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Research Article

Noninvasive Superoxide Monitoring of *In Vitro* Neuronal Differentiation Using a Cell-Based Biosensor

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Membrane-engineered cells bearing superoxide dismutase (SOD) molecules on their surface offer the capability of ultrarapid and ultrasensitive detection of the superoxide anion (O_2^-) through the measurement of changes of their cell membrane potential. We herewith report the application of this technology for the noninvasive determination of superoxide levels during the *in vitro* differentiation of PC12 cells. We were able to detect changes in O_2^- accumulation in the culture medium, which were closely associated with the progress of neuronal differentiation.

1. Introduction

Superoxide anion, which is mainly generated as a by-product of the oxidative phosphorylation by the electron transport chain of the mitochondria, is released to the mitochondrial matrix where it is converted immediately to hydrogen peroxide. Mitochondrial hydrogen peroxide can then diffuse to the cytosol and the nucleus and react with other free radical species, modulate signalling pathways, or cause cellular damages [1]. Together with other free radical species, superoxide has been found to mediate neural differentiation in several *in vitro* models [2–5]. However, the measurement of O_2^- concentration in biological systems is a challenging analytical problem. Physiological concentrations of superoxide are in the range of 100 pM [6], thus requiring a sensitivity of detection in the range of nanomolar or even picomolar sensitivity to effectively quantify changes in the concentrations of this radical species. It is also desirable to measure O_2^- in a noninvasive and rapid manner, so that measurements reflect the actual cellular processes to the best achievable degree.

Membrane-engineering is a generic methodology for increasing the selectivity of a cell biosensor against a target molecule, by electroinserting target-specific receptor molecules on the cell surface. We have previously reported the construction of an ultrasensitive (detection limit: 1 pM) O_2^- sensor based on immobilized cells, which have been membrane-engineered with superoxide dismutase (SOD) [7, 8]. Electroinserted SOD molecules retained their characteristic catalytic properties, as proven by selective inhibition assays. Superoxide dismutation triggered changes to the membrane potential of membrane-engineered fibroblast cells, which were associated with changes in $[Ca^{2+}]_{cyt}$ as revealed by the selective inhibition of intracellular calcium ion traffic.

In the present work, we demonstrate the application of the biosensor for monitoring changes in O_2^- concentration during the *in vitro* differentiation of PC12 pheochromocytoma cells. We demonstrate that, based on this approach, it is possible to efficiently and reliably monitor *in vitro* neuronal differentiation on a day-to-day basis. The perspectives for high throughput, noninvasive physiological profiling of cell cultures using this novel tool are discussed.

2. Materials and Methods

2.1. Chemicals. All solvents and chemicals used were of analytical quality. Water was double distilled. Vero cell and PC12 (pheochromocytoma) cell cultures were originally purchased from LGC Promochem (Teddington, UK). Dulbecco's Modified Eagle's Medium, fetal bovine serum, horse serum, L-glutamine, penicillin/streptomycin, and trypsin/EDTA

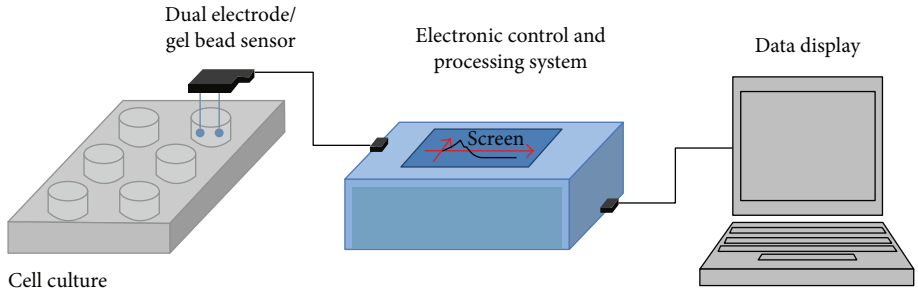


Figure 1: Schematic outline of the cell-based superoxide biosensor system.

