TECHNICAL SPECIFICATION

ISO/TS 21633

First edition
2021-08

Label-free impedance technology to assess the toxicity of nanomaterials in vitro

Technologie de l'impédance électrique sans marqueur pour évaluer la toxicité des nanomatériaux in vitro

Reference number
ISO/TS 21633:2021(E)

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 229, *Nanotechnologies.*

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Label-free impedance technology to assess the toxicity of nanomaterials in vitro

1 Scope

This document describes a methodology of a label free and real-time detection for non-invasive monitoring of cell-based assays to assess toxicity of nanomaterials to eukaryotic and prokaryotic cells.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO/TS 80004-1, *Nanotechnologies — Vocabulary — Part 1: Core terms*

ISO/TS 80004-2, *Nanotechnologies — Vocabulary — Part 2: Nano-objects*

ISO/TS 10993-1, *Biological evaluation of medical devices — Part 1: Evaluation and testing within a risk management process*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

3.2
nanomaterial
NM
material with any external dimension in the *nanoscale* (3.1), or having internal structure or surface structure in the nanoscale

Note 1 to entry: This generic term is inclusive of *nano-object* (3.3) [and *nanostructured material* (3.4)].

3.3
nano-object
discrete piece of material with one, two or three external dimensions in the *nanoscale* (3.1)

3.4
nanostructured material
material having internal nanostructure or surface nanostructure

3.5
nanoparticle
NP
nano-object (3.3) with all external dimensions in the *nanoscale* (3.1) where the lengths of the longest and the shortest axes of the nano-object do not differ significantly

[SOURCE: ISO/TS 80004-2:2015, 4.4, modified — Note 1 to entry has been deleted.]

3.6
test sample
material, device, device portion, component, extract or portion thereof that is subjected to biological or chemical testing or evaluation

3.7
cell index
CI
dimensionless parameter obtained from the electrochemical impedance measurement

3.8
electrochemical impedance
effective resistance of an electric circuit or component to alternating current, arising from the combined effects of ohmic resistance and reactance.

3.9
impedance-based flow cytometry
IFC
technique used to detect and measure physical and chemical characteristics of a population of cells or particles

Note 1 to entry: A sample containing cells or particles is suspended in a fluid and injected into the flow cytometer instrument.

3.10
electrochemical impedance spectroscopy
EIS
method that measures the impedance of a system in dependence of the AC potentials frequency and therefore that determines both the resistive and capacitive (dielectric) properties of materials

3.11
electrical impedance tomography
EIT
technique in which electrical measurements between many pairs of appropriately positioned surface electrodes are used to produce images of underlying body structures

4 Abbreviations

AC	Alternating current
AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles
BSA	Bovine serum albumin
CB	Carbon black
CI	Cell index
CeO_2	Cerium oxide

CuO	Copper oxide
DIC	Differential interference contrast
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
ECIS	Electric cell-substrate impedance sensing
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EIS	Electrochemical impedance spectroscopy
EIT	Electrical impedance tomography
EMEM	Eagle's minimum essential medium
Fe_2O_3	Ferric oxide
FBS	Fetal bovine serum
HTS	High throughput system
IC_{50}	half-maximal inhibition concentration
IFC	Impedance-based flow cytometry
IMEs	Interdigitated microelectrodes
Mn_2O_3	Manganese oxide
NCI	Normalized CI
Ni	Nickel
PBS	Phosphate buffered saline
QD	Quantum dot
RTCA	Real-time cell analyzer
RPMI	Roswell park memorial institute medium
SiO_2	Silicon dioxide
SPR	Surface plasmon resonance
TiO_2	Titanium dioxide
ZrO_2	Zirconium oxide
ZnO	Zinc oxide

5 Background

5.1 General

Several in vitro assay systems that have been developed for the assessment of the toxicity of different chemical compounds have also been implemented to assess the toxicity of NMs. Due to their physicochemical properties, NMs may behave differently than the chemical compounds for which these assay systems were developed and therefore, when they were used with NMs, discrepancies in results among assays were often observed [1]. As a result, investigators were prompted to consider the interaction of NMs with the assay systems as a possible source for the observed discrepancies.

The detection systems of these toxicity assays are mostly optical in nature and rely on absorbance, luminescence or fluorescence to quantify the products of the assay systems (e.g. tetrazolium salts). NMs may therefore interfere directly with the assay readout by altering the absorbance, luminescence or fluorescence of the products of these assay systems [2]. Depending on their material, shape and size, certain NMs may absorb, scatter and emit light at the assay detection wavelength. Carbon-based NPs, for example, CB are known to absorb light in the visible spectrum whereas metal oxides, metal hydroxides, and metal carbonate NPs are known to scatter light [3]. AuNPs with a strong SPR absorb more light than iron oxide NPs and larger NPs absorb more light than smaller NPs [1]. Similar to AuNPs, AgNPs also have strong plasmon resonances [4]. Such absorptive abilities of these NPs may therefore interfere with the absorptive properties of products obtained from different assay systems. NMs may also interfere directly with the assay by interacting with the chemical reaction product. Due to their large surface area per unit mass and surface reactivity, compared to large particles NMs may also display an increased adsorption capacity thereby increasing the possible interaction between nanoparticles and assay components [3][5]. Finally, NMs may also catalyse the conversion of substrate to product. The large surface area per unit mass and surface reactivity may lead to an excess in surface energy with subsequent enhancement in the catalytic activity of NMs [6].

This document is therefore based on current information about electrochemical impedance technique that does not rely on optical measurements to determine the degree of cell viability or cytotoxicity and also provide kinetic information non-invasively and with high temporal resolution through recorded growth curves. The electrochemical impedance technique can therefore be used as an alternative assay system for the study of the viability and toxicity of NMs in vitro with no interference. It also directs further studies into the mode of action (toxicity) of a NM.

