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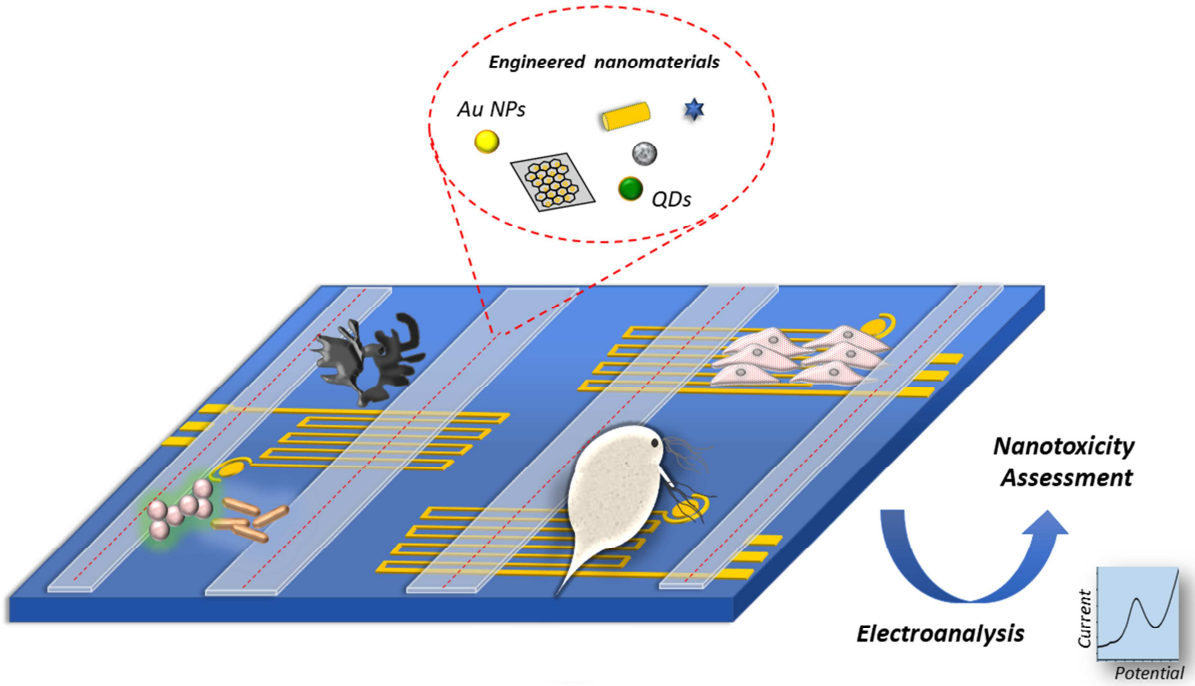

Nanotoxicity assessment: a challenging application for cutting edge electroanalytical tools

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Abstract

In the recent years, the number of commercial products containing engineered nanomaterials (ENMs) has increased exponentially. Consequently, the toxicological profile of ENMs on the ecosystems as well as on human health has to be carefully evaluated. Nanotoxicology, an interdisciplinary research area devoted to assessing the hazards associated with ENMs, is expanding rapidly. Many physicochemical techniques and biochemical methodologies have been proposed and are currently used to characterize nanomaterials from a toxicological point of view. Electroanalytical and bioelectrochemical methods can be useful in expanding the repertoire of accessible nanotoxicity-assessment technologies and to accelerate the testing and screening of the toxicological effects of ENMs. These methods can be used for elucidating the toxicological behavior of ENMs at single cell, cell population and whole-organism levels, for *in vitro* and *in vivo* measurements, respectively. The aim of this review is to provide an overview on the bioelectrochemical approaches that have been proposed for ENMs toxicity assessment. Furthermore, an overview on cutting-edge electroanalytical devices with a potential impact to this peculiar application is provided.

Keyword: nanomaterial, nanotoxicity, electroanalysis, biosensor, high throughput screening techniques, single cell, cell population, whole-organism

1. Introduction

Currently there are thousands of nanotechnology-based consumer products on the market, including cosmetics, food, food containers, sports equipment, and even textiles, just to mention a few of them [1],[2],[3],[4]. The amount of engineered nanomaterials (ENMs), i.e. nanomaterials intentionally produced for specific applications, ranges from several million tons/year (e.g. carbon black for car tires) to milligram quantities (e.g. quantum dots for biochemical applications like fluorescent labels) [5]. Indeed, the unique physicochemical properties of ENMs give rise to the exploitation of ENMs in many different commercial products. However, these properties can also lead to unanticipated effects on the environment and on human health. For this reason, ENMs are considered as emerging pollutants [6]. Workers and consumers may be exposed to potentially hazardous ENMs when they come in contact with nanomaterials and nanomaterial-containing products. Moreover, the release of ENMs into the environment creates associated risks that are more difficult to monitor than those previously encountered with airborne nanoparticles (ultrafine nanoparticles), which are chemically heterogeneous, polydisperse materials that bear little resemblance beyond their physical size to most engineered nanoparticles [7],[8]. Thus, nanotoxicology has been proposed as a new branch of toxicology to address the adverse effects on human health caused by nanoparticles and nanomaterials [8],[9],[10],[11], as well as the impact of the release of ENMs in the environment (nanoecotoxicology) [12].

Indeed, extensive research is currently being carried out in order to assess nanotoxicity of different nanomaterials in various exposure environments and to define nanotoxicity-risk factors. Basically, as reported in Figure 1, with the purpose of correlating the biological response obtained with well-characterized ENMs, the first step in nanotoxicity studies involves the physico-chemical characterization of the nanomaterials. This characterization is aimed to define core composition, size, shape, solubility, surface functionalization, surface area and charge, crystallinity, as well as agglomeration/aggregation state. The different techniques for physico-chemical characterization of nanomaterials are well summarized in several reports [13],[14],[15],[16],[17],[18] and are not discussed in this review.

Once the preliminary physico-chemical characterization has been performed, cell-based tests are used, as *in vitro* assessment platforms, to elucidate the toxic impact of ENMs at a cellular level. Nanotoxicity *in vitro* assessment is basically performed following conventional procedures that measure a particular cellular endpoint [16],[19]. Some of the most commonly used *in vitro* tests are summarized in Table 1. We ask the reader to refer to detailed book and review paper for the specific features of these different assays [15],[16],[20],[21],[22].

Finally, an *in vivo* evaluation of nanotoxicity is performed, which usually happens by testing organ

toxicity and other biological responses induced by nanomaterial exposure. These studies are performed in model animals.

These three key steps were formulated mainly from the perspective of the potential effects of nanomaterials on humans. When the whole ecosystem is considered, the effects of ENMs on bacteria, algae, plants, invertebrates (e.g. crustacean *Daphnia Magna*) and other biota organisms also have to be evaluated [12]. Moreover, ecotoxicological assessment should be conducted mimicking real environmental conditions, performing various ecotoxicity tests in laboratory setting and in higher complex settings (i.e. microcosm and mesocosm, respectively).