were purchased from Biochrom AG (Berlin, Germany). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) and MitoSOX were purchased from Invitrogen (Carlsbad, CA, USA). Coomassie brilliant blue R was purchased from MP Biomedicals (Solon, OH, USA). Finally, nerve growth factor-β (NGF-β) and all other reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Creation of SOD-Membrane-Engineered Vero Cells (Vero-SOD). Membrane-engineered cells were created by electroinserting SOD into the membrane of Vero cells following a modified protocol of Zeira et al. [9] and Moschopoulou and Kintzios [7]. Briefly, the cells were centrifuged at 100 g for 6 min and then resuspended in PBS (pH 7.4). Subsequently, cells were incubated together with CuZnSOD (EC 1.15.1.1) for 20 min on ice. Then, the cells-SOD mixture was transferred to appropriate electroporator (Eppendorf Eporator, Eppendorf AG, Germany) cuvettes. Electroinsertion was performed by applying an electric field (four pulses) at 1800 V cm^{-1}. After electroinsertion, cells were incubated at 37°C for 1 hour. Finally, cells were centrifuged at 100 g for 6 min and resuspended in PBS (pH 7.4). This step was repeated twice.

2.3. Sensor Fabrication from "Membrane-Engineered" Cells. The engineered cells were incorporated in a biosensor system according to the principles of the Bioelectric Recognition Assay [8]. Sensors were fabricated from membrane-engineered cells according to the following procedure: cells (1 mL) were mixed with 2 mL of 4% (w/v) sodium alginate solution and then the mixture was added dropwise, by means of a 22 G syringe, in 0.8 M $CaCl_2$. Cells were immobilized in calcium alginate beads at a density of 3×10^6 mL^{-1}. In this way, each of the resulting calcium alginate beads had an approximate diameter of 2 mm and contained approximately 75×10^3 cells.

2.4. Recording and Data Processing. A dual (working and reference) electrode system was used. Both electrodes were made from pure silver, electrochemically coated with an AgCl layer and having a diameter of 0.2 mm. The working electrode was connected to a cell-bearing bead (cell sensor) while the reference electrode was attached to a cell-free bead. Electrodes were connected to the recording device, which comprised the PMD-1608FS A/D card (Measurement Computing, Middleboro, MA). The software responsible for the recording of the signal and processing of data was InstaCal (Measurement Computing) (Figure 1).

For each assay, the sensor system, comprising of the two beads attached to the working and the reference electrode, respectively, was immersed into the culture medium. The response of each sensor was estimated by recording the change of the sensor potential after biosensor sinking to well culture and until the response was stabilized. A stable response of each biosensor was achieved within three min. The average of the sensor potential of each assay was considered as the numerical value of each response. The measured values represented the relative superoxide concentration, which is the fraction of the actual superoxide concentration in the culture medium reaching the measuring electrode.

The performance of the biosensor was checked prior to each experimental session by calibrating the system against a wide range of superoxide ion concentrations (1 pM–10 nM), generated by the oxidation in aqueous solution of xanthine by xanthine oxidase, as reported previously [7, 8].

2.5. Cell Culture. Pheochromocytoma (PC12) cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) horse serum, 5% (v/v) fetal bovine serum, L-glutamine, and penicillin/streptomycin, in a standard incubator (95% humidity 5% CO_2) and subcultured three times per week. PC12 cells were plated onto 24-well tissue culture plates coated with 50 μg/mL of poly-l-lysine. Cells were plated at density of 4×10^4 cells/well. After waiting four hours for cell attachment and stabilization, the used medium was replaced with fresh medium (control) and with medium supplemented with 100 nM NGF for seven days (changing the medium every two days).