5.2 Electrochemical impedance technique

ECIS was developed in the 1980s for studying cellular processes in real time [7][8][9]. In this assay, cells are cultured in wells, which contain a large reference electrode as well as number of detection electrodes that cover 80 % of surface area of each well bottom. Upon application of the low amplitude sinusoidal potential, the electrochemical impedance between the electrodes is measured. As the cells attach and spread on the electrode surface, they alter the effective area available for current flow causing an increase in the impedance of the system [10]. Increase in impedance is possible due to insulating properties of cellular bilipid membranes which act as dielectric objects and therefore it should correlate to the number of cells on the electrodes. Subsequently, the technique was applied to assess cellular toxicity [11][12][13][14] as well as motility [7][10][15]. Impedance spectroscopy of cellular activity was also developed based on this same impedance technique [12][16][17][18][19][20].

Based on this ECIS technique, a new electronic sensing RTCA was developed with improvements on the electrode structure to allow detection of almost all cell types in a culture well [21][22][23][24][25][26][27][28]. Here, cells are allowed to grow and attach to the electrodes and with change in the flow of current around and through cells, concomitant increase in impedance may ensue and thus providing information on their count, morphology and viability. Upon addition of a test material, cells may detach causing a drop of impedance indicating toxicity through reduction in cellular viability [11][12][29]. To ensure that cells do not detach due to overcrowding, a cell proliferation assessment should be performed prior to experimentation to determine an ideal seeding concentration for the cell type in question. In addition, untreated control cells should be assayed alongside the treated cells to monitor confluency.

In the past, an impedance measurement technique was applied to quantitatively monitor cell number and cell viability in monolayer cultures through various impedance measurements of cellular responses, such as proliferation and toxicity, in a real-time and label-free manner [30][31][32][33][34][35][36][37][38][39]. With this technique, it is also possible to assess cell differentiation, cell invasion and migration, cell-cell interaction using co-cultures, and cellular mechanistic investigation such as intracellular levels of calcium and DNA damage [40][41][42][43]. The electrochemical impedance technique was also used successfully for in situ monitoring of NM-induced cellular toxicity and other aspects of cell physiology such as proliferation, morphology, attachment, and intercellular adhesion [44]. For example, electrochemical impedance-based monitoring was used to investigate the cytotoxic effects of various carbon-based NMs with different cell lines, see Table 1.

Table 1 — Cell types used for the toxicity assessment of nanomaterials using the impedance technique

Cell type	Nanomaterials	Reference
endothelial EA.hy926 (ATCC ® CRL-2922™) cells	Carbon nanotubes	[45][46][47]
L929 (ATCC® CCL-1™) and V79-4 (ATCC® CCL-93™) fibroblasts		
GC-2spd(ts) (ATCC® CRL-2196™) cell line, derived from immortalized mouse spermatocyte		
THP-1 (ATCC® TIB-202™) human monocytic cells		
Human glioblastoma U87-MG (ATCC® HTB-14™)	Graphene	[48]
Rat astrocytes (CRL-2006) and mouse endothelial (ATCC® CRL-2583™) cells		
Hepatoma C3A (ATCC® CRL-10741™) cells		[49]
Epithelial lung cell line, A549 (ATCC® CCL-185™)	CuO and TiO_2	[50]
16HBE14o, an adherent, immortalized human bronchial epithelial cell line	CeO_2, SiO_2, Fe_2O_3, Mn_2O_3, ZnO and ZrO_2	[51]
Human epithelial intestinal HT-29 (ATCC® HTB-38™) cell line	SiO_2	[52]
Human intestinal Caco-2 (ATCC® HTB-37™) cell line	AgNPs	[53]
Bronchial epithelial BEAS-2B (ATCC® CRL-9609™) cell line	AuNPs	[54]
Chinese hamster ovary cell line CHO (CRL-12023)		
Human embryonic kidney cell line HEK 293 (ATCC® CRL-1573™)		
Hepatocellular carcinoma cells (SMMC-7721)	Ni NPs	[55]
HeLa (ATCC® CCL-2™) human cervix epithelia cell line	Polymeric nanoparticles	[56]

6 Basic principles, instruments

6.1 Basics of electrochemical impedance technique

In the EIS system, an electrical equivalent circuit is used to curve fit the experimental data. For cellular detection, number of electrical equivalent circuits were proposed (Reference [57] and its impedance spectrum for IMEs were reported [58]). In a simplified electrical equivalent circuit, it is suggested that two identical double layer capacitances at each electrode (C_{di}) are connected to the medium resistance (R_{sol}) in series, where the dielectric capacitance of the medium (C_{di}) is introduced in parallel with these series elements [30]. In this equivalent circuit, there are two parallel branches namely C_{di} and $C_{di} + R_{sol} + C_{di}$ where the impedance Z_1 and Z_2 in each branch can be expressed with Formula (1) for branch $C_{di} + R_{sol} + C_{di}$: and with Formula (2) for branch C_{di}:

$$|Z_1| = \sqrt{R_{sol}^2 + \frac{1}{(\pi f C_{di})^2}} \tag{1}$$

$$|Z_2| = \sqrt{\frac{1}{(2\pi f C_{di})^2}} \tag{2}$$

As biological cells are very poor conductors at frequencies below 10 kHz [32] the presence of the electrically insulated cell membranes alters the C_{di}. The conductivity of the cell membrane is around 10^{-7} S/m, whereas the conductivity of the interior of a cell can be as high as 1 S/m ([59]). Therefore, cell proliferation can be estimated by the total impedance at low frequencies [30].

6.2 Types of instrument

6.2.1 Electrochemical impedance-based instruments for in vitro analysis of toxicity on cell monolayers

The xCELLigence®, CellSine, and ECIS (ECIS Zθ)[1] systems are the examples of current commercially available electrochemical impedance-based instruments for in vitro analysis of toxicity where they use cell monolayers to monitor the changes in impedance properties of cells after exposure to bioactive agents including NMs.

The design of the cell culture plates with gold-plated electrodes attached to the bottom of the wells implemented in HTS format making it possible for real-time observations to be made of cell changes throughout an experiment without the need for destructive cell sampling [14][15][29][60]. Information may thus be provided on cell proliferation and their reaction to the bioactive agent including NMs in question [61].

A portable automated bench-top mammalian cell-based toxicity sensor that incorporates enclosed fluidic biochips containing endothelial cells monitored by ECIS technique was also developed to assess the toxicity of drinking water [62].