Both *in-vitro* and *in-vivo* toxicity assays are required to evaluate the toxicity of ENMs, in order to design a complete risk assessment and to develop new safe strategies for the fabrication, the application and disposal of nanomaterials. Actually, the cost, the low throughput and the ethical concerns of *in vivo* and *ex vivo* assays pushes in developing *in vitro* systems (by using physiologically relevant cell architectures) [23] coupled to computational studies (such as quantitative structure-activity relationship models). In view of the large number of ENMs currently in use, high throughput screening (HTS) techniques, i.e. measurement tools aimed at facilitating rapid execution of a large number and a variety of biological assays that may include several substances in each assay, are clearly needed [24], [25], [18], [26]. For HTS purposes, *in vitro* methods have the advantages of simplicity, economy, and shorter time required for investigation; they can help in revealing general mechanisms underlying the effects of ENMs on cells, and can provide a basis for evaluating potential risks of exposure. Ideally, when considering HTS screening of novel ENMs for toxic effects a combination of *in vitro* methods simulating *in vivo* conditions as closely as possible should be used.

Recent years have seen an increasing activity in the use of electrochemical devices and techniques for investigating the properties of single cells, populations of cells, as well as whole-organisms. Many reports discussed the application of electrochemical techniques in the studying of cellular properties [27],[28],[29],[30],[31]. Moreover, over the past decades, the field of electrochemical biosensors has expanded exponentially and many examples of the determination of biomolecules related to cell analysis have been reported [32].

Indeed, electrochemical methods possess particularly useful features for studying some of the critical mechanisms involved in ENMs toxicity [33]. Electrochemical methods can be applied in a variety of environments and sample types, both *in vivo* and *in vitro*. Electrochemistry can be used for sensitive, rapid, multiplexed and ultimately high throughput analysis of electrochemically active molecules. Moreover, the detection of non-electroactive molecules can be performed, by designing selective biosensing tools. The high sensitivity of the electrochemical techniques allows us to

investigate the influences of external parameters, such as exposure to ENMs, on cell exocytosis as well as on the release of molecular markers of cell toxicity.

The aim of this review is to summarize the different electrochemical methods for evaluating nanomaterial toxicity. The review is organized in two distinct sections. First, an updated literature survey of the novelty emerged in the field of the (bio)electrochemical and biosensing methods devoted to nanotoxicity assessment is presented. Then, the second section is devoted to discussing the electroanalytical cutting-edge technologies that might have an impact on nanotoxicity assessment in the next years.

2. Electrochemical methods for *in vitro* nanotoxicity evaluation

In a tiered approach for the safety assessment of nanomaterials [26], the molecular initiating events are the first molecular levels of interaction that contributes to adverse outcomes. One of the generally accepted toxicity mechanisms of ENMs exposure is attributed to the production of an excess of reactive species leading to oxidative stress. Molecular initiating events result in the activation of toxicity signaling pathways. For the determination of the electroactive molecules involved in the molecular initiating events or in the toxicity signaling pathways, electroanalytical techniques can be applied directly; in the case of electrochemically inert molecules (e.g., cytokines and other toxicity biomarkers) or to increase selectivity and sensitivity of the measurement, biosensors can be used [34].

2.1 Direct monitoring of electrochemically active molecules involved in nanotoxicity events

Oxidative stress is commonly defined as an imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and the cell's ability to detoxify the reactive intermediates or to repair the resulting damage. In normal conditions ROS are reduced into H_2O by an interacting network of enzymes. Several enzymes participate in this detoxification pathway protecting the cells against oxidative stress. Superoxide Dismutase catalyzes the dismutation of ion superoxide ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) and oxygen (O_2), while Catalase and Glutathione Peroxidase react with H_2O_2 and generate H_2O by two different mechanisms, respectively. Disturbance in the normal redox state of tissues may cause toxic effects through the production of peroxides and free radicals exerting deleterious effects on the cell through a direct attack on the DNA, on proteins or membrane lipids (including mitochondrial lipids). These oxidative damages lead to cellular death. Some of the reactions involved in oxidative cellular damage are summarized in Figure 2, with superoxide and nitric oxide as primary species of oxidative and nitrosative stress, respectively.

Electrochemical methods have been proposed for monitoring oxidative stress at living cells for decades [29],[27],[35],[36],[37]. Basically, electroactive species involved in the oxidative stress process can be directly monitored by applying a proper potential value at a bare carbon, platinized carbon or platinum electrode surface, properly positioned at micrometers distance over the living cells. In this arrangement single living cells can be monitored. Due to the presence of oxygen in the measurement medium, the direct measurement of a reduction reaction is very challenging and is generally performed only in an indirect scheme by using electrochemical mediators. Indeed, oxygen reduction takes place at a low cathodic potential, thus it acts as an interferent, and the reduction signal of any other electroactive species would be convoluted with and masked by oxygen reduction signal. Therefore, the oxidation current of some of the electroactive species reported in Figure 2 is generally monitored, by applying an appropriate anodic potential value, (i.e. around + 0.1 V vs. Ag/AgCl in the case of superoxide or around + 0.7V vs. Ag/AgCl for nitric oxide determination). Micro- and nanoelectrodes are used in order to maximize the signal-to-noise ratio. Microelectrodes positioned at micrometric or submicrometric distance from a living cell (as reported in Figure 3A) allow the determination of the molecules released by the cell in a restricted extracellular volume, avoiding excessive dilution and ensuring a quantitative collection efficiency from the different release points located on the cell surface. Cell dimensions are in the micrometer range; thus, in the same order of magnitude of the microelectrode. Consequently, the micrometric dimension of the electrode surface area helps to control the noise (i.e. capacitance through the conducting surface area) while maximizing the analytical information (i.e. faradaic current) arising only from the surface area exposed to the cell release. The electrochemical monitoring of the electroactive molecules involved in the oxidative stress is very challenging and we remand to specific and exhaustive reviews on this topic for specific details [29].

Quite recently, nanoelectrodes have emerged as important tools for studying single cell events [28], [38], [39], [40], increasing spatial resolution and sensitivity. Moreover, a nanoelectrode can also be inserted inside a cell or tissue without disturbing the cellular function [41], [42],[43].

Regarding the specific application of nanotoxicity assessment, the overproduction of ROS/RNS species induced by ENMs has been pointed out by several studies. Type, size, shape and surface coating of the ENMs greatly influence the extent of the induced oxidative stress. Erofeev et al. [44] developed a platinized carbon nanoelectrode with a cavity on the tip integrated into a micromanipulator on an upright microscope to study intracellular levels of ROS measured in different cancer cells before and after exposure to 10 nm size iron oxide nanoparticles. Less than 30 minutes were needed for assessing nanoparticle toxicity by monitoring ROS production with the proposed method. It must be noted that a potential of + 0.8 V vs. Ag/AgCl is applied to the

nanoelectrode, meaning that the whole amount of ROS, including superoxide, hydroxyl radical and peroxide, are monitored non-specifically. Examples of the current profiles recorded in living cells exposed to different type of iron oxide NPs are shown in Figure 3B.