2.6. Coomassie Brilliant Blue Staining. Every day of culture in the presence or the absence of NGF, as described above, cells were fixed and stained with coomassie brilliant blue. Briefly, cells were fixed with 90% (v/v) methanol in PBS at −20°C, stained for 1 min at room temperature with a coomassie brilliant blue solution (1.25% (w/v) coomassie brilliant blue R-250, 40% (v/v) methanol, and 20% (v/v) acetic acid), washed with PBS, and observed in a MBL3200 Krüss inverted microscope (A. Krüss Optronic, Germany). Neurite length was measured by the use of software ImageJ 1.47v (USA). The rate of neurite elongation on a certain day (x) was

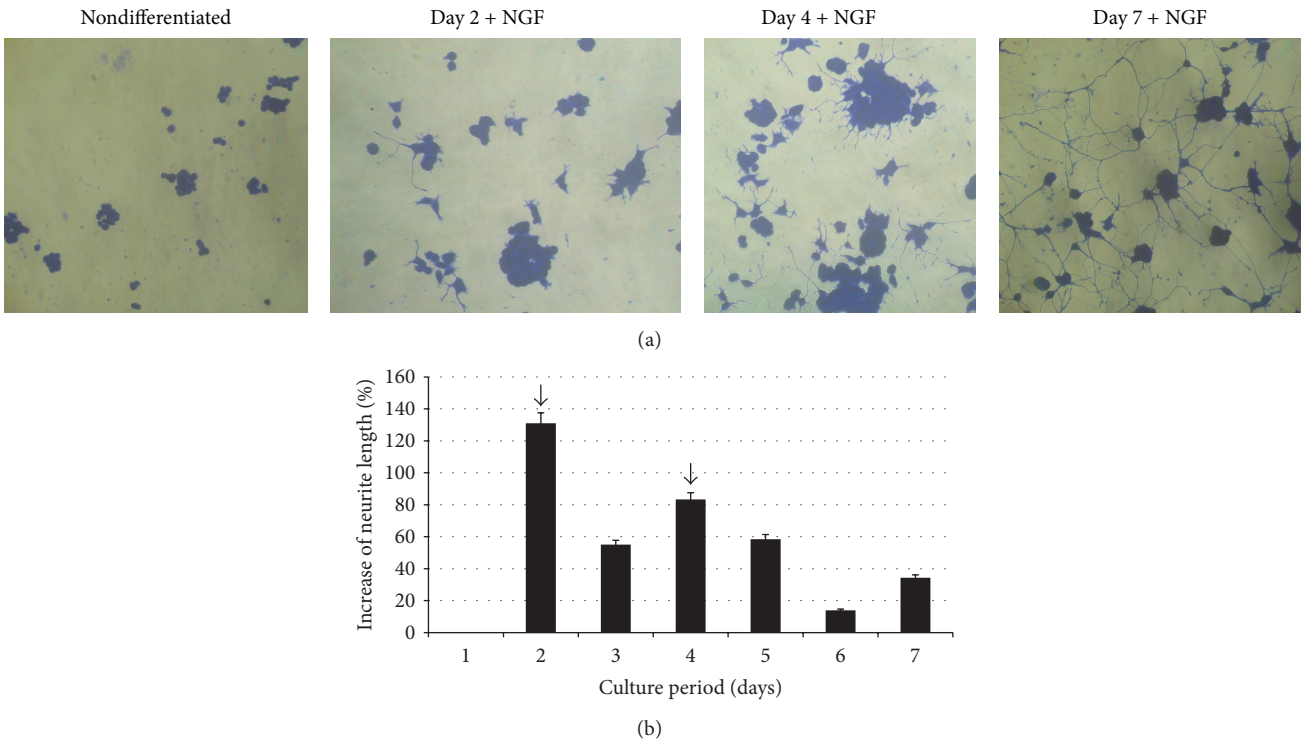


Figure 2: (a) Images of PC12 cells stained with coomassie brilliant blue and differentiated in response to NGF. (b) Progress of PC12 differentiation during the culture period as expressed by the relative rate of neurite length changes day by day during the seven days of *in vitro* differentiation with NGF. Maximum values on days 2 and 4 are indicated with arrows. Average results from replicate experiments ($n = 3$).

expressed as the percentage of change relative to the previous day ($x - 1$).

