After application of five µl of the dye, the fluorescence of the specimens was recorded for five minutes at 10 s intervals. Slides with stained cells were mounted on a Zeiss Axiolab fluorescent microscope equipped with a BP-546 excitation filter and an FT-580 chromatic beam splitter. A digital camera (SONY S75 digital still camera) was attached to the microscope with adjustable BP-546/FT-580 excitation filter/chromatic beam splitter combinations. In order to control photobleaching, we kept specimen exposure times at a minimum level. No significant alteration of the intensity of the fluorescence was observed during the observation of the specimens.

2.9. Experimental design

Both biosensor and conventional sample analysis were conducted according to a double-blind protocol. Experiments were set up in a completely randomized design and each experiment was repeated three times. In each application, a set of five biosensors was tested against each individual sample. Data means among different days were compared using Duncan’s multiple range test (with significance at p<0.05).
Figure 2. Biosensor response against standard solutions containing carbaryl (A) or chlorpyriphos (B) at various concentrations. Sensor response is expressed as a change in the membrane potential of immobilized cells. ($n = 15$ replications (different sensors) for each sample and error bars represent standard errors of the average value of all replications with each sample). Columns marked with different letters indicate statistically different values.

MRL = 1.5 ppm

MRL = 0.5 ppm
3. Results

3.1. Response of the sensor to standard pesticide solutions

The results of the assay of standard pesticide solutions at different concentrations with the BERA neuroblastoma biosensors are shown in Figure 2. When no pesticide was present in the sample (control sample), a constant sensor response of -0.019 ± 0.002 mV was observed. The sensors responded to carbaryl by considerable negative increase of the sensor’s potential (Figure 2A). This response was concentration-dependent in the range of 100 ppt to 1 ppm ($r^2=0.7432$), so that both the qualitative and the quantitative detection of the pesticide was possible, at least at concentrations lower or closer to the Minimum Residue Level (MRL) (1.5 ppm). A similar, concentration-dependent pattern ($r^2=0.8558$) was observed in the sensor’s response against chlorpyriphos (Figure 2B), although the sensor’s response did not change at the 0.0001-0.01 ppm range. The biosensor response against either pesticide was quite reproducible, with 8.6/7.8 % variation from the average in the response observed against carbaryl/chlorpyriphos, respectively.

Figure 3. Biosensor response against thirteen different tobacco leaf samples containing chlorpyriphos and carbaryl at various concentrations. Samples have been ranked in the order of ascending cumulative pesticide residue concentration. Sensor response is expressed as a change in the membrane potential of immobilized cells. (n = 15 replications for each samples and error bars represent standard errors of the average value of all replications with each sample). Samples 12 and 13, marked with pink, contain pesticide residues at concentrations equal or above the official Minimal Residue Levels.
3.2. Residue profile by conventional analysis of tobacco leaf samples

The organophosphate chlorpyrifos and the carbamate carbaryl were detected by conventional analysis in the thirteen tobacco leaf samples. The concentrations of each pesticide residue in the individual samples are presented in Table 1, while the cumulative pesticide residue concentration in each sample is presented in the x axis of Figure 3. The biosensor response against each sample is also presented in the same figure. Pesticide residues in tobacco samples caused a considerable cell membrane hyperpolarization on neuroblastoma cells immobilized in the sensor, as indicated by the increase of the negative sensor potential, which was clearly distinguishable from the sensor’s response against pesticide-free control samples. There were no false-negative measurements. The observed response was quite reproducible, with an average variation of ±5.6%. In spite of the observed correlation, the biosensor-based assay can presently be considered only qualitative.

Table 1. Pesticide residue concentration (ppm) in each assayed tobacco leaf sample.

| Sample | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 |
|--------|----|----|----|----|----|----|----|----|----|----|----|----|----|
| chlorpyrifos | 0.04 | 0.07 | 0.06 | 0.05 | 0.06 | 0.05 | 0.04 | 0.16 | 0.21 | 0.15 | 0.14 | 0.36 | 0.27 |
| Carbaryl  | 0.23 | 0.42 | 0.21 | 0.48 | 0.25 | 0.2 | 0.97 | 1.4 | 1.1 | 0.64 | 0.94 | 2.60 | 1.6 |

