An impedance-based high-throughput method for evaluating the cytotoxicity of nanoparticles

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Abstract. Impedance-based assays can constitute a reliable alternative to the conventional methods used in nanotoxicology due to the important advantages of being label-free and monitoring the cells in real-time. In this study, the suitability of impedance-monitoring for the screening of nanoparticle (NP)-induced cytotoxicity was assessed. The effect of titanium dioxide (TiO₂)-NPs on cellular proliferation, viability, spreading, and detachment from substrate was evaluated by continuous impedance-based measurements made with an xCELLigence system. Fibroblasts seeded in microelectrode-embedded E-plates were exposed to spherical anatase nano-TiO₂ (5, 10, and 40 nm in diameter) for up to 120 h. An alternative excitation signal (20 mV control voltage amplitude) was applied at 10, 25, and 50 kHz to the microelectrodes in the E-plates. Cells attached to the electrode surfaces act as insulators and lead to an increase in impedance. For validating the impedance-method, Trypan Blue exclusion and ultrahigh resolution imaging (URI) were employed. The general trend observed was a decrease in impedance following exposure to TiO₂-NPs. Impedance-based results were in most instances in accordance with those from the Trypan Blue exclusion and URI assays indicating that the impedance-based approach has merit. Further studies are needed to validate it as a high-throughput method for evaluating NPs’ cytotoxicity.

1. Introduction

A reliable and efficient evaluation of the biological effects of nanomaterials requires the development of appropriate high-throughput screening methods [1, 2]. In general, the same methods that have been used for in vitro toxicity testing of soluble and macro-scale substances are also employed for evaluating the toxicity of NPs [3, 4]. A considerable number of nanotoxicity studies have generated confounding or conflicting data, indicating that due to their physicochemical properties, NPs interfere...
with assay components and/or detection systems [4]. Label-free techniques would eliminate the interferences between NPs, substances used for labeling and the detection systems [4]. Another common problem shared by traditional methods is that they assess the effects only at a specific point in time and do not give any information on the biokinetic behavior and the interaction between living cells and toxicants over time [5]. As a result, the need for label-free real-time detection methods for investigating the toxicity of NPs on living systems has emerged.

Assays that measure the electrical properties of cells, such as impedance-based assays, are label-free and can represent a reliable alternative to the traditional methods [6]. These methods analyze matter either by their active or passive electrical properties [7]. A method that takes advantage of the passive properties is impedance-based monitoring which measures how much an object impedes a flow of electrical current. In impedance-based monitoring, an alternating current is used to generate information about the properties of the cell as a function of signal frequency. A variety of cellular properties such as proliferation, growth, morphology, and adhesion can be measured by applying an electric source to an electrode covered by cells, and measuring how the cells impede the current [7].

Titanium dioxide (TiO$_2$) NPs are widely used in paints, pharmaceuticals, food colorants, and cosmetics. Titanium and its alloys are commonly used as implant materials [8] and TiO$_2$ NPs can be released from implants and cause toxic effects [9, 10, 11]. In this study, the suitability of impedance-monitoring for the screening of NPs-induced cytotoxicity was assessed. Impedance-based monitoring of L929 fibroblasts exposed to TiO$_2$ NPs was performed by using an xCELLigence system, which is able to monitor cells “in situ” without the use of markers. The system monitors electrical impedance by sending an alternative current through microelectrodes at the bottom of the wells. When the cells cover the microelectrodes, the impedance goes up. The increase in impedance leads to an increase in voltage drop across the electrodes. Based on the measured impedance, it can be inferred whether the amount of cells is increasing. The number, the development, morphology, strength and the degree of adhesion of the cells can also be deduced by the instrument to a varying degree. To validate the method, cellular proliferation and viability were evaluated by manual and automated cell counting (the Trypan Blue assay) and by ultrahigh resolution microscopy (URI).