6.2.2 Impedance-based flow cytometry

In addition to surface-attached cell-based electrochemical impedance technique, an IFC system, a microfluidic chip-based IFC, can analyze single cell impedance without specific sample preparation [63] This technique is also able to provide information on size and number of cells as well as on their membrane capacitance and cytoplasmic conductivity.

1) xCELLigence®, CellSine, and ECIS (ECIS Zθ) are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

6.2.3 Electrochemical impedance-based spectroscopy

EIS is a non-invasive, label-free method to measure the dielectric properties of samples while applying a varying frequency AC electrical field by means of electrodes. EIS was initially developed to elucidate double-layer capacitance but it is now used to characterize electrochemical processes under complex potential modulation [64][65]. With EIS, in addition to monitoring cell growth rate in a non-invasive manner, it is also possible to obtain high-resolution imaging of non-adherent or suspended cells [66][67].

EIS is a label-free, non-invasive analysis method for a wide variety of biological samples, ranging from single cells to multicellular aggregates and organisms. For example, real time cell viability and toxicity of test materials can be assessed [16][20]. Moreover, it was also shown that with EIS it is possible to measure the alterations in morphology of cell aggregates due to necrosis and apoptosis by hydrodynamically positioning cell spheroids in a capillary featuring a four-electrode measurement setup [68][69]. EIS measurements was used in environmental toxicology to characterize and manipulate multicellular organisms, such as trapping, sorting, and counting of *Caenorhabditis elegans* [70][71][72][73] or measuring the responses of fish embryos to cryoprotective chemicals, such as methanol and DMSO [74][75]. Moreover, EIS measurements can also be used for the detection of parasites in drinking water [76] and for testing anthelmintic drugs by monitoring parasite motilities [77][78].

Using EIS, the toxicity of silica nanowires on breast epithelial cancer cells [79] and of QD and AuNPs to fibroblastic V79 V794 (e.g. ATCC® CCL93™[2]) cells [80] can be assessed.

6.2.4 Electrical impedance tomography

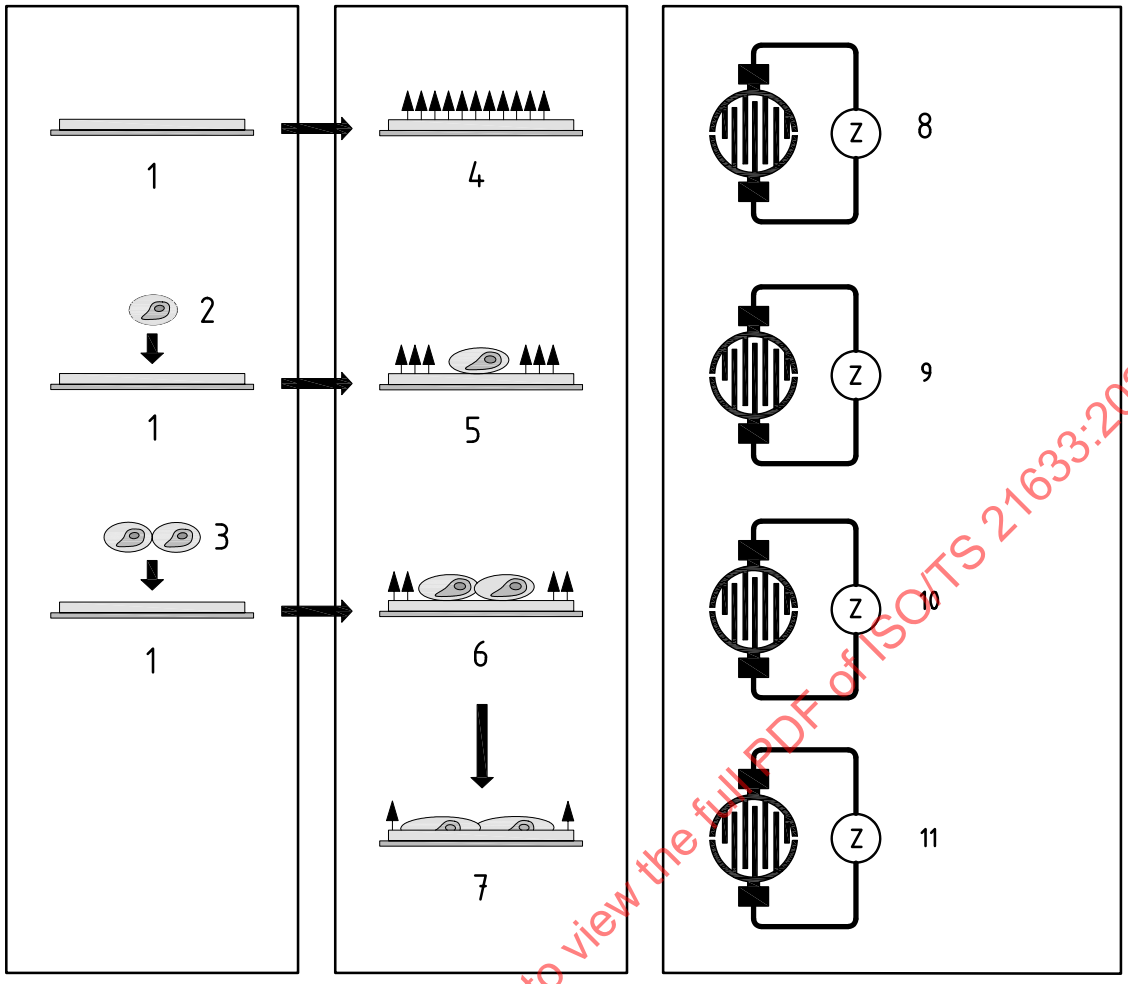
EIT, in addition for its possible clinical applications [81], is also used to assess cell growth and cell activity in live 3D structures as well as cell viability that can spatially resolve cell viability for single 3D spheroids [82].

7 Application for in vitro toxicity assessment

7.1 General

It is possible to apply the electrochemical impedance-based technique in the assessment of the toxicity of NMs. Cells are cultured and when adherent cells attach and spread across the sensor surface of an electrode, increases in impedance are recorded. Conversely, cells that round up or detach even for a short time will cause impedance values to drop. The presence of cells on top of the E-Plates electrodes, a multiple-well electronic microtiter plates, affects the local ionic environment at the electrode/solution interface, leading to a change in circuit impedance (see Figure 1).