Figure 4. Results of the acetylcholinesterase inhibition assay. AChE activity in N2a cells was reduced after the addition of carbaryl (pink) or chlorpyrifos (yellow), compared to control cells (blue).
Figure 5. Changes (expressed as differences in fluorescence intensity) of the cytoplasmic calcium ion concentration before (A) and after (B, C, D, E) treatment of N2a cells with chlorpyrifos or carbaryl (A) Untreated cells (control) (B, C) Cells treated with 0.03 or 0.3 µM chlorpyrifos, respectively (D, E) treated with 0.03 or 0.3 µM carbaryl, respectively.
3.3. *Acetylcholinesterase inhibition assay.*

As presented in Figure 4, AChE activity in N2a cells was reduced after the addition of carbaryl or chlorpyriphos, compared to control cells. Therefore, it was clearly demonstrated that functional AChE units exist on the surface of N2a cells, which can be inhibited by either pesticide. The inhibition was greater in the case of carbaryl.

3.4. *Effect of pesticides on calcium uptake by N2a cells*

Neuroblastoma cells responded to the presence of either chlorpyrifos or carbaryl by a rapid and very considerable increase of their intracellular Ca$^{2+}$ stores (Fig. 5), compared to untreated cells. However, the intensity of this effect was not depended on the type or the concentration of the pesticide.

4. Discussion

The operating principle of a cellular biosensor is entirely different from conventional methods for pesticide residue detection: cell sensors respond to different molecules according to their bioactivity rather than their structure or physical chemical properties (as assayed, for instance, by mass spectrometry or chromatography) [6, 8]. Additionally, biosensors rarely provide a full spectrum of the compounds present in a sample [1, 5, 24]. One of the traits that make cell biosensors attractive as a clinical analytical tool is their considerable sensitivity, which is assumed to enable, in some cases, the possibility to detect just a single target molecule [13, 14]. This theoretical assumption is based on the intricate interaction, which has emerged as a result of relentless trial-and-error testing through countless millennia and allows cells to respond to individual molecules and organisms in rather precise and reproducible ways. This ability, however, comes at a cost, or, rather, a compromise: because cells can react in roughly the same manner against an amazingly large number of different molecules, cell sensors can exhibit a very poor selectivity. This is a very common problem in toxicity assays, where cell sensors successfully detect the presence of a toxic (or genotoxic) compound, but generally fail to determine the exact nature of the toxic analyte [10, 11, 28].

Despite this, the present biosensor system offers a number of distinct performance characteristics for pesticide residue detection, including:

- Ultra-high speed: an assay can be completed in less than 3 min.
- High sensitivity, with the ability to detect pesticides at concentrations as low as 1 ppb (for carbaryl).
- High reproducibility.
- Low cost, not exceeding 30-50 € per assay batch.
- The ability to monitor at real-time conditions.
- Finally, the ability to detect toxicity with complex, “real world” mixtures of toxicants and interfering compounds.

From a practical point of view, these traits could turn the novel biosensor into an attractive future technology for routine, field-based pesticide assays. Providing novel solutions for food quality assurance can be a very critical issue, especially in view of the new EU [29] and international
regulations for minimal residue concentration in marketed food and agricultural products. The development of the novel sensor concept is still at a preliminary stage: this means that further experiments are required in order to define the linear range of the response and the possible interference of different pesticides on the selectivity of the sensor. In the same context, it may be worth mentioning that, within the framework of the present study, standard solutions of pesticides for calibrating the novel biosensor were prepared by spiking residue-free tobacco, in order to avoid the so-called “matrix effect”, i.e. the interference of sample-specific components on the sensor performance. Different samples, different types of extract, different commodities and different “concentrations” of matrix may exhibit matrix effects of different magnitudes. Matrix effects are variable in occurrence and intensity and calibration should be matrix-matched routinely [30]. For example, when we prepared pesticide standard solutions by directly dissolving carbaryl or chlorpyriphos in a solvent, rather than by spiking them in tobacco, an entirely different pattern of sensor response to either compound was observed (analytical results not shown).

According to the working hypothesis of our method, inhibition of AChE by either pesticide may have caused excessive stimulation of N2a cells by Ach, thus resulting to membrane depolarization [22]. The considerably increased concentration of intracellular Ca\(^{2+}\) stores after treating neuroblastoma cells with the pesticides provides an indication that this hypothesis may be true, although it is yet uncertain whether calcium ions are the sole contributors to the observed depolarization effect. In various cell types (including neural and cancer cells) membrane polarization events are associated with other stress-induced changes, such as an increase in cytoplasmic free calcium [31]. Whelan and Zare [27] have previously shown that receptor-like interactions between molecules on the cell surface and target analytes resulted in a detectable change in the concentration of cytosolic Ca\(^{2+}\), while an association between conformational changes of electroinserted SOD molecules, calcium ion efflux and cell membrane potential has been demonstrated by Moschopoulou and Kintzios [32]. In this way, cells with a particular type of surface-bound receptor serve as recognition platforms for homologous target molecules, whereas the translation of the recognition reaction into potential changes makes the assay much more rapid (and, in some cases, more sensitive) than any conventional methods.