2. Material and Methods

2.1. TiO$_2$ nanoparticles

Three spherical anatase TiO$_2$-NPs (Nanostructured & Amorphous Mat., USA) with diameters of 5 (Ti1), 10 (Ti2) and 40 nm (Ti3), were characterized by X-ray diffraction, transmission electron microscopy (TEM), N$_2$-BET adsorption, dynamic light scattering, and electrophoretic mobility [11] (table 1). NP suspensions were prepared as described by Allouni et al. [11, 12]. The concentrations used, i.e., 0.05, 0.5, and 5 mg/L were in the range of those that have been reported in serum, blood, and in dry tissue surrounding Ti-based implants [13, 14, 15].

2.2. Cell culture

L929 mouse fibroblasts (CCL-1; ATCC, Manassas, VA, USA) were cultured in 25 cm$^2$ flasks (Costar, Switzerland) in RPMI 1640 medium (BioWhittaker, Lonza, Belgium) supplemented with 10% heat inactivated (30 min, 56°C) fetal calf serum (FCS) (Millipore, MA, USA), 2% Penicillin/Streptomycin/Amphotericin, and 1% L-glutamine (BioWhittaker, Lonza, Belgium). The cells were kept at 37°C in humidified air with 5% CO$_2$. Only cells from cultures with a viability >90% (tested by exclusion of 0.2% Trypan Blue) were used in the experiments. All subsequent experiments were run in duplicate and repeated three times.

2.2. Impedance-based monitoring of cells

The system used in this study was the xCELLigence Real Time Cell Analyzer Dual Plate instrument (RTCA DP) (Roche Diagnostics GmbH, Mannheim, Germany). The system measures electrical impedance across interdigitated microelectrodes situated at the bottom of culture wells. The RTCA-DP Analyzer has three stations for electrode-plates (E-plates). The measurements are done by applying
an alternative excitation signal (20 mV control voltage amplitude) at three different frequencies (10, 25 and 50 kHz) through the microelectrodes in the E-plates while monitoring the voltage drop across the electrodes where the quotient voltage/current yields the impedance. It was previously demonstrated that the excitation signal used does not affect the physiological state of the cells [16]. The E-plate 16 is a single-use plate with 16 wells (5 mm diameter) with microelectrodes which cover about 80% of the bottom of the wells. The RTCA DP Analyzer was located inside an incubator at 37°C in humidified air with 5% CO_2. The RTCA DP Analyzer is connected to a control unit consisting of a computer with software installed for controlling the unit and for receiving, analyzing and displaying the data obtained from the measurements. The software displays the measured impedance, which is normalized by a frequency factor Z_n, as a Cell Index (CI) for each point in time. The CI is a measure of the electrical impedance both in the presence and absence of cells (equation 1):

\[
CI = \frac{R(f_n,t) - R(f_n,t_0)}{Z_n}
\]

where \( f_n \) is the frequency at which the impedance measurement is carried out, \( R(f_n,t) \) is the measured impedance at frequency \( f_n \) at time-point \( t \), \( R(f_n,t_0) \) is the measured impedance at frequency \( f_n \) at \( t_0 \) (time of background measurement), and \( Z_n \) is the corresponding frequency factor of \( f_n \). The xCELLigence system measures the impedance at three discrete frequencies: \( f_1 = 10 \text{ kHz} \), \( f_2 = 25 \text{ kHz} \) and \( f_3 = 50 \text{ kHz} \). The corresponding frequency factors are \( Z_1 = 15 \Omega \), \( Z_2 = 12 \Omega \), and \( Z_3 = 10 \Omega \) respectively. The CI is a dimensionless number since the measured impedance is divided by the frequency factor. A change in CI can be indicative of changes in cell number, cell adhesion, spreading or cell morphology.

When cells are not present the impedance measured from the microelectrodes in the wells is dependent on the electrode geometry, the ion environment and the electrical properties of the material in the well. When cells are attached to the sensor surfaces of the microelectrodes they act as insulators, and lead to an increase in impedance.