2) ATCC® CCL93™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.



Key

1	electrode	7	electrode with two strongly-attached cells
2	cell	8	$z = z_0$ baseline
3	cells	9	$z = z_{\text{cell } 1}$ impedance
4	electrode without cell	10	$z = z_{\text{cell } 2}$ impedance doubled
5	electrodes with one cell attached	11	$z = z_{\text{cell } 3}$ impedance further increased
6	electrode with two cells attached		

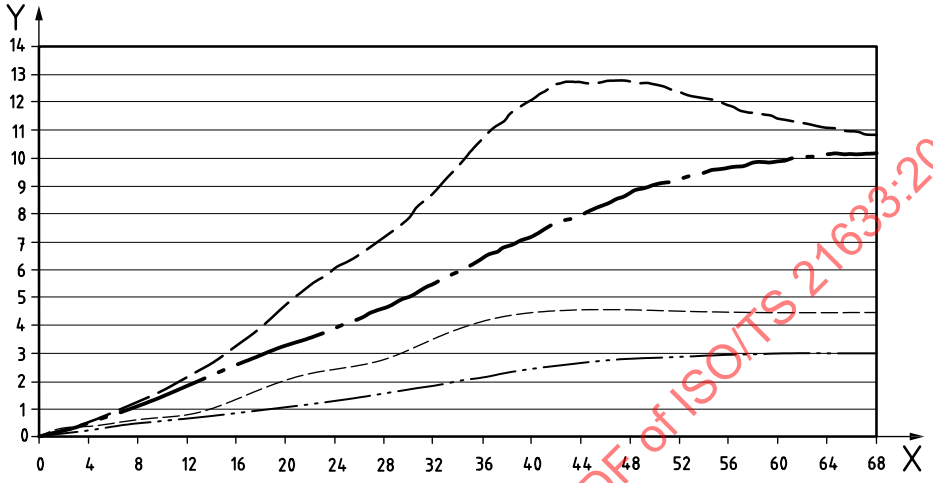
Figure 1 — Schematic illustration of the biological cell electrochemical impedance measurement and integrated microelectrodes on the well bottom[83]

Cell death leads to the release of cells from the surface of the measurement electrode that induces the decrease of the impedance measured across the electrodes. Recording of the impedance across electrodes at the bottom of the plate (a complex resistance in Ohm's law) is expressed as a parameter in different systems described in 6.1. For example, it is presented as CI with xCELLigence, Ziec (Ω) with CellKey™[3], Δ|Z| (Ω) with CellSine, and Impedance (Ω) with ECIS Zθ system.

The following discussion provides an example of how the xCELLigence system calculates and utilizes the cell index. As the cells interact with the biocompatible microelectrode surface in the E-Plate well with the aid of specialized software, the electrochemical impedance signal is converted to a specific parameter called the CI.

3) CellKey™ is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

Monitoring of cell viability is critical and the cell impedance system enables continuous measurement and quantification of cells [84][85]. The CI reflects the cell viability, which in turn measures cell number, attachment quality and cell type [25][26]. The CI, a unit-less parameter, is derived to represent cell status based on the measured relative change in impedance that occurs in the presence and absence of cells in the wells [13][86]. Figure 2 represents four different cell lines displaying different proliferation curves with different cell indices. This in turn, indicates that cell index relates to cell type where each has a relatively unique lag phase, exponential phase and stationary phase.



Key

X time (h)

Y cell index

MCF 7 (30 000 cells)

COS 7 (6 250 cells)

HT 29 (50 000 cells)

PC3 (6 250 cells)

NOTE Cell lines were seeded in triplicate to calculate the average and ± SD of CI values of corresponding cell lines [83].

Figure 2 — Cell proliferation curves of four different cell lines displayed as cell index on the cell impedance system

The instrument software converts impedance in ohms (Ω) into a CI value, where CI = Ω/15. Cell impedance is displayed as CI values and can be used to monitor cell viability, proliferation, degree of cell adhesion, cell number and morphology. Low CI values, compared to untreated cells, indicate cytotoxicity while high CI values indicate cell adhesion and proliferation. It is indicated that impedance change may occur depending on mainly two factors:

a) the number of cells attached to the electrodes where, if there are no cells on an electrode surface, the sensor's electronic feature is not be affected and the impedance change is 0;

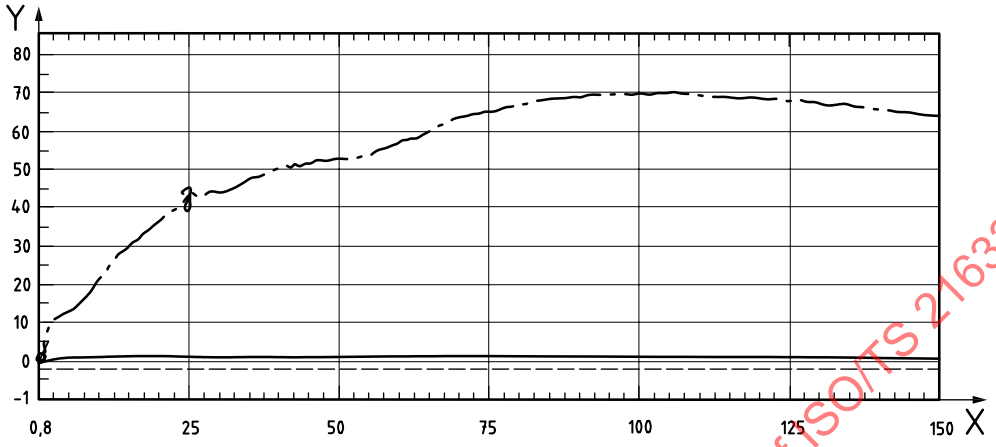
b) the dimensional change of the attached cells on the electrodes where despite the same cell numbers, dimensional changes such as an increase in cell adhesion or cell spread of the attached cells on the electrodes leads to a higher CI value. [87]

On the other hand, a decrease in CI value indicates cell detachment due to a decrease in resistance measured by the electrodes.