2.7. Fluorescence-Based ROS and Superoxide Assays. PC12 cells were cultured in the presence or in the absence of the NGF, as described in Section 2.5. Superoxide and ROS concentrations were assessed by staining with 5 μM MitoSOX and 10 μM H_2DCFDA, respectively. Briefly, cells were incubated with the dye for 30 min and 45 min, respectively, at 37°C and subsequently washed twice with PBS. Fluorescence analysis was performed on an Infinite 200 Pro fluorescence plate reader (TECAN, Switzerland) at 510 nm excitation and 580 nm emission for MitoSOX and at 492 nm excitation and 520 nm emission for H_2DCFDA.

2.8. Experimental Design. 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.

3. Results and Discussion

In response to treatment with NGF, PC12 cells demonstrated differentiation through axonal neurite prolongation (Figure 2(a)), also in accordance with previous reports [3]. No differentiation was observed in the absence of NGF. The rate of neurite elongation during the seven-day culture period was fluctuating, with maximum values observed on days 2 and 4 (Figure 2(b)). During the same period, ROS accumulation was generally increased, reaching a maximum value on days 3 and 7, while higher ROS concentrations were determined in NGF-treated cultures compared to control (Figure 3). Therefore, the pattern of total free radical species accumulation could not be clearly associated with PC12 differentiation, expressed through neurite development.

A different pattern was observed for the concentration of superoxide anion, as measured with the fluorescent dye MitoSOX: maximum accumulation was observed on day 2 (coinciding with the maximum rate of neurite elongation) and, again, day 7 (Figure 4). Similar to ROS measurements, an almost identical pattern was observed for superoxide accumulation in both NGF-treated and untreated PC12 cells, with higher O_2^- concentrations being associated with the differentiation process.

Maximum concentrations of superoxide in the culture medium on days 2 and 4 were determined with the biosensor-based assay, while a third peak was observed again on day 7 (Figure 5). Therefore, only by using the cell-based biosensor, we were able to more precisely associate the pattern of superoxide production with measurable changes in the expression of neuronal differentiation. Contrary to the MitoSOX-based assay, no changes in O_2^- accumulation were observed in the control culture medium.

Previous studies have reported the involvement of superoxide and other ROS species in the elicitation of neuronal differentiation, for example, through activation of the MAPK

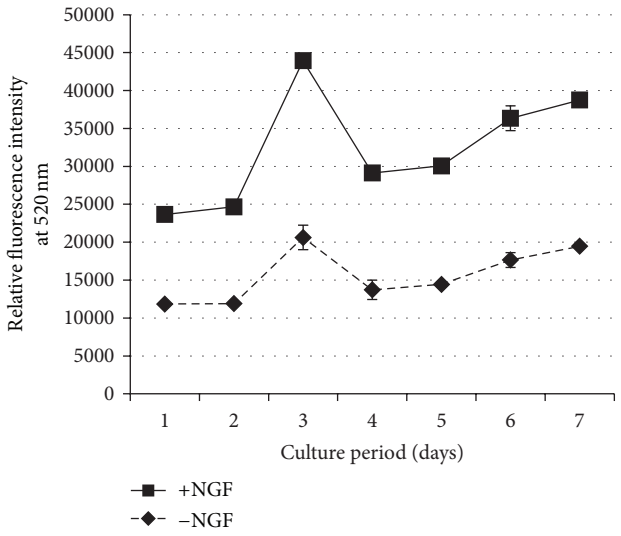


Figure 3: Relative ROS accumulation (expressed as relative fluorescence intensity of H_2DCFDA-stained PC12 cells) during the seven-day period of *in vitro* differentiation with NGF (solid line). *Control:* dashed line. Average results from replicate experiments ($n = 3$).