Further increase in the sensor sensitivity could arise by increasing the concentration of immobilized cells per sensor, whereas the selectivity of the sensor’s response against different pesticides could be increased by developing genetically engineered cell lines expressing different AChE isoforms on their surface. In addition, the utilization of artificial neural network technology [33] for rapid, automated pattern recognition of the sensor’s response could transform the method into a powerful, high-throughput analytical tool. These approaches are being currently tested by our research group.

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References

1. Bachmann, T.T.; Leca, B.; Villate, F.; Marty, J.L.; Fournier, D.; Schmid, R.D. Improved multianalyte detection of organophosphates and carbamates with disposable multielectrode biosensors using recombinant mutants of Drosophila acetylcholinesterase and artificial neural networks. *Biosens. Bioelectron.* **2000**, *15*, 193-201.

2. Nunes, G.S.; Montesino, T.; Marques, P.B.O.; Fournier, D.; Marty, J.L. Acetylcholine enzyme sensor for determining metamidophos insecticide. Evaluation of some genetically modified acetylcholinesterases from *Drosophila melanogaster*. *Anal. Chim. Acta.* **2001**, *434*, 1-8.

3. Drossopoulos, J.B.; Bouranis, D.L.; Kintzios S., Aivalakis, G.; Karides, J.; Chorianopoulou, S.N. Distribution profiles of selected macro-nutrients in oriental field grown tobacco plants as affected by nitrogen fertilization. *J.Plant Nutrition* **1998**, *21*, 1391-1406.

4. Haib, J.; Hofer, I.; Renaud, J.-M. Analysis of multiple pesticide residues in tobacco using pressurized liquid extraction, automated solid-phase extraction clean-up and gas chromatography-tandem mass spectrometry. *J. Chromatogr. A* **2003**, *1020*, 173-187.

5. Sherma, J. Review of advances in the thin layer chromatography of pesticides: 2004-2006. *J Environ Sci Health B.* **2007**, *42*, 429-40.

6. Kintzios, S. Cell-based sensors in clinical chemistry. *Mini Rev. Clin. Chem.* **2007**, *7*, 1019-1026.

7. Cairns, J. Jr.; Mount, D.I. Aquatic toxicology. *Environ. Sci.Technol.* **1990**, *24*, 154-161.

8. Daunert, S.; Barrett, G.; Feliciano, J. S.; Shetty, R.S.; Shrestha, S.; Smith-Spencer, W. Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. *Chem. Rev.* **2000**, *100*, 2705-2738.

9. Kintzios, S.; Bem, F.; Mangana, O.; Nomikou, K.; Markoulatos, P.; Alexandropoulos, N.; Fasseas, C.; Arakelyan, V.; Petrou, A-L.; Soukouli, K.; Moschopoulou, G.; Yialouris, C.; Simonian, A. Study on the mechanism of Bioelectric Recognition Assay: evidence for immobilized cell membrane interactions with viral fragments. *Biosens. Bioelectron.* **2004**, *20*, 906-916.

10. Kaiser, K.L.E.; Palabrica, V.S. Photobacterium phosphoreum Toxicity Data Index. *Water Pollut. Res. J. Can.* **1991**, *26*, 361-432.

11. Riska, P.F.; Su, Y.; Bardarow, S.; Freundlich, L.; Sarkis, G.; Hatfull, G.; Carrière, C.; Kumar, V.; Chan, J.; Jacobs, W.R. Jr. Rapid Film-Based Determination of Antibiotic Susceptibilities of *Mycobacterium tuberculosis* Strains by Using a Luciferase Reporter Phage and the Bronx Box. *J. Clin. Microbiol.* **1999**, *37*, 1144-1449.

12. Choi, S.H.; Gu, M.B. A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria. *Biosens. Bioelectron.* **2002**, *17*, 433-440.

13. Gu, M.B.; Min, J.; Kim, E.J. Toxicity monitoring and classification of endocrine disrupting chemicals (EDCs) using recombinant bioluminescent bacteria. *Chemosphere* **2002**, *46*, 289-294.