The CI values are then calculated as follows [88]:

$$I_{Ci} = \max_{i=1,\ldots N}\left[\frac{R_{\text{cell}}(f_i)}{R_b(f_i)} - 1\right] \tag{3}$$

where



$R_{\text{cell}}(f_i)$ is the frequency-dependent electrode impedance;

$R_{\text{b}}(f_i)$ is the background impedance measured at the initial time without cells;

N is the number of frequency points at which the impedance is measured.

Key

X time (h)

Y cell index

BEAS-2B (10 000 cells)

particle control (media and particles only)

media control (media only wells)

NOTE Cell viability curve of human bronchial epithelial (BEAS-2B) cells, media control and particle control displayed as cell index after exposure to 14 nm AuNPs [89].

Figure 3 — Real-time monitoring of cell viability

As can be seen in Figure 3, where there are no cells or only nanoparticles added, the CI value is zero because $R_{\text{cell}}(f_i)$ = Rb(f_i) in Formula (3) indicating no interference by the nanoparticles tested. On the other hand, when there is an increase in cell attachment, the CI values are larger. Thus, CI is related to the cell number present in a well. Moreover, changes in CI values will give an indication in changes in cell morphology, cell adhesion, or cell viability [36].

7.2 Normalized cell index

In order to allow comparisons amongst various treatments, it is required to normalize the CI of the wells to minimize inter-well variability at the time point just before NP addition.

The NCI value is calculated as given in Formula (4):

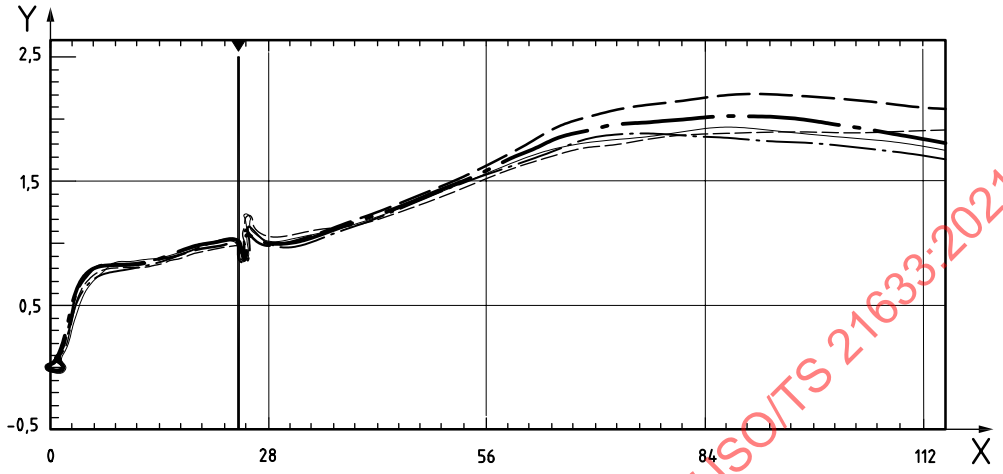
$$I_{Ci,\text{N}} = \frac{I_{Ci}(t)}{I_{Ci}(t_{\text{of dose}})} \tag{4}$$

where

$C_{Ci}(t)$ is CI at any time;

$I_{Ci}(t_{of\ dose})$ is CI at the time of NP dosing.

The NCI for all wells is therefore set to one at the normalization time point, as illustrated in Figure 4.



Key

X time (h)

Y normalized cell index

cell line 1

cell line 2

cell line 3

cell line 4

cell line 5

NOTE The normalization time point was chosen at the time just before the addition of NPs, as indicated by the black vertical line [54].

Figure 4 — NCI of BEAS-2B cells treated with AuNPs

A procedure for use of the xCELLigence system is given in Annex A and case studies using standard operating procedure for setting up an xCELLigence experiment with various cellular models are given in Annex B.

8 Technical limitations

Cell adhesion is required for the application of the impedance technique. Adhesion of cells in suspension is achieved by using a suitable ECM to facilitate this attachment. These include collagen ([90]), fibronectin [91] or poly-L-lysine [92].

However, a limitation of the ECIS technique was said to include the provision of an average measure of adhesion changes over all cells present on the microelectrode surface with no information about the precise nature of adhesion or morphological changes at the level of individual cells. It was therefore proposed that a live cell DIC microscopy and image analysis will provide a complementary measure of adhesion changes and will reveal the morphological changes of individual cells [91].

It is also important not to overload the electrodes and therefore assess interference of NMs tested by conducting experiments without cells at the concentrations of NMs used for the toxicity assessment to different cells, as indicated in Figure 3.

Annex A
(informative)

Basic procedures using the xCELLigence system

A.1 General

According to the literature, currently the most widely used electrochemical impedance system to assess the toxicity of NMs is the xCelligence instrument. It is therefore justifiable to describe in more detail the methodologies involved in the assessment of the toxicity of NMs.

A.2 System validation

The RTCA system cannot be validated; however, system verification with a dummy electrochemical cell can be performed.