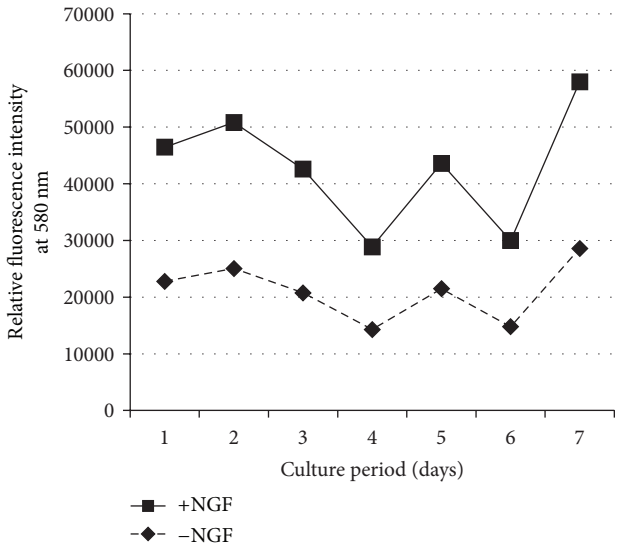


Figure 4: Relative superoxide accumulation in mitochondria of PC12 cells (expressed as relative fluorescence intensity of MitoSOX-stained cells) during the seven-day period of *in vitro* differentiation with NGF (solid line). *Control:* dashed line. Due to very small standard errors, SE bars are not clearly shown.

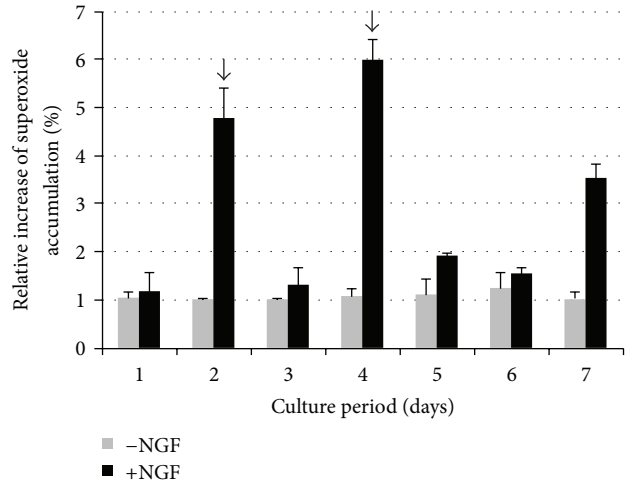


Figure 5: Relative increase (% control) of superoxide accumulation in the culture medium during the seven-day period of *in vitro* PC12 differentiation with NGF (black column), as determined with the cell-based superoxide biosensor. *Control:* grey column. Maximum values on days 2 and 4 are indicated with arrows. Average results from replicate experiments ($n = 3$).

pathway [2–5, 10], the hydrogen peroxide-dependent phosphorylation of ERK, and heme oxygenase induction in differentiating N2a neuroblastoma cells [11, 12].

The association between superoxide production and neuronal cell differentiation has been recently demonstrated in N2a neuroblastoma by Valero et al. [5], who suggested that differentiation and mitochondrial biogenesis are part of a hormetic response which is triggered by a modest increase of superoxide anion concentration within the mitochondria. Regarding PC12 cells, it is known from previous studies that during differentiation pheochromocytoma cells increase the expression of cholinergic receptors, which may render differentiated cells more sensitive to oxidant stimuli than undifferentiated ones [13]. Another line of evidence for a role of superoxide as a downstream regulator of differentiation has been provided by Cassano et al. [14], who showed that NGF-induced differentiation in PC12 affects mitochondrial metabolism by reducing mitochondrial-produced reactive oxygen species through the stimulation of mitochondrial manganese superoxide dismutase (MnSOD) both transcriptionally and posttranscriptionally via Ki-Ras and ERK1/2. The authors concluded that low and spatially restricted levels of H_2O_2 induce and maintain long term ERK1/2 activity and ultimately differentiation of PC12 cells. In this context, the present study provides data further supporting the hypothesis of a functional link between changes in the pattern of superoxide anion accumulation and differentiation in PC12 cells.

The use of living cells for fast specific and nonspecific chemical sensing is an area of increasing importance. The purpose of the present study was to demonstrate the applicability of the biosensor system, based on the bioelectric patterning of membrane-engineered cells, in determining the *in vitro* O_2^- accumulation in a noninvasive and rapid manner. In parallel, the assay was compared with a standard fluorescence-based method.