Monitoring of cell's exocytotic behavior following nanomaterial exposure can be a useful way to evaluate the activation of some toxicity signaling pathways. Exocytosis is a highly conserved cellular function across cell types and species. During exocytosis, intracellular vesicles fuse with the plasma membrane and the vesicle contents are released, enabling communication between cells through secreted chemical messengers (Figure 3C). Basically, the cell's exocytosis process consists of five discrete steps that culminate in secretion [45], any of which is susceptible to perturbation by internalized nanoparticles (Figure 3C). First, vesicles are mobilized to the release zone via the actin-myosin cytoskeletal system. Second, the vesicles arrive in the release zone via interaction with specific proteins. The most known interactions are with the membrane receptor SNARE (Soluble Nsf (N-ethylmaleimide sensitive factor) Attachment Protein Receptor) or with the Sec1/Munc18 (SM) protein complex. Third, in an ATP-dependent priming step, a conformation change of the SNARE and/or SM protein complex makes the docked vesicle fusion competent. Fourth, high intracellular Ca^{2+} concentrations trigger the protein-complex conformation change that drives fusion of the vesicle and intracellular lipid bilayers. Finally, the intravesicular matrix expands and expels the vesicle contents into the extracellular space. A complete or a partial release of the vesicle content is possible (Figure 3C).

Electrochemical detection of electroactive signaling molecules secreted through exocytosis relies on the oxidation of these molecules at a microelectrode surface. Indeed, as already mentioned in the case of oxidative-stress monitoring, reduction-current detection is generally prohibited because O_2 is in high or comparable concentrations *vs.* the released species [29]. The recorded oxidative currents thus evidence the nature of the molecules released and their quantities as well as the dynamics of the release itself. Generally, electrochemical detection of the secreted compounds are achieved with bare carbon fiber microelectrodes (CFME) (with a diameter ranging from 1 to 10 μm) located close to the cell (Figure 3A). Most frequently, amperometry and fast voltammetry are used [29] for monitoring the release of electrochemical species. Practically, a glass micropipette is positioned near the cell investigated and is used to administer stimulating agents that trigger exocytosis (for instance, nicotine, Ba^{2+}, etc. [29]).

Exocytosis events can be detected as distinct spikes in a current versus time trace (Figure 3D). Integration of the spikes gives the total charge transferred (Q) during the event. The Q value allows for quantification of the number of moles released by the Faradays law: $N = (Q)/(nF)$, where n is the number of electrons transferred during the redox reaction, and F is Faraday's constant. Due to

the high temporal resolution (10– 200 μs), detailed information about the dynamics of the release process can be obtained [31], [30].

ENMs induced changes in cell exocytosis can be quantified by measuring the concentration of secreted redox-active molecules and the kinetics of individual secretion events; the latter is calculated from the frequency and half-width of current spikes. Mast cell exocytosis and secretion of electroactive chemical messengers is a good model for assessing the effects of nanoscale materials [45]. Maurer-Jones et al. showed that serotonin exocytosis from murine peritoneal mast cells decreased in frequency and increased in duration following exposure to titania (TiO_2) NPs; lower total release of serotonin was also observed. These effects were attributed to increased concentrations of intracellular ROS measured following nanoparticle exposure [46]. Love et al. showed that murine adrenal medullary chromaffin cells exposed to PEG-functionalized Au NPs decreased the exocytosis of epinephrine and norepinephrine, while cells exposed to Ag NPs showed unaltered exocytosis, despite significant cellular internalization [47].

Notably, direct monitoring of oxidative stress and exocytosis by using micro- and nano-electrode, as reported above, can be studied at single cell level [46]. Chip-technology is proposed for monitoring 3D cell aggregates [48] and cell population [49], as described in section 4. However, to the best of our knowledge, applications to nanotoxicology have not reported yet.

2.2 Electrochemical biosensors for monitoring of nanotoxicity molecular events

Electrochemical biosensors have been proposed for the detection of electrochemically inert molecules excreted or processed internally by cells [32] or to increase the selectivity and the sensitivity of the measurement of electroactive molecules. A typical biosensor consists of (a) a biorecognition element that ensures the specificity of the interaction with the target analytes and (b) a transducer that converts the interaction between the biorecognition element and the target analyte into a detectable signal. Electrochemical transducers are considered particularly interesting due to enhanced sensitivity and scalability of readout devices in comparison to optical-based detection methods and other detection techniques.

Based on the biochemical recognition element and the biochemical specificity conferring mechanism, biosensors can be broadly classified as catalytic or affinity biosensors. Catalytic systems integrate, within a transducer, enzymes, tissues or whole cells that act as biological catalysts for particular reactions. Molecules such as glucose or lactate can be monitored by using glucose oxidase and lactate oxidase modified electrodes, while ROS/RNS can be monitored by using cytochrome c (Cyt c) modified electrodes [50], [51]. As an example, Cyt c immobilized on a gold electrode has been used to monitor CeO_2 NPs effect on ischemic brain slices [52]. Cyt c is

reduced by superoxide and the reduced Cyt c is regenerated electrochemically at the electrode surface. The concentration of superoxide was quantified by constant potential amperometry with the electrode poised at the potential of + 0.15 V vs. Ag/AgCl, which corresponds to the oxidation potential of Cyt c. The exposure to CeO_2 NPs induced an immediate decrease in the superoxide signal under control conditions. Recently, a whole cell biosensor has been developed to evaluate CdTe Quantum Dots (QDs) nanotoxicity [53]. This biosensor was capable to measure changes in intracellular acid phosphatase upon exposure to CdTe QDs. Acid phosphatase converts 2-naphthyl phosphate to 2-naphthol, which is oxidized at a carbon electrode surface and the current recorded by voltammetry; this approach was validated against the MTT assay.

On the other hand, affinity biosensors, take advantage of different biorecognition elements, such as antibodies or aptamers. In affinity biosensors, the binding between the target analyte and the immobilized biomolecule on the transduction element are governed by an affinity interaction under thermodynamic considerations, like the antigen-antibody (Ag-Ab) or the protein-aptamer binding.