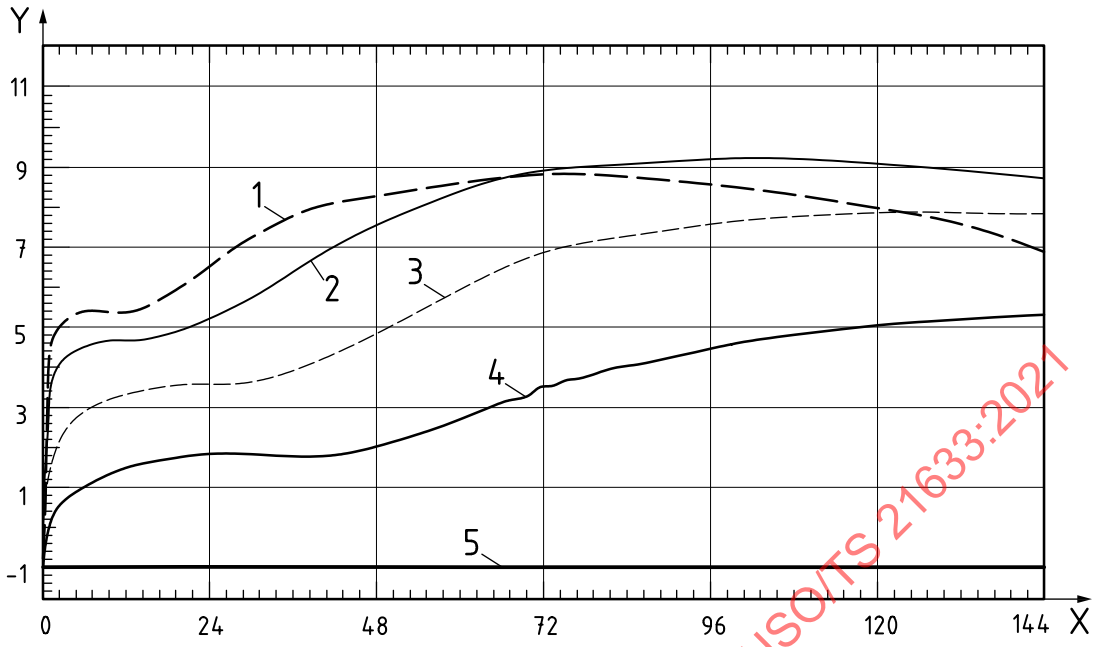
A.3 System verification

The xCELLigence system has a resistor plate that is used to verify the instrument. The resistor plate has the same size as the corresponding e-plate, i.e. the same number of wells. Where, instead of a micro sensor array, a resistor array is placed at each well position in such a way that it shows resistance values close to that of the corresponding e-plate when only cell media are put into each well. Therefore, system functionality can be verified. Resistor plates are not instrument-specific. Each resistor plate can be used to perform resistor plate verification on another station (the dedicated place that accommodates the electronic microtiter plate (e-plate) of the same type).

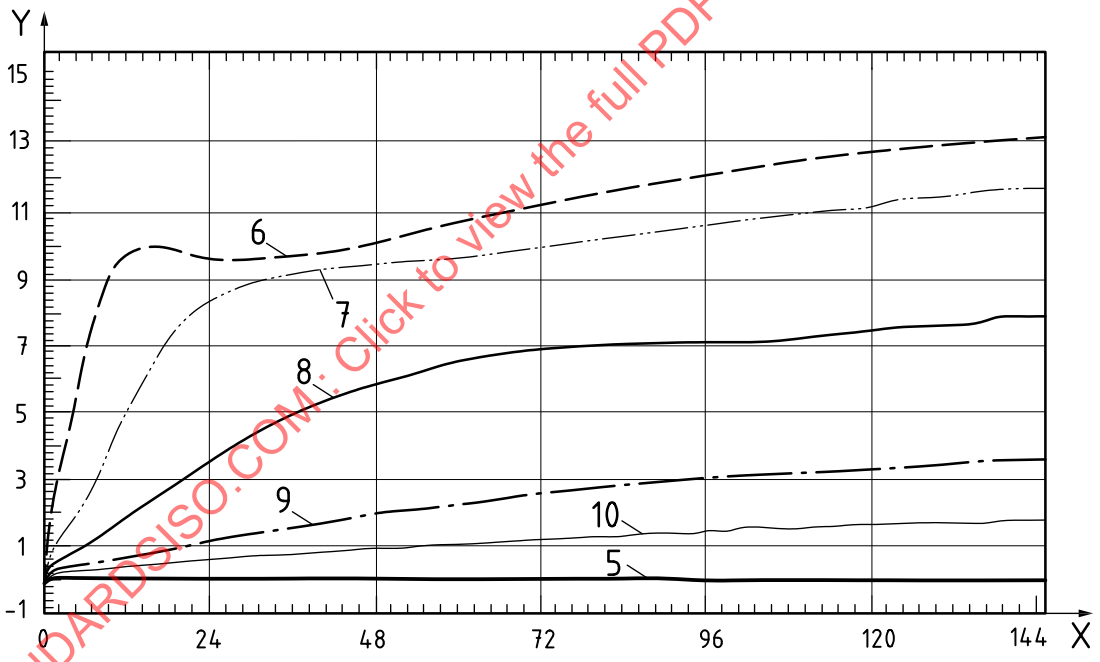
A.4 Cells and cell culture — Testing for seeding concentration

In both 16-well and a 96-well electronic microtiter plate, the volume per well is 243 µl. It is however advisable not to exceed 200 µl per well. To determine the optimal cell seeding concentration and cell growth within this specified volume, preliminary “cell titration” experiments should be done to determine the optimized cell seeding density and the preferable treatment point in the exponential growth phase. Once the seeding density has been established, this information is used for toxicity assessments of the NMs. One would however, include ‘control untreated cells’ that would confirm expected cell growth and confluency during the toxicity testing experiments.

It is important to note that different seeding densities will produce different proliferation curves (see Figure A.1) and this would influence at which time point the cells will be treated [90][93]. As shown in Figure A.1, the growth profile and optimal seeding density for the two cell lines varies. In Figure A.1a, a density of 20 000 cells can be used for experimentation where the cells per well are allowed to recover for 24 h followed by treatment for up to 72 h. In Fig. A.1b, a density of 6 250 cells can be used where the cells are seeded and allowed to recover for 24 h followed by treatment for up to 5 d.



a) Adhesion of HMEC-1 endothelial cells



b) NTera2/D1 (NT2)-astrocytes

Key

X time (h)
Y cell index
1 40 000 cells
2 20 000 cells
3 10 000 cells
4 5 000 cells
5 0 cells
6 12 500 cells
7 6 250 cells

8 3 125 cells
9 1 500 cells
10 750 cells

NOTE Curves represent the mean cell index value from > 4 wells ±SD [90].

Figure A.1 — Optimization of ideal seeding quantities

A.5 Reagents

Each instrument requires a particular disposable plate for cell measurements. Test cells are maintained using growth conditions and supplements required for the cell line and using optimal culture conditions. Test substances for growth assays are purchased as required and used with appropriate solvent controls.