As shown in the present study, employment of the biosensor for superoxide determination in the culture medium of differentiating PC12 provided more accurate information regarding the differentiation process than fluorescence-based methods. It should be also noted that fluorescent probe-based determination of superoxide anions in cell cultures is usually coupled with high performance liquid chromatography in

order to provide a rigorous, high throughput assay system, which is inevitably associated with considerable cost and time considerations [15]. In addition, the biosensor does not interfere with the assayed cellular parameters, as it may happen with some fluorescent dyes [16]. Moreover, there is no need of disrupting the sensor, which would cause a delay between the actual cellular event and its measurement. We should also mention that the biosensor described in the present study assays the total concentration of superoxide released from the cells into the culture medium; that is, it is not restricted to measure O_2^- production at a certain subcellular compartment or organelle (as is the case with MitoSOX).

Real-time measurement of O_2^- dynamics *in vitro* is also feasible with amperometric methods, but not with the same sensitivity as with the cell-based sensor, even if O_2^- is measured in isolated mitochondria [17]. Chang et al. [18] used an electrochemical approach in order to measure the response of A172 glioblastoma cells to various chemicals. They developed an amperometric sensor array custom-embedded in a 24-well cell culture plate format, which required washing of the cell cultures with Hank's Balanced Salt Solution, thus necessitating an additional process step. In addition, biosensor responses were strongly dependent on the density of cells in the assayed culture system. Quite recently, Gómez-Mingot et al. [19] reported the use of electrochemical biosensors for the real-time, *in situ* determination of superoxide anions in culture medium. Satisfactory biosensor selectivity over potential interferants, such as glucose, lactic acid, pyruvic acid, ascorbic acid, citric acid, lipoic acid, and various amino acids, was demonstrated. However, the authors did not test the system with actual cell cultures, whereas a potential drawback of their approach is related to the requirement of a daily preparation of fresh sensor elements, that is, a low storability of the biorecognition element. On the contrary, immobilized cells used in our approach have been repeatedly shown to retain their functionality even after 3-4 weeks. In addition, the system described in the present study does not suffer from other limitations of various electrochemical systems, such as the requirement of a flow technique for maintaining close contact of O_2^- molecules to the working electrode or the presence of other cells and tissue components in the sample [20].

The ability to monitor, in real time, superoxide anion production, in neural cells and tissues, opens fascinating new perspectives in neurobiology research. Not only are free radicals involved with neuronal differentiation, as shown in the present study and elsewhere [5]; they are also associated with the basic functions of the neurons, as demonstrated by Ganesana et al. [21] who used a miniaturized electrochemical cytochrome c (Cyt c) biosensor in order to monitor superoxide production in mouse hippocampal slices. They were able to establish the transmembrane flux of superoxide anions into the extracellular space through voltage-dependent anion channels (VDACs), in other words, key neurotransmission elements. Boulton et al. [22] evaluated the neurotoxicity of 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline (TaClo) in SH-SY5Y neuroblastoma cells by assaying superoxide production from isolated mitochondria with an amperometric biosensor. It should be noted, however, that, contrary to our approach, this method required the fractionation of cellular organelles and as such could not be used for continuous monitoring of superoxide anion production from intact cells.

4. Conclusion

In conclusion, the early detection of changes in the pattern of superoxide accumulation may be used for monitoring neuronal cell differentiation in response to the application of different chemical agents. On a clinical level, there is a documented association between excesses in blood concentration and postoperative care, traumatic brain injury, and diabetes mellitus-related cell injury [23, 24]. Taking into account that it is currently feasible to scale up the screening capability of the sensor platform up to 96 tests in approximately 70 min [25], there is an apparent opportunity to employ the biosensor as an advanced companion diagnostics system for the high throughput evaluation of novel compounds with neuron-differentiating properties.

Conflict of Interests

The authors declare that they have no conflict of interests.