Table 1. Physiochemical characteristics of TiO_2 NPs used in the study

| Sample | \( S_{BET}^a \) (m\(^2\)/g) | Crystal Structure | \( \zeta \)-potential ± SD [mV] | IEP\(^b\) | Hydrodynamic diameter (PDI) (nm) (after 24 h) |
|--------|-----------------|-----------------|-----------------|------|----------------------------------|
| Ti1    | 131             | 86.6% A. 13.4% R. | -13.2 ± 0.6     | 3.52 | 423.7 ± 9.7 (408.0 ± 10.0)       |
| Ti2    | 105             | 100% A.        | -11.8 ± 1.1     | 6.88 | 463.1 ± 12.1 (445.5 ± 10.9)      |
| Ti3    | 20              | 81% A. 18.9% R. | not given       | 3.45 | 446.2 ± 6.1 (400.0 ± 9.2)        |

\(^a\) Specific surface area. \(^b\) Isoelectric point from titration curve: zeta potential vs. pH in aqueous solution of 0.14 M NaCl. \(^c\) DLS measurements in RPMI cell culture medium with supplemented 10% FBS, immediately after dispersing the particles, and after 24 hours (from Allouni et al. [11]).
2.2.1. **Determination of optimal cell density.** A titration experiment was performed in order to
determine the cell concentration that gave optimum cell viability and proliferation. The following cell
densities were tested: 3125, 6250, 12500, 25000, 50000, and 100000 cells/cm$^2$.

2.2.2. **Impedance-based monitoring of cells exposed to TiO$_2$ nanoparticles.** L929 cells (100,000
cells/cm$^2$) needed 48 h to reach the log phase, which was the time-point when they were exposed to
0.05, 0.5, and 5 mg/L suspensions of TiO$_2$ NPs in cell culture medium. Impedance measurements were
carried out at 15 min intervals for a total period of 7 days, including the initial attachment phase.

2.2.3. **Trypan Blue assay**
The viability of the cells was evaluated at the beginning and end of each experiment by Trypan Blue
(0.2%). The cells were seeded in 24-well plates (TPP, Switzerland) and exposed to TiO$_2$ NPs under
the same conditions as in the impedance assay. The viable and total cell number was counted
manually with a Neubauer chamber and with the Countess Automated Cell Counter (Invitrogen, CA,
USA). The cells on the E16 plates were trypsinised at the end of each assay and their viability
evaluated by manual counting.

2.3. **Ultra-high resolution microscopy**
For investigating morphological changes after interactions with NPs, an Ultrahigh Resolution Imaging
(<100 nm) (URI) system (CytoViva™, Auburn, USA) was used [17]. The cells were fixed and then
visualized with a 100x oil immersion objective as described by Allouni et al. [11].

2.3.1. **Data analysis**
The Mann-Whitney U test for paired comparison of independent samples (p<0.05) and the SPSS
statistical software were used. The Cl-values of both the control and treated cells were compensated
for the impedance of the medium and for the impedance of the NPs plus medium, respectively by
performing the following subtractions at each time-point:

$$Cl(\text{control cells}) = Cl(\text{control cells} + \text{medium}) - Cl(\text{medium})$$

$$Cl(\text{treated cells}) = Cl(\text{treated cells} + \text{medium} + \text{NPs}) - Cl(\text{medium} + \text{NPs})$$

For cells exposed to TiO$_2$ NPs, the Mean Cell Index (MCI) values for all experiments were calculated
at each time-point by using equation (2):

$$MCI = \frac{1}{N} \sum_{i=1}^{N} CI_i$$

(2)

where $N$ was the number of measurements and $CI_i$ were the individual measurements of the CI at a
given time. The MCI-values were normalized by choosing the MCI at 7 h after exposure as the basis
for normalization. This was done to correct for the differences which occurred before the
normalization time. The reason for choosing this time-point for normalization was that variations due
to changes in the temperature had settled down at this time. The Normalized Cell Index (NCI) was
calculated as follows:

$$NCI = \frac{MCI \ (\text{specific time})}{MCI \ (\text{normalization time})}$$

A Mean Normalized Cell Index (MNCI) for the different control cells and treated cells was
subsequently calculated for all time-points $t$ up to $t_{\text{final}}$, as follows:
where $N$ was the number of measurements, $N_{CI_i}$ were the NCIs for each time-point $t$, and $\Delta t$ was the time between the time-point of normalization and the time-point where the cells started to plateau $t_{final}$. The calculated MNCI was used to determine the impact of TiO$_2$ NPs cell proliferation.