As an example, to perform a basic cell titration or toxicity experiment the following reagents are required:

— cell line of interest, e.g., BEAS-2B cells, CHO cells, HEK 293 cells, HepG2 cells, U937 monocyte-derived macrophages;

— ECM molecule of choice, e.g. fibronectin, collagen, poly-L-lysine (when necessary);

— appropriate growth medium for culturing cell line of interest, for example, DMEM, EMEM and RPMI;

— appropriate base medium to be used in experiment;

— L-Glutamine (when needed);

— PBS;

— FBS (when needed);

— trypsin-EDTA for cell culture;

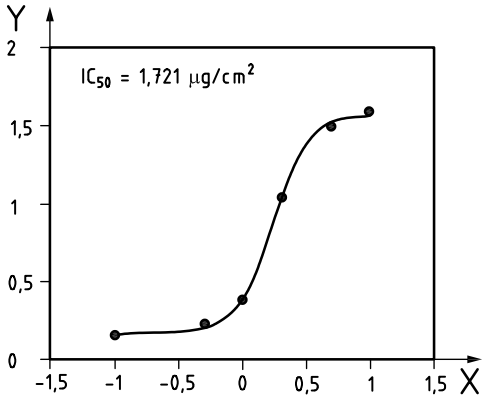
— ≥96 % pure BSA (when needed).

A.6 Growth measurements

Growth assay measurements are carried out as per manufacturer's instructions. As an example, this is determined by a change in the impedance values, where proliferation and attachment of cells will be displayed as an increase in CI. The measurements can be set on the "Schedule" page. Experiments can be divided into multiple steps, and each step consists of a minimum of one sweep. Each sweep is one measurement across all selected wells and can be set at a desired interval.

A.7 IC_{50} estimation

The system allows for the calculation of time dependent IC_{50} values in real-time (see Figure A.2)[26]. For example, where there are no cells or there is no cell adherence, the CI value is zero. On the other hand, when there is an increase in cell attachment, the CI values are larger. Thus, CI is related to cell number present in a well. Moreover, changes in CI values give an indication of the changes in cell morphology, cell adhesion, or cell viability (see Reference [89]). Using the instrument software, it is possible to display the standard deviation of the CIs of replicate samples as well as plotting of a dose response curve and IC_{50} value.



Key

X log of concentration (µg/cm^2)

Y normalized cell index

Figure A.2 — Dose-response curve and IC_{50} value of BEAS-2B cells treated with AgNPs [26]

The software is able to calculate and display IC_{50} values of data obtained, where cells were treated with a compound at different concentrations and can be derived using various methods.

— From the dose response curve, this can be achieved by using the "sigmoidal dose-response equation" to apply curve-fitting to the data points, from which the IC_{50} values are calculated.

— The time dependent change in IC_{50} values over a time interval following a compound treatment, providing kinetic data on the effectiveness and potency of a compound (e.g. compound-induced cytotoxicity).

A.8 Testing

When adding a test compound (e.g. NM), prepare the dispersion, at a desired concentration, followed by carefully removing and replacing the old media with fresh media containing the test compound.

The test sample should be pre-equilibrated to room temperature to minimize handling time (to preferably less than 5 min) as cooling the cells to room temperature will have an impact on cellular behavior and the CI values. Solution volume in wells should not exceed 200 µl/well.

A.9 Basic experimental design

— Prior to every experiment, control wells should be included to assess whether nanoparticles and nanofibres cause direct changes of the readings, i.e. in the absence of cells.

  — Wells with media only.

  — Wells with media and dispersion medium (if not growth media).

  — Well with media and the dispersed NM (at the highest concentration).

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the e-plate, prior to cell seeding.

— Seeding of different cellular models to a final volume of 150 µl supplemented media.

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to proliferate/mature prior to treatment for appropriate time (the optima cell number is determined in cell titration experiment), during which time CI scans are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (maximum volume 200 µl).

— Return the E-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media must be included.

  — The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

A.10 Data output

Some of the parameters that can be obtained for cell-based assays include:

— average CI (line graph – CI versus time);

— standard deviation of the CI;

— IC_{50} values for experimental data, including various methods for obtaining the IC_{50} values;

— time dependent IC_{50} value;

— dose-response curves;

— graphical representation of all data indicated.

A.11 Maintenance and cleaning

The manufacturer's instructions for the particular instrument should be followed for maintenance and cleaning.

Annex B
(informative)

Case studies using standard operating procedure for setting up an xCELLigence experiment with various cellular models

B.1 Human bronchial epithelial (BEAS-2B) cells — Basic experimental design

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the e-plate, prior to cell seeding.

— Seeding of different cellular models in a final volume of 150 µl supplemented media: BEAS-2B cells are seeded at 10 000 cells/well (5×10^4 cells/cm^2).

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to proliferate prior to treatment for 24 h, during which time CI readings are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (final volume of 150 µl).

— Return the e-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media and wells containing highest NP concentration in supplemented media must be included.

— The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

B.2 Chinese hamster ovary (CHO) cells — Basic experimental design

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the e-plate, prior to cell seeding.

— Seeding of different cellular models in a final volume of 150 µl supplemented media: CHO cells are seeded at 3 000 cells/well ($1{,}5 \times 10^4$ cells/cm^2).

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to proliferate prior to treatment for 24 h, during which time CI readings are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (final volume of 150 µl).

— Return the E-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media and wells containing highest NP concentration in supplemented media must be included.

— The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

B.3 Human embryonic kidney 293 (HEK 293) cells — Basic experimental design

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the e-plate, prior to cell seeding.

— Seeding of different cellular models in a final volume of 150 µl supplemented media: HEK 293 cells are seeded at 50 000 cells/well ($2{,}5 \times 10^5$ cells/cm^2).

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to proliferate prior to treatment for 24 h, during which time CI readings are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (final volume of 150 µl).

— Return the e-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media and wells containing highest NP concentration in supplemented media must be included.

— The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

B.4 Human liver carcinoma (HepG2) cells

B.4.1 Poly-L-lysine coating of seeding platform (e-plate)

The seeding platform (e-plate) should be coated with poly-L-lysine to aid the adherence of the HepG2 cells.

— Coat the E-plate by adding 100 µl of the 150 µg/ml poly-L-lysine solution to each well.

— Allow the plate to incubate at room temperature overnight in a biosafety cabinet.