References

[1] D. C. Wallace and W. Fan, "Energetics, epigenetics, mitochondrial genetics," *Mitochondrion*, vol. 10, no. 1, pp. 12–31, 2010.

[2] H. L. A. Vieira, P. M. Alves, and A. Vercelli, "Modulation of neuronal stem cell differentiation by hypoxia and reactive oxygen species," *Progress in Neurobiology*, vol. 93, no. 3, pp. 444–455, 2011.

[3] J. C. Schwamborn, R. Fiore, D. Bagnard, J. Kappler, C. Kaltschmidt, and A. W. Püschel, "Semaphorin 3A stimulates neurite extension and regulates gene expression in PC12 cells," *The Journal of Biological Chemistry*, vol. 279, no. 30, pp. 30923–30926, 2004.

[4] H. Wu, S. Ichikawa, C. Tani et al., "Docosahexaenoic acid induces ERK1/2 activation and neuritogenesis via intracellular reactive oxygen species production in human neuroblastoma SH-SY5Y cells," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1791, no. 1, pp. 8–16, 2009.

[5] T. Valero, G. Moschopoulou, L. Mayor-Lopez, and S. Kintzios, "Moderate superoxide production is an early promoter of mitochondrial biogenesis in differentiating N2a neuroblastoma cells," *Neurochemistry International*, vol. 61, no. 8, pp. 1333–1343, 2012.

[6] E. Cadenas and K. J. A. Davies, "Mitochondrial free radical generation, oxidative stress, and aging," *Free Radical Biology & Medicine*, vol. 29, no. 3-4, pp. 222–230, 2000.

[7] G. Moschopoulou and S. Kintzios, "Application of 'membrane-engineering' to bioelectric recognition cell sensors for the ultrasensitive detection of superoxide radical: a novel biosensor principle," *Analytica Chimica Acta*, vol. 573-574, pp. 90–96, 2006.

[8] G. Moschopoulou, T. Valero, and S. Kintzios, "Superoxide determination using membrane-engineered cells: an example of a novel concept for the construction of cell sensors with customized target recognition properties," *Sensors and Actuators B: Chemical*, vol. 175, pp. 78–84, 2012.

[9] M. Zeira, P.-F. Tosi, Y. Mouneimne et al., "Full-length Cd4 electroinserted in the erythrocyte-membrane as a long-lived

inhibitor of infection by human-immunodeficiency-virus," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 10, pp. 4409–4413, 1991.

[10] X. Wang, Z. Wang, Y. Yao et al., "Essential role of ERK activation in neurite outgrowth induced by α-lipoic acid," *Biochimica et Biophysica Acta*, vol. 1813, no. 5, pp. 827–838, 2011.

[11] U. Kemmerling, P. Muñoz, M. Müller et al., "Calcium release by ryanodine receptors mediates hydrogen peroxide-induced activation of ERK and CREB phosphorylation in N2a cells and hippocampal neurons," *Cell Calcium*, vol. 41, no. 5, pp. 491–502, 2007.

[12] N. Shinjyo and K. Kita, "Relationship between reactive oxygen species and heme metabolism during the differentiation of Neuro2a cells," *Biochemical and Biophysical Research Communications*, vol. 358, no. 1, pp. 130–135, 2007.

[13] M. Pera, P. Camps, D. Muñoz-Torrero, B. Perez, A. Badia, and M. V. Clos Guillen, "Undifferentiated and differentiated PC12 cells protected by huprines against injury induced by hydrogen peroxide," *PLoS ONE*, vol. 8, no. 9, Article ID e74344, 2013.

[14] S. Cassano, S. Agnese, V. D'Amato et al., "Reactive oxygen species, Ki-Ras, and mitochondrial superoxide dismutase cooperate in nerve growth factor-induced differentiation of PC12 cells," *The Journal of Biological Chemistry*, vol. 285, no. 31, pp. 24141–24153, 2010.