14. Kim, B.C.; Park, K.S.; Kim, S.D.; Gu, M.B. Evaluation of a high throughput toxicity biosensor and comparison with a Daphnia magna bioassay. *Biosens. Bioelectron.* **2003**, *18*, 821-826.

15. Knight, A.W.; Keenan, P.O.; Billington, N.; Cahill, P.A.; Dalrymple, I.M.; Hawkyard, C.J.; Stratton-Campbell, D.; Walmsley, R.M. Clear and present danger? The use of a yeast biosensor to monitor changes in the toxicity of industrial effluents subjected to oxidative colour removal treatments. *J. Environ. Monit.* **2007**, *9*, 1394-1401.
16. Slavik, J. *Fluorescent probes in cellular and molecular biology*, CRC Press: Boca Raton, 1993.

17. Peechatinikov, V.A.; Rizvanov, F.F.; Pletnev, V.V. Permeability of sarcoplasma reticulum membrane for monovalent cations. *Stud. Biophys.*, 1983, 28, 669-673.

18. Eddy, A. Use of carbocyanine dyes to assay membrane potential of mouse ascites tumor cells. *Methods in Enzymol.* 1989, 172, 95-101.

19. Dixit, R.; Cyr, R. Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. *Plant J.* 2003, 36, 280-290.

20. Kintzios, S.; Pistola, E.; Panagiotopoulos, P.; Bomsel, M.; Alexandropoulos, N.; Bem, F.; Varveri, C.; Ekonomou, G.; Biselis, J.; Levin, R. Bioelectric Recognition Assay (BERA). *Biosens. Bioelectron.* 2001, 16, 325-336.

21. Kintzios, S.; Pistola, E.; Konstas, J.; Bem, F.; Matakadiadis, T.; Alexandropoulos, N.; Biselis, J.; Levin, R. Application of the Bioelectric recognition assay (BERA) for the detection of human and plant viruses: definition of operational parameters. *Biosens. Bioelectron.* 2001, 16, 467-480.

22. Hoang, B.X.; Graeme Shaw, D.; Pham, P.; Levine, S.A. Neuro-bioenergetic concepts in cancer prevention and treatment. *Medical Hypotheses* 2007, 68, 832-843.

23. Anonymous. *International Standard (ISO) 4389: Tobacco and tobacco products – Determination of organochlorine pesticide residues – gas chromatographic method*. ISO, Geneva.

24. Djordjevic, M.V.; Fan, J.; Hoffmann, D. Assessment of chlorinated pesticide residues in cigarette tobacco based on supercritical fluid extraction and GC-ECD. *Carcinogenesis* 1995, 16, 2627-2632.

25. Ellman, G.L.; Courtney, K.D.; Andres, V.; Featherstone, R.M. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 1961, 7, 88-98.

26. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976, 72, 248-254

27. Whelan, R.J.; Zare, R.N. Single-cell immunosensors for protein detection. *Biosens. Bioelectron.* 2003, 18, 1065-1072.

28. Van der Lelie, D.; Regniers, L.; Borremans, B.; Provoost, A.; Verschaeve, L. The VITOTOX® test, an SOS bioluminescence Salmonella typhimurium test to measure genotoxicity kinetics. *Mutat. Res.* 1997, 389, 279-290.

29. Anonymous. Commission recommendation concerning a coordinated Community monitoring programme for 2007 to ensure compliance with maximum levels of pesticides residues in and on cereals and certain other products of plant origin and national monitoring programmes for 2008. *OJ L* 2007, 96, 21-27.

30. Anonymous. European Union Document: Quality control procedures for pesticide residue analysis. 2006, 10232, 1-30.

31. Krnjevic, K. Early effects of hypoxia on brain cell function. *Croat. Med. J.* 1999, 40, 375-380.

32. Moschopoulou, G.; Kintzios, S. Application of "membrane-engineering" to bioelectric recognition cell sensors for the detection of picomole concentrations of superoxide radical: a novel biosensor principle. *Anal. Chimica Acta* 2006, 573-574, 90-96.
33. Frossyniotis, D.; Anthopoulos, Y.; Kintzios, S.; Perdikaris, A.; Yialouris, C.P. A multisensor fusion system for the detection of plant viruses by combining artificial neural networks. *Lectures Comp. Sci.* **2006**, *4132*, 401-409.

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