— Following the coating step the E-plate should be washed, twice, with 1 × PBS and allowed to dry overnight prior being used.

B.4.2 Basic experimental design

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the E-plate, prior to cell seeding.

— Seeding of different cellular models in a final volume of 150 µl supplemented media: HepG2 cells are seeded at 10 000 cells/well (5×10^4 cells/cm^2).

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to proliferate prior to treatment for 24 h, during which time CI readings are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (final volume of 150 µl).

— Return the E-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media and wells containing highest NP concentration in supplemented media must be included.

— The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

B.5 U937 monocyte derived macrophages

B.5.1 Fibronectin coating of seeding platform (e-plate)

The seeding platform (e-plate) should be coated with poly-L-lysine to aid the adherence of the U937 macrophages (semi-adherent cell line).

— The e-plate should be coated with 5 µg/cm^2 fibronectin.

— Allow the plate to incubate for 1 h, at room temperature, in a biosafety cabinet.

— Following the coating step, the e-plate should be washed, once, with 1 × PBS and allowed to dry overnight prior being used.

B.5.2 Basic experimental design

— Establish a baseline reading for the instrument, by adding 50 µl of supplemented media into each well of the e-plate, prior to cell seeding.

— Seeding of different cellular models in a final volume of 150 µl supplemented media: U 937 monocyte are stimulated with 32 nM PMA per ml of cell suspension and seeded at 25 000 cells/well ($1{,}25 \times 10^5$ cells/cm^2).

— Allow cells to equilibrate for approximately 30 min prior to being placed into the RTCA station in an incubator.

— The cells are then allowed to mature into macrophages prior to treatment for 72 h, during which time CI readings are acquired every 15 min.

— Treatment of cells with the NPs, over the selected concentration range, must be done in fresh supplemented media (final volume of 150 µl).

— Return the e-plate to the RTCA station at 37 °C with 5 % CO_2 in a humidified atmosphere.

— Readings are acquired every 5 min for the first 24 h and every 15 min for the remainder of the experiment.

  — Untreated cells receive fresh supplemented media only.

  — Control wells containing supplemented media and wells containing highest NP concentration in supplemented media must be included.

  — The CI values should be normalized, at the treatment point, to minimize inter-well variability and allow comparison between wells, for data analysis.

Bibliography

[1] Oostingh G. J., Casals E., Italiani P., Colognato R., Stritzinger R., Ponti J., Pfaller T., Kohl Y., Ooms D., Favilli F., Leppens H., Lucchesi D., Rossi F., Nelissen I., Thielecke H., Puntes V. F., Duschl A., Boraschi D.2011. , Problems and challenges in the development and validation of human cell-based assays to determine nanoparticle-induced immunomodulatory effects. Part Fibre Toxicol, **8**, 8.

[2] Guadagnini R., Halamoda Kenzaoui B., Walker L., Pojana G., Magdolenova Z., Bilanicova D., Saunders M., Juillerat-Jeanneret L., Marcomini A., Huk A., Dusinska M., Fjellsbo L. M., Marano F., Boland S.2015. , Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic *in vitro* tests. Nanotoxicology, **9** Suppl 1, 13-24.

[3] Kroll A., Pillukat M. H., Hahn D., Schnekenburger J.2009. , Current *in vitro* methods in nanoparticle risk assessment: limitations and challenges. Eur J Pharm Biopharm, **72**, 370-7.

[4] Amendola V., Bakr O. M., Stellacci F.2010. , A Study of the Surface Plasmon Resonance of Silver Nanoparticles by the Discrete Dipole Approximation Method: Effect of Shape, Size, Structure, and Assembly. Plasmonics, **5**, 85–97.

[5] Forest V., Figarol A., Boudard D., Cottier M., Grosseau P., Pourchez J.2015. , Adsorption of lactate dehydrogenase enzyme on carbon nanotubes: how to get accurate results for the cytotoxicity of these nanomaterials. Langmuir, **31**, 3635-43.

[6] Belyanskaya L., Manser P., Spohn P., Bruinink A., Wick P., 2007. The reliability and limits of the MTT reduction assay for carbon nanotubes-cell interaction. 45, 2643-2648.

[7] Giaever I., Keese C. R.1991. , Micromotion of mammalian cells measured electrically. Proc Natl Acad Sci U S A, **88**, 7896-900.

[8] Tiruppathi C., Malik A. B., Del Vecchio P. J., Keese C. R., Giaever I.1992. , Electrical method for detection of endothelial cell shape change in real time: assessment of endothelial barrier function. Proc Natl Acad Sci U S A, **89**, 7919-23.

[9] Alexander F. A.jr, Celestin M., Price D. T., Nanjundan M., Bhansali S.2013. , Design and validation of a multi-electrode bioimpedance system for enhancing spatial resolution of cellular impedance studies. Analyst, **138**, 3728-34.

[10] Giaever I., Keese C. R.1993. , A morphological biosensor for mammalian cells. Nature, **366**, 591-2.

[11] Xiao C., Luong J. H.2003. , On-line monitoring of cell growth and cytotoxicity using electric cell-substrate impedance sensing (ECIS). Biotechnol Prog, **19**, 1000-5.

[12] Xiao C., Lachance B., Sunahara G., Luong J. H.2002. , Assessment of cytotoxicity using electric cell-substrate impedance sensing: concentration and time response function approach. Anal Chem, **74**, 5748-53.

[13] Xing J. Z., Zhu L., Jackson J. A., Gabos S., Sun X. J., Wang X. B., Xu X.2005. , Dynamic monitoring of cytotoxicity on microelectronic sensors. Chem Res Toxicol, **18**, 154-61.

[14] Ceriotti L., Ponti J., Broggi F., Kob A., Drechsler S., Thedinga E., Colpo P., Sabbioni E., Ehret R., Rossi F.2007a. , Real-time assessment of cytotoxicity by impedance measurement on a 96-well plate. Sensors and Actuators B: Chemical, **123**, 769-778.

[15] Scrace S., O'Neill E., Hammond E. M., Pires I. M.2013. , Use of the xCELLigence system for real-time analysis of changes in cellular motility and adhesion in physiological conditions. Methods Mol Biol, **1046**, 295-306.