[15] J. Zielonka, J. Joseph, A. Sikora, and B. Kalyanaraman, "Real-time monitoring of reactive oxygen and nitrogen species in a multiwell plate using the diagnostic marker products of specific probes," *Methods in Enzymology*, vol. 526, pp. 145–157, 2013.

[16] R. Dixit and R. Cyr, "Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy," *The Plant Journal*, vol. 36, no. 2, pp. 280–290, 2003.

[17] J. R. Henderson, H. Swalwell, S. Boulton, P. Manning, C. J. McNeil, and M. A. Birch-Machin, "Direct, real-time monitoring of superoxide generation in isolated mitochondria," *Free Radical Research*, vol. 43, no. 9, pp. 796–802, 2009.

[18] S.-C. Chang, N. Pereira-Rodrigues, J. R. Henderson, A. Cole, F. Bedioui, and C. J. McNeil, "An electrochemical sensor array system for the direct, simultaneous *in vitro* monitoring of nitric oxide and superoxide production by cultured cells," *Biosensors and Bioelectronics*, vol. 21, no. 6, pp. 917–922, 2005.

[19] M. Gómez-Mingot, S. Griveau, F. Bedioui, C. E. Banks, V. Montiel, and J. Iniesta, "Electrochemical devices for monitoring biomarkers in embryo development," *Electrochimica Acta*, vol. 140, pp. 42–48, 2014.

[20] M. Fujita, R. Tsuruta, S. Kasaoka et al., "In vivo real-time measurement of superoxide anion radical with a novel electrochemical sensor," *Free Radical Biology & Medicine*, vol. 47, no. 7, pp. 1039–1048, 2009.

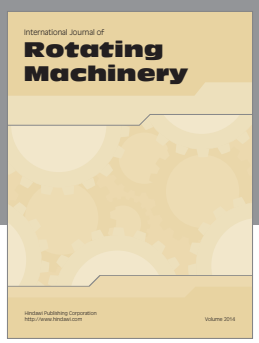
[21] M. Ganesana, J. S. Erlichman, and S. Andreescu, "Real-time monitoring of superoxide accumulation and antioxidant activity in a brain slice model using an electrochemical cytochrome c biosensor," *Free Radical Biology & Medicine*, vol. 53, no. 12, pp. 2240–2249, 2012.

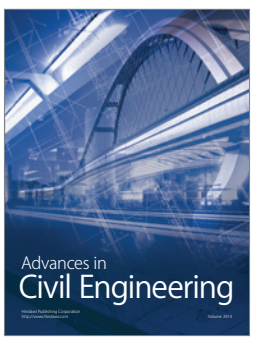
[22] S. J. Boulton, P. C. Keane, C. M. Morris, C. J. McNeil, and P. Manning, "Real-time monitoring of superoxide generation and cytotoxicity in neuroblastoma mitochondria induced by 1-trichloromethyl-1,2,3,4-tetrahydro-beta-carboline," *Redox Report*, vol. 17, no. 3, pp. 108–114, 2012.

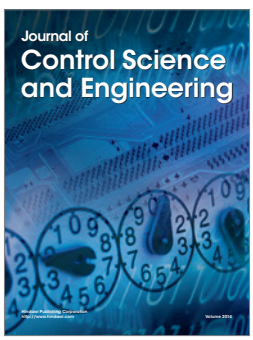
[23] E. Crimi, V. Sica, S. Williams-Ignarro et al., "The role of oxidative stress in adult critical care," *Free Radical Biology & Medicine*, vol. 40, no. 3, pp. 398–406, 2006.

[24] K. Maiese, S. D. Morhan, and Z. C. Zhao, "Oxidative stress biology and cell injury during type 1 and type 2 diabetes mellitus," *Current Neurovascular Research*, vol. 4, no. 1, pp. 63–71, 2007.

[25] A. Perdikaris, N. Vassilakos, I. Yiakoumettis, O. Kektsidou, and S. Kintzios, "Development of a portable, high throughput biosensor system for rapid plant virus detection," *Journal of Virological Methods*, vol. 177, no. 1, pp. 94–99, 2011.







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