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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

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Foreword

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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.