Some ENMs, even though they have no obvious effects on cell viability, could induce pro-inflammatory effects by secretion of cytokines, which influence cell functions and injury mechanisms [26]. Cytokines are soluble proteins produced by many cells of the immune system (neutrophils, monocytes, macrophages, B-cells, and T-cells) to regulate immune responses. These proteins are widespread in mammals and interestingly, have recently been discovered in invertebrates [54]. There are several different families of cytokine proteins and the number of identified proteins continues to grow. For example the interleukin family is categorized in numerical order (i.e. IL-2) and other families include those describing functional activity such as the tumor necrosis family. Cytokines differ not only in their function, but they also have a wide variety of molecular weight ranges from approximately 6 to 70 kDa. Cytokines act as mediators and modulators within highly localized environments and regulate immunological responses, hematopoietic development and cell-to-cell communication as well as host responses to toxicants. One of the most common and earliest approaches for cytokine determination is the enzyme-linked immunosorbent assay (ELISA) [55]. Recently, electrochemical affinity biosensors have been proposed for cytokine determination, by using a variety of biorecognition elements, including antibodies [56],[57],[58],[59],[60] as well as aptamers [61],[62] and engineered proteins [63]. However, to the best of our knowledge, biosensors have not yet been used in the determination of cytokines released as a consequence of ENMs exposure.

2.3 DNA damage and genotoxicity

As already discussed, some nanomaterials have the potential to damage the cellular DNA.

Some commonly used *in vitro* methods to evaluate genotoxicity induced by nanomaterials are reported in Table 2. Actually, DNA-based electrochemical sensors have been reported as very effective bioanalytical tools for investigating of the behavior of a surface-attached DNA in relation to its damage [64], [65], [66], [67]. In the development of an electrochemical DNA sensor for damage evaluation, the genomic DNA, derived from calf thymus, red blood cells or salmon sperm, as well as plasmid DNA or synthetic double-strand DNA structures (including complementary target and capture oligonucleotides or stem loop structures) can be used as surrogate (Figure 4). DNA may be damaged by a number of different types of compounds with a range of mechanisms. The main types of DNA damage include interruptions of the sugar-phosphate backbone (strand breaks), release of bases due to hydrolysis of *N*-glycosyl bonds and a variety of nucleotide lesions resulting from reactions of the DNA with a broad range of chemical agents, including alkylating agents and oxidants. Moreover, specific binding of a molecule by intercalation can cause a lengthening of the DNA helix, perturbation of the phosphate backbone, and even untwisting of the double helix. Thus, in addition to the changes of covalent bonds, the term DNA damage is also used to describes alterations of the DNA structure induced by non covalent binders which could affect the biological function of the DNA. Altered chemical, physico-chemical, and structural properties of the damaged DNA are reflected in its behavior at the transducer, and this fact can be used for DNA damage detection (Figure 4). A wide spectrum of transducers can be used [68],[69],[65,66,70,71], [72] with designs that allow multiplexed analysis [73],[74]. Among others, electrochemical transducers are particularly interesting, since the damage to the DNA causes a change in the intrinsic electrochemical signal of the DNA (i.e. guanine and adenine redox signals) [75].

Recently, the effect of photoactivated CdTe QDs leading to damage to salmon sperm DNA and calf thymus DNA immobilized at glassy carbon electrodes has been evaluated [76]. Depending on the size of the QDs, a significant effect on the rate of the degradation of dsDNA by UV-C light and by visible light has been found. The behavior of CdS QDs was evaluated in another report [77]. CdS QDs contribute to the effect of UV-C irradiation of DNA, which results in deep DNA degradation leading to the opening of the helix structure and to strand breaks with releasing the DNA fragments. These data were corroborated by electrophoretic experiments. The potential toxic effect of the CdS nanoparticles is size dependent and becomes deeper with time of exposure to UV-C radiation. Small size QDs possessing the highest fluorescence were significantly more effective than the larger ones with the lowest fluorescence. The effect of CdS on DNA from different sources (plasmid DNA, calf thymus and salmon sperm DNA) have also been evaluated [78].

Electrochemical hybridization-based biosensors, also known as genosensors

[79],[80],[81],[82],[83],[84],[84], can be theoretically used to evaluate nanomaterial genotoxicity, by monitoring ENMs-induced modification at a genomic, epigenetic or transcriptomic level, even if, to the best of our knowledge, this application has not been reported yet.

2.4 Monitoring cell damage trough electrochemical impedance

The dielectric properties of living cells, with their insulating bilipid membranes, can alter the impedance of metallic electrode on which they grow [85], [86], [87] (Figure 5A). Monitoring the growth of a cell culture is thus possible by simply measuring the electrode impedance, which will change with cell surface coverage, or will dynamically fluctuate with cell motility or with subtle changes in cellular metabolism as a reaction to a toxicant. When cells die, they detach from the electrode surface, causing a drop in the recorded impedance, which indicates a reduction in the number of viable cells. Thus, impedimetric measurements may provide a continuous, real-time, label-free approach of cell count, cell morphology, cell motility and viability. Nowadays there are some commercially available impedance-based instruments for *in vitro* evaluation of cell toxicity, (i.e. xCELLigence® or ECIS® systems). These systems essentially work on the same principle [25], [88]. They utilize cell culture well plates with gold-plated electrodes attached to the bottom of the wells and measure the real-time effect of the seeded cells to the applied alternate current, providing information about the cells' attachment to the well, their proliferation and their reaction to a possible toxicant (Figure 5A). Different formats of cell culture plates are possible, with a different number of wells (from 16 to 384 wells), allowing the screening of a large number of ENMs and of different type of cells.

Examples of impedimetric-based methods capable of monitoring the behavior of different cell types (fibroblast, macrophages, neuronal cells, lung cells, etc) exposed to different nanomaterial types can be found in literature. As an example, human lung fibroblasts and rainbow trout gill epithelial cells, exposed to AuNPs and AgNPs, single walled carbon nanotubes (SWCNTs) and cadmium oxide (CdO) NPs [89], have been monitored by a multiplexed impedimetric chip. Among these ENMs, exposure to CdO NPs exhibited the fastest rate of cytotoxicity. An impedance-based monitoring system was used to screen TiO_2 NPs-induced cytotoxicity on fibroblasts [90]. A real-time method for profiling the cytotoxicity of TiO_2 and Ag NPs on different cell lines (two human lung carcinoma cell lines (A549 and SK-MES-1, respectively) and a normal mammalian cell line, CHO-K1) was developed by Moe et al. [91]. The obtained cytotoxicity profile was concentration-, time-, particle-, and cell-dependent in agreement with the data obtained by neutral red assay.

Otero-Gonzalez et al. [92] tested a group of 11 inorganic nanomaterials (Ag^0, Al_2O_3, CeO_2, Fe^0, Fe_2O_3, HfO_2, Mn_2O_3, SiO_2, TiO_2, ZnO, and ZrO_2) for potential cytotoxicity to a human bronchial

epithelial cell, by using an impedance-based real time cell analyzer. The obtained data were in agreement with data obtained using the conventional MTT assay.

However, a limitation of such impedance-based devices is that they provide a cellular response to a toxicant without giving any indication of how the effects took place. At the same time, an important advantage is that they do not need destructive cell sampling and allow real-time observations of cell changes. A very interesting feature of impedance-based methods is that they are easy to implement and can be used in HTS fashion for testing many nanomaterials at different concentrations simultaneously.

Impedance measurements have also been applied for the analysis of a single cell instead of a population of cells, providing information regarding the type, size, and dielectric properties in single cells. There are two different ways to employ impedance measurement on the single cell level: the analysis of immobilized cells and cytometric flow-through analysis [93]. For instance, Shah et al. [94] described an impedimetric method for cytotoxicity evaluation by using an immobilized single cell on a microelectromechanical system (MEMS) device. The same MEMS device was used to study a population of a limited number of cells. The controlled capture of a different number of cells in different-sized microwells (Figure 5B-C) was obtained by using a combined method of surface functionalization and dielectrophoresis. The nanotoxicity assessment was performed by monitoring impedance over time, before and after exposure to CuO NPs. The comparative study showed that the small population of cells was more sensitive to toxicity when compared to a single cell; however, in both cases a toxic effect was assessed. In impedance-based flow cytometry chip, the changes of the electrical resistance of a fluidic medium when cells pass through the applied electric field are measured. A high sensitivity can be achieved by adjusting the channel dimensions close to the size of the measured cell type, increasing thereby the ratio between cell and detection volume. Micro fluidic chips for impedance based flow cytometry are also commercially available (Amphasys AG).

Impedance-based methods have been compared with conventional cytotoxicity assays, examining the effects on various types of cells induced by ENMs as well as other chemicals [25]. Toxicological information obtained by impedance-based methods was found to be consistent with results obtained via conventional methods. Importantly, impedance-based methods allow in situ monitoring of not just cytotoxicity but also other aspects of cell physiology such as proliferation, morphology, attachment, and intercellular adhesion. To date, several studies using cell impedance have demonstrated its ability to differentiate cytostatic from cytotoxic effects and also to predict different outcomes in cell-based functional assays. In conclusion, cellular impedance provides qualitative and quantitative cytotoxicity data, and also drives further studies into the mode of action

(toxicity) of ENMs [25].

3. *In vivo* evaluation of nanotoxicity parameters

Other than *in vitro*, electrochemistry can be used for *in vivo* monitoring. Electrochemical *in vivo* measurements of neurological activity in invertebrates exposed to ENMs have also been reported. Indeed, recently invertebrates have been found attractive for use as model systems for *in vivo* monitoring [95],[96],[97], because they can provide significant insights into the mechanisms of developmental and physiological processes in humans [95]. Moreover, they are interesting models from a nanoecotoxicology point of view as well, because they are part of the trophic chain, revealing important information about the accumulation of toxic effects within the rest of the organisms in the ecosystem. Other interesting aspects are that they have a rapid life cycle, there is no need to refer them to bioethical committees and they allow HTS. Andreescu's group studied the chemical reactivity of CeO_2 NPs *in vivo*, by measuring intestinal serotonine levels after CeO_2 NPs exposure to whole zebrafish embryos [98]. They monitored the concentration of serotonin in the intestine using implanted CFMEs and differential pulse voltammetry [98]. Intestinal serotonin levels decreased with multiday exposure to 20−50 ppm of CeO_2 NPs, probably due to depletion of serotonin from tissues by adsorption to the nanoparticle surface. Correlated in vitro experiments using UV−Vis and FT-IR spectroscopies indicated that serotonin had adsorbed to the nanoparticle surface.

Özel and colleagues quantified electrochemically the intestinal nitric oxide (NO) concentration in live zebrafish using a microelectrode implanted in the intestine of embryos [99]. Changes in NO concentration following exposure to copper oxide (CuO) and cerium oxide (CeO_2) nanoparticles (NPs) were monitored and correlated with results from conventional assays based on fluorescent dyes specific for NO. CuO NPs were found to increase NO concentration to almost 4 times higher compared with unexposed embryos, suggesting thus an intestinal oxidative damage, while low concentrations of CeO_2 NPs showed a scavenging effect against NO. The increase in intestinal nitric oxide concentrations after exposure to CuO NPs was attributed to the release of copper(II) from the NPs as a result of dissolution; control experiments showed that embryo exposure to $CuSO_4$ also increased NO levels. Low exposure concentrations of CeO_2 NPs decreased intestinal NO concentrations, probably due to radical scavenging as a result of redox reactions with exposed Ce^{3+}/Ce^{4+} sites on the nanoparticle surface. However, the scavenging effect was concentration-dependent, as exposure to higher nanoparticle concentrations increased intestinal nitric oxide concentrations.

4. Innovative electroanalytical technologies: the perspective in nanotoxicity assessment

Nowadays, it is clear that there is a need for reliable and efficient analytical methods for nanotoxicological studies because the evaluation of the possible adverse effects of ENMs is a major challenge. Although electrochemistry is a well established, easy-to-use and relatively inexpensive family of techniques that can be used in a variety of sample types, both *in vivo* and *in vitro*, there are surprisingly few reports on the use of electrochemical methods in nanotoxicity research.

In the previous paragraphs, we have reported and discussed the electrochemical approaches found in literature for nanotoxicity assessment. The different electrochemical strategies have been reviewed considering a tiered format for *in vitro* toxicity evaluation, starting from molecular initiating events, then toxicity signaling pathways and finally cell damage. Indeed, from the literature survey, it becomes clear that even though the number of possible approaches, up to now only few electrochemical tools have been used. Basically, these methods can be summarized as the following: fast voltammetry and amperometry at micro and nanoelectrodes and electrochemical impedance spectroscopy.

Moreover, only few examples of catalytic and affinity biosensors have been reported for the evaluation of the oxidative stress, for the monitoring of specific signaling molecules and for genotoxicity studies, despite the huge possibilities offered by this technology.

In our opinion, in the near future cutting-edge electrochemical tools [34], currently proposed for other applications (i.e. clinical diagnostic or drug discovery), could be used to elucidate the mechanism of ENMs toxicity and thus to perform nanotoxicity assessment studies.

As an example, a number of biosensors and bioassays approaches are already reported in the literature for studying cell functionality and metabolism, including the interruption of ionic homeostasis, endoplasmic reticulum stress, mitochondria dysfunction and related impairment of cellular ATP production [100],[101],[102],[97],[48],[103]. Recently, an ATP biosensor has been developed by filling the two channels of a double-barrel nanopipette with pyrolytic carbon nanoelectrodes as source and drain of a nanometric field-effect transistor (FET) (Figure 6A). The tops of both electrodes were coated with electrodeposited polypyrrole (PPy), which forms a transistor channel. Hexokinase was immobilized on the nano-FET, catalyzing the conversion of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) and causing the phosphorylation of glucose and the release of a proton. This resulted in the protonation of the PPy layer increasing the PPy conductivity to record ATP concentrations near and inside living cells [104]. As reported above, monitoring the intracellular ATP concentration, as well as monitoring many other cell parameters involved in the cellular response to ENMs exposure, is of crucial importance; thus, the bioelectrochemical approaches proposed to monitor these processes could be used to evaluate the

role of ENMs in many initiating biomolecular events and toxicity signaling pathways.

Notably, with the advancement in micro- and nanofabrication, new platforms have emerged as suitable tools for single cell analysis which could be used in nanotoxicity studies. Recently, Ewing's group has described the possibility to study the mechanism of exocytosis obtaining information about the vesicular content by using intracellular vesicle impact electrochemical cytometry [31],[105],[106],[107]. By etching a cylindrical carbon fiber microelectrode, a thin needle shape electrode is obtained with a tip diameter of down to 50–100 nm. This nano-tip electrode penetrates the cell membrane without causing damage to the cell. Inside the cytosol, vesicles are adsorbed, electroporated, and the content is oxidized, allowing a quantification of the vesicular content inside the natural environment of the cell. This approach is promising for observing the cellular response of a stimulus (i.e. ENMs exposure) on the vesicular content and the vesicular process; indeed it can help in the definition of molecular initiating events and signaling pathways.

Scanning electrochemical microscopy (SECM) and scanning ion conductance microscopy (SICM) are other powerful electrochemical platforms for cell analysis. In SECM, a micro/nanoelectrode is passed over a sample surface to detect local chemical reactions. Although a probe device with a single electrode is generally used for SECM, several types of probe devices have been proposed [108],[109],[110],[111]. In SICM, an ionic current is driven by an electric field generated between an internal and an external quasi-reference counter electrode. The resistance between the two electrodes increases inversely with the gap between the nanopipette and sample. SICM can be used to extract the local surface charge of biological specimens from the measured properties of the diffuse double layer at a live cell-electrolyte interface [112],[113],[114]. Both SECM and SICM may provide information at a single living cell helping in elucidating toxicity mechanism of ENMs at a single cell level.

However, one of the major challenges for nanotoxicity studies is the requirement of high-throughput screening methods, as the toxic effects of a nanomaterial may be dependent on the core composition, size, shape, and surface modification that a material possesses, suggesting that a high number of materials should be tested. The many different cell and tissue types of the human body can react in different ways to any given nanomaterial, meaning that an ideal toxicology screening should test every unique cell type. Thus, the possibility to create a fast, stable screening process for the maximum number of combinations of nanoparticles and human cells is of great interest. To this end, microfluidic approaches seem particularly interesting [115],[93],[116], especially in combination with electrochemical chip devices, containing multiple microelectrodes for array-based cell monitoring [48]. Chip devices and the incorporated microstructures, such as microwells and

microfluidics, are generally produced by thin-film technologies (i.e. photolithography). Different chip designs are possible, with different electrode connections, data recording processes, electrical settings that ultimately influence measurement times and sensitivity. Readers are asked to refer to selected reports regarding chip development using different techniques and procedures [117],[48],[108],[118],[119],[120],[121]. Recently, a microfluidic chip-based platform was proposed by Amatore's group to monitor electrochemically four primary ROS and RNS [49] released by a population of cell. The chip is based of an upstream microchamber hosting the cell culture and four parallel microchannels located downstream for the multiplexed and separated determination of H_2O_2, $ONOO^-$, $NO^\cdot$ and NO_2^-. The advantage of this device is the effective and noninvasive detection of ROS and RNS from a large population of cells. Separating emitting cells from the detection area seems a relevant approach to avoid any perturbations of cell behaviors by electrode operations. In comparison with the micro- nano-electrode configuration, the collection efficiencies of the short-lived chemical species were slightly lower, as a result of their kinetic decomposition. However, a relevant number of measurements could be obtained from only a few experiments, obtaining a rapid insight about biologically relevant effects of oxidative stress on average cellular behaviors.

Moreover, a large-scale integration (LSI)-based chip, containing about 400 Pt or Au microelectrodes, [122] which enables both rapid imaging for monitoring fast, transient biological phenomena such as exocytosis events and the simultaneous analysis of multiple molecules, has been proposed by Matsue. Two different potentials, as a checkered potential pattern, are applied to each electrode to acquire the oxidation and reduction currents from two different electroactive species derived from the cells, simultaneously. The two electrochemical responses consisting of reduction or oxidation currents are image-processed to acquire an electrochemical image consisting of two signal scales for the oxidation and reduction currents, providing a multicolor electrochemical image (Figure 6 B-C).

Chip technology was also proposed to electrochemically study the viability of invertebrates. The correlation of locally induced convective disturbances on the diffusion-limited steady-state currents with the movements of *Daphnia magna* was evaluated in [123] by using an electrochemical chip (Figure 6B). The device consisted of an array of 20x20 Au or Pt electrodes with a pitch of 250 mm. A sample solution was introduced into the device. Ag/AgCl reference and Pt counter electrodes were inserted into the solution. An electrochemical image, consisting of 400 pixels displaying current values, was obtained every 200 ms. The convective flow caused by the motion of the microorganism supplies redox species to the microelectrodes, resulting in an increase in redox current at the microelectrode near the motion areas. Thus, flow was converted to electrochemical

signals, resulting in the capture of the motion of microorganisms in electrochemical images. The use of difference imaging helped in obtaining high-contrast motion and helped to avoid the influence of electrode fouling by adsorbents. The motions of the microorganism (*Daphnia*) were clearly tracked based on the electrochemical oxidation of $[Fe(CN)_6]^{4-}$ and reduction of O_2. When the microorganism stopped moving, the supply of redox species to the microelectrodes was blocked by the microorganism, and the currents decreased. The decrease in current may have also been caused by fouling of the electrode surface by compounds secreted from the microorganism upon its death. O_2 detection was suitable for the detection of such secreted compounds, because O_2 reduction is sensitive to electrode fouling. Thus, a decrease in the signal can be used for the detection of compound secretion. This way, electrochemical imaging can potentially provide information that cannot be acquired by optical images. The motion of a single *Daphnia magna*, swimming in a confined volume of water, was also reported by Compton's group, by using current–time transients recorded by photoelectrochemistry measurement [124]. Using an array of individually-addressable electrodes under illumination, the motion of the daphnid was detected by means of measuring "dark" transients as the shadow cast by the moving sphere passed over each electrode.

Viability monitoring of microorganisms is an end point in ecotoxicological assays. Thus, the presented method is expected to be useful for the investigation of biological functions, and the assessment of nanomaterial toxicity toward the ecosystems.

Nanotoxicity assessment could benefit from another cutting edge electrochemical-based technology in the near future. Indeed, electrode arrays are expected to be widely utilized in base devices for organs-on-a-chip, which comprise of microfluidic and cell culture components and simulate tissue- and organ-level physiology with continuous perfusion [125], [126]. This application is envisaged to be important for nanotoxicity assessment in HTS screening format, avoiding the *in vivo* testing in living animals.

5. Conclusions

In conclusion, electroanalysis can provide useful information in the determination of the toxicological impact of ENMs at a cellular and a whole-oganism level. However, at the moment only few examples have been reported. In our opinion the unprecedented technological advancement in electronics and in micro- and nanofabrication of electrodes and microfluidics should give rise in the near future to a broadening in the scope of bioelectrochemical methods for toxicity assessment and their consolidation as promising tools for developing reliable nanotoxicity assays in an HTS format and organs-on-a-chip configuration. Furthermore, impedance methods

have been largely exploited and have been proven to allow HTS analysis.

On the other hand, the application of electroanalytical tools to non-invasive single cell monitoring has been already demonstrated by different techniques. The importance of studies at a single cell level, due to the heterogeneity of cell populations and nanoparticle distributions has been pointed out in recent review papers [3]. The use of nanoelectrode-based technologies will enable the monitoring of intracellular molecular events. Indeed, one of the major challenges in nanotoxicity evaluation is the understating of causal relationship among biological events. The Adverse Outcome Pathways (AOPs) is a recently proposed conceptual framework that links the biological events between the molecular initiating events to the adverse outcomes that are relevant to risk assessment [127]. Electroanalytical methods can be used to monitor different key events at different biological levels thus being useful in an AOP-based approach.

Thus, although a number of challenging issues remain to be addressed (i.e. fouling of the electrode surface due to the measurement in a biological specimen and thus the need of electrode regeneration) the possibility to use electroanalytical tools as screening methods will help in classify the toxicological profile of ENMs.

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Table 1

Table 1: Some examples of conventional tests used for *in vitro* toxicity assessment

Test	Evaluation endpoint
Dichlorodihydrofluorescein diacetate (DCFH-DA)	Oxidative stress (direct method)
Nitroblue tetrazolium (NBT) assays	Oxidative stress (direct method)
Glutathione (GSH) content	Oxidative stress (indirect method)
Malondialdehyde (MDA) formation	Oxidative stress (indirect method)
Superoxide dismutase (SOD) activity	Oxidative stress (indirect method)
Flow cytometry with annexin-V assay	Apoptosis (programmed cell death) induced by ENMs
Measurement of the mitochondrial membrane potential	Apoptosis induced by ENMs
Protein markers of apoptosis, including Bax, Bcl2, p53 and caspases	Apoptosis induced by ENMs
Adenosine triphosphate (ATP) luminescence assays	Measurement of cellular metabolic activity
3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay	Measurement of cellular metabolic activity
Lactate dehydrogenase (LDH)	Assessment of membrane integrity and cell necrosis (accidental cell death)
Vital dyes (e.g., trypan blue and propidium iodide)	Assessment of membrane integrity and cell necrosis (accidental cell death)
Neutral red test	Assessment of membrane integrity and cell necrosis (accidental cell death)
Cytokine release	Inflammatory reaction and phagocytosis
Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)	Evaluation of Cell internalization of nanomaterials
Confocal microscopy	Evaluation of Cell internalization of nanomaterials
Inductively-coupled plasma mass spectrometry (ICP-MS)	Evaluation of Cell internalization of nanomaterials

Table 2

Table 2: Some examples of conventional test used for genotoxicity evaluation

Test	Description	Endpoint
Comet assay	Electrophoretic separation of DNA fragments from lysed cells in agarose gel coupled fluorescence microscopy following staining with a fluorescent dye	The extent of migration is finally interpreted as a reflection of the DNA damage
TUNEL (Terminal deoxynucleotidyl transferase deoxyuridine triphosphate Nick End Labeling	Fluorescent dye for labeling the 3'- hydroxyl termini in the double-strand DNA breaks generated during apopotosis	DNA damage-induced apoptosis mechanisms
Micronucleus assay	Micronucleus visualization using different methods	Mutagenesis
Ames Test	Cell culture	Mutagenesis

Figure 1

Figure 1: Scheme of the workflow involved in nanotoxicity and nanoecotoxicity evaluation. Redrawn with permission from ref. [16]

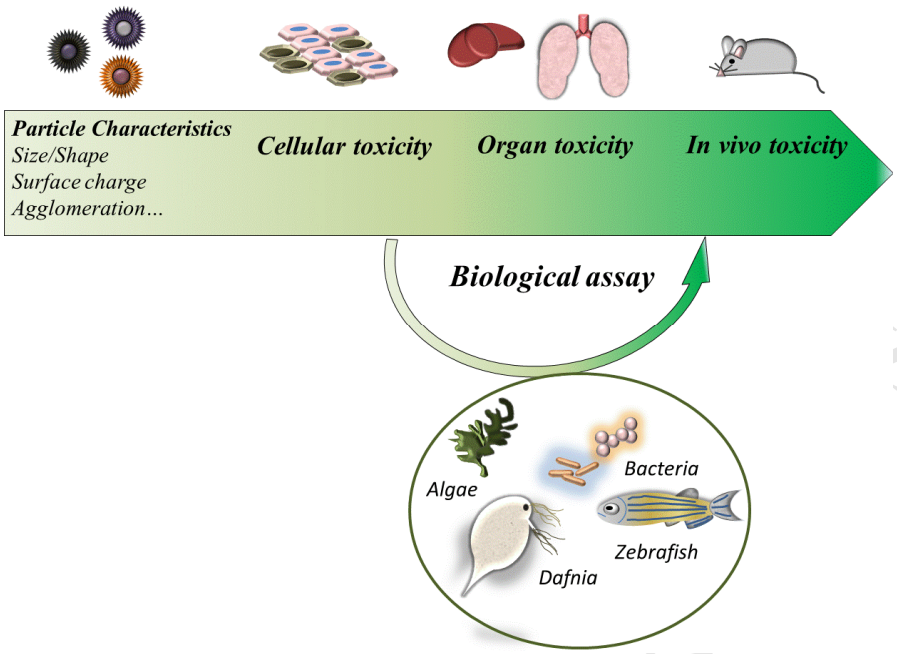


Figure 2

Figure 2: Main reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the oxidative stress pathway. Redrawn with permission from ref. [29] (Copyright (2008) American Chemical Society).

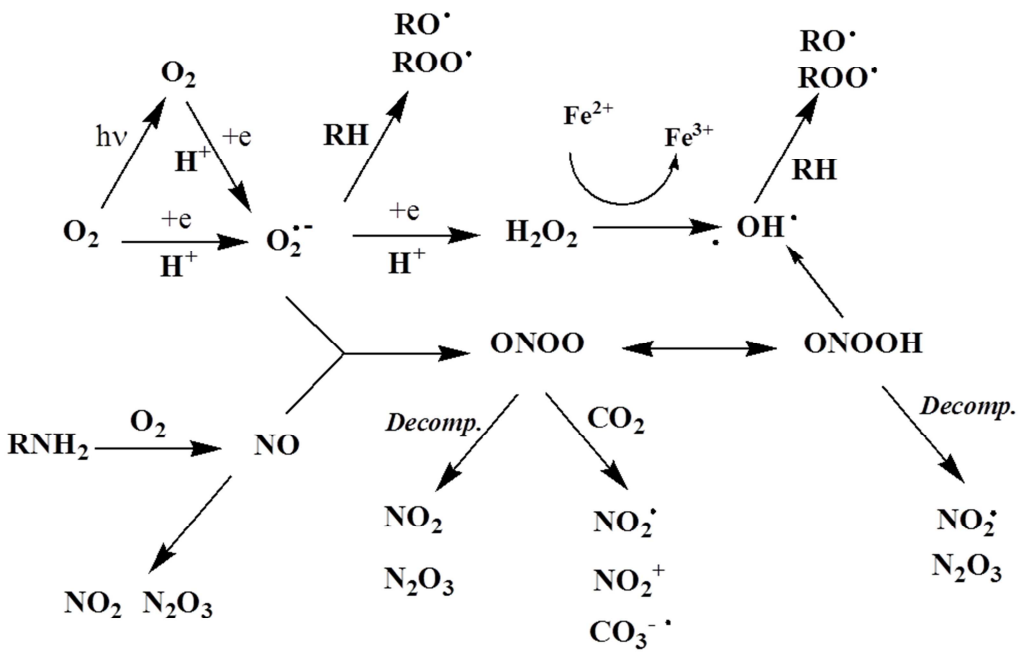
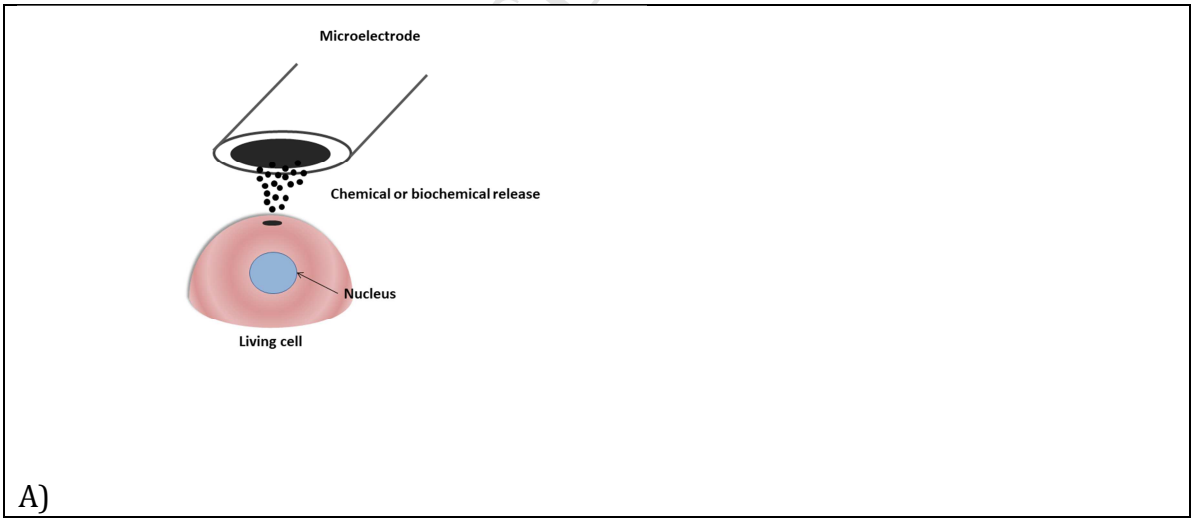
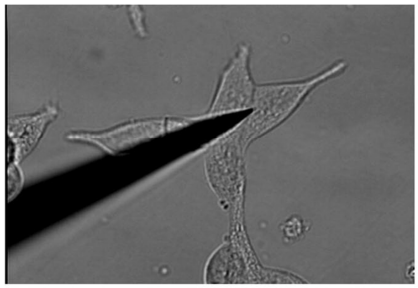


Figure 3

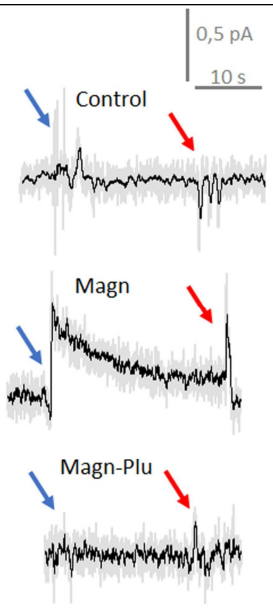
Figure 3: Some examples of methods used for the electrochemical monitoring of ENMs toxicity. A) Schematic representation of the measurement using a microelectrode positioned near a living cell releasing chemical or biochemical species. Redrawn with permission from ref. [29] (Copyright (2008) American Chemical Society); B) Photomicrograph of intracellular measurement of ROS in living cells by nanoelectrode (i) and example of ROS intracellular measurements in individual living cells (ii). Representative current traces of a nanoelectrode polarized at + 800 mV vs Ag/AgCl inside and outside living cells. The arrows indicated, respectively, the moment of penetration and retraction of the nanoelectrode. Control – ROS intracellular measurements in pure cells after 3 h in buffer; Magn - ROS intracellular measurements in cells incubated with iron-oxide NPs during 3 h, Magn-Plu - ROS intracellular measurements in cells incubated with iron oxide NPs modified with layers of biocompatible Pluronic F127 during 3 h. Grey curves are pure records, black curves are records with 100 averaging. Redrawn with permission from ref. [44]; C) The main phases of exocytosis process. Redrawn with permission from ref. [29] (Copyright (2008) American Chemical Society); D) Example of amperometric traces of exocytosis from stimulated mast cells exposed to 1-0 nM of 28±7 nm diameter Au NPs for 48 h (i) and control plates (ii). The 3 s secretagogue stimulation pulse is indicated by the bold line below each trace. The lower inset displays an expanded view of an individual amperometric spike including a prespike foot event. Redrawn with permission from ref. [45] (Copyright (2008) American Chemical Society)



A)

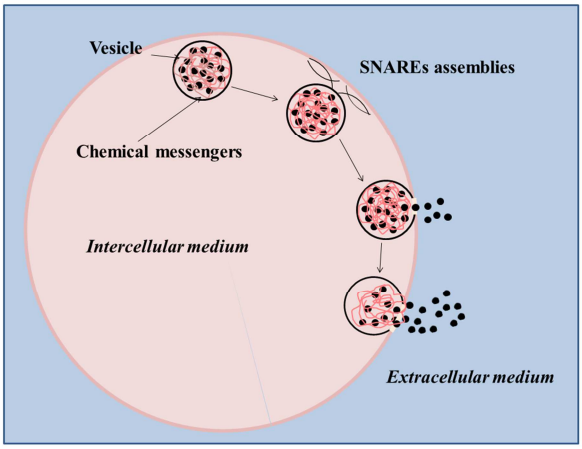


i)