3. Results

3.1. Determination of optimal cell density

The MCI reached a peak 2-3 h after cell seeding and then declined and reached a minimum after about 20 h (figure 1). The MCI then started to rise as a result of cells attaching to the surface and dividing. No growth could be seen for 3,125 and 6,250 cells/cm$^2$, while a minimal growth was noted at 12,500 cells/cm$^2$. Tested densities leading to the highest rate of cell growth were 50,000 and 100,000 cells/cm$^2$.

3.2. Impedance-based monitoring of cells exposed to TiO$_2$ nanoparticles.

Plots of MCI vs. time and NCI vs. time are shown for cells exposed to Ti3 in figures 2 and 3. In the first plot, the MCI vs. time is shown for the different treatments for the duration of the experiment (figure 2). The second plot shows the NCI starting at 7 h after the exposure to TiO$_2$ NPs (figure 3). The MNCI was calculated starting at 7 h after exposure and until the cells reached a plateau.

At 12 h, cells started to spread and divide and differences between the curves became visible, which are possibly due to small differences in seeding densities. TiO$_2$ NPs were added at 48 h when cells were in the log phase. When NPs were added, a double peak was observed (B). The time at 55 h was selected as the time point for normalizing the MCI-curves from which the relative growth of the cells can be observed (figure 3). As mentioned earlier, this specific time-point was chosen for normalization because peaks due to changes in the temperature had settled down at this time. By normalization, it was possible to correct for the different MCI of the cells that were observed before the exposure. The relative growth of the NCI for each of the curves was plotted from the normalization point at 55 h onward up to $t_{final}$ (figure 3). The highest NCI at the end was observed for the control, while the rest distributed according to the concentration of TiO$_2$ NPs added. It could be seen that the increased NPs concentration induced a decrease in NCI.
3.3 Effects of TiO2 NPs on the Mean Normalized Cell Index.
For comparing the effects of the NPs, the MNCI was calculated for the interval of 55-130 h. The endpoint of the interval was considered to be where cell proliferation reached a plateau. All exposures induced a decreased MNCI compared to controls (figure 4). For Ti1, the MNCI increased with an
increase in the concentration of particles. For the remaining particles, the only trend that could be observed was that the treatments had lower MNCIs than the controls.

Figure 4. MNCIs of L929 cells exposed to TiO\textsubscript{2} NPs 48 h after being seeded at a density of 100,000 cells/cm\textsuperscript{2}. The average cell indexes were calculated starting 7 h after exposure until the cells plateaued at 75 h postexposure to NPs. The cell indexes were normalized based on the MCI 7 h after exposure to NPs. The results are expressed as mean ± SD of the mean. (*) shows statistical significance (p<0.05) compared to the control.

Figure 5. Total number of viable L929 fibroblasts after 120 hrs of incubation with Ti1, 2 and 3. Cells were seeded at an initial density of 100,000 cells/cm\textsuperscript{2}, allowed to attach during 48 hrs before NPs were added for 120 hrs. At the end of incubation, cells were trypsinized, stained with Trypan Blue and the number of viable cells was counted using the Countess automated cell counter as well as by manual counting using a Neubauer counting chamber. Total cell count was normalized to control for each NP type. (*) shows statistical significance (p<0.05) compared to the control.
The most common trend appears to be that the MNCl (impedance measurements) and the number of viable cells (Trypan Blue) decrease after exposure to particles. Statistically significant differences could be noted for Ti1 at 0.5 and 5 mg/L and for Ti3 at all concentrations in the impedance assay. In the Trypan Blue assay, significant differences were noted only for Ti2 5 mg/L and Ti1 0.05 mg/L. Even for the same assay, i.e., Trypan Blue, differences could be seen between the results obtained by manual and automated counting. The impedance measurements were more in accordance with the ones obtained by manual counting.

3.4. URI microscopy.
The uptake of TiO$_2$ NPs by L929 fibroblasts and putative changes in cellular morphology were explored by URI-microscopy. Agglomerates of TiO$_2$ NPs could easily be observed due to their intense light scattering and were associated with cells at all three concentrations [11]. The particles were situated on the membrane and inside the cells. The cells exposed to 0.5 mg/L and 5 mg/L were rounder and smaller than the control cells, which could be indicative of cellular death by apoptosis (figure 6).

Figure 6. URI images of L929 cells at 100x magnification after 24 h exposure to Ti1: A) unexposed cells. B) 0.05 mg/L. C) 0.5 mg/L. D) 5 mg/L. The bright spots are TiO$_2$ NPs, either attached to the cell membrane or internalised by the cells.

4. Discussion
The objectives of this study were to evaluate the impedance-based sensing approach for nanotoxicity assays and to investigate the effect of TiO$_2$ NPs on cellular viability and proliferation.

The xCELLigence system monitored the proliferation and adhesion of cells positioned on microelectrodes by measuring the cell-sensor impedance. Several studies have indicated that the xCELLigence system is advantageous for testing the cytotoxicity of compounds compared to end-point assays [16, 18, 19]. Nevertheless, the need for validating the xCELLigence results with results obtained from traditional assays has also been pointed out [18]. For evaluating the impedance-based approach in this study, cell proliferation and viability measured by the Trypan Blue assay were used for comparison. To control for possible interferences of NPs, both manual and automated (Countess, Invitrogen) counting were employed. URI was used as a qualitative assay for visualizing the putative modifications in cellular morphology after exposure to TiO$_2$ NPs.
The variations of the MCI represented in the xCELLigence plots as curves, could be explained as follows: after being seeded on the E-plates, the cells sedimented to the bottom of the wells and started to attach to the surface of the sensors. This was observed as a sharp increase in the MCI around 2-3 h after cell seeding. This was followed by a lag phase with cells preparing to enter cell division. This was seen as an early plateau in the MCI plots. Once the cells found their place, they attached better to the surface and started to stretch and spread, and then entered the log phase where they proliferated. The proliferation was reflected by a steep MCI increase. Then, the curves reached a final plateau, also known as the stationary phase, where the cells stop growing due to constraints like too little space or cell culture medium. During this phase, some cells will die and MCI consequently declines. Nanoparticles can affect the adhesion and spreading of cells as well as cell growth and viability. Changes in the cell surface/electrode sensor contact will lead to a change in the measured impedance [20]. When cells die by apoptosis, they round up and detach from the substrate and the measured impedance decreases. If cells die by necrosis, their membranes become permeable and the measured impedance decreases as in the case of apoptosis.

To correct for differences in CI before exposure, the MCI was normalized. The MNCI was used as a measure for the overall proliferation of the cells. The lowered MNCI is mainly attributed to cellular shrinking and cell detachment, which corroborated with the reduced proliferation and viability seen in the Trypan Blue assay suggesting that the cause for the lower impedance was cell death. Morphological modifications were observed in the cells exposed to TiO$_2$ NPs by URI microscopy. It was observed that, while the control cells were well spread and stretched, the treated cells appeared to be smaller and rounder, signs indicative of apoptosis [21, 11]. Observations made by URI, as well as those for viability, indicate that cell death, both by apoptosis and necrosis, occurred. These results are in accordance with results from previous studies [10, 11, 22, 23, 24]. In the absence of a clear dose-response effect and given the fact that a relatively high standard deviation was seen in the manual counting, especially for Ti3, a definitive conclusion could not be drawn with regard to the validity of the impedance-based assay for nanotoxicity testing. However, the fact that all the methods showed the same low level of toxicity induced by the chosen nano-TiO$_2$ indicates that the impedance-based approach deserves to be taken into consideration.

5. Conclusions
Impedance-based monitoring provided valuable information, in real-time, about the effects of TiO$_2$ NPs on fibroblasts’ proliferation and viability. The results from impedance measurements were in most instances in accordance with those given by the Trypan Blue assay, proving that the impedance-based approach for nanotoxicity has merit. Further studies are needed in order to validate it as a high-throughput method for evaluating the cytotoxicity of NPs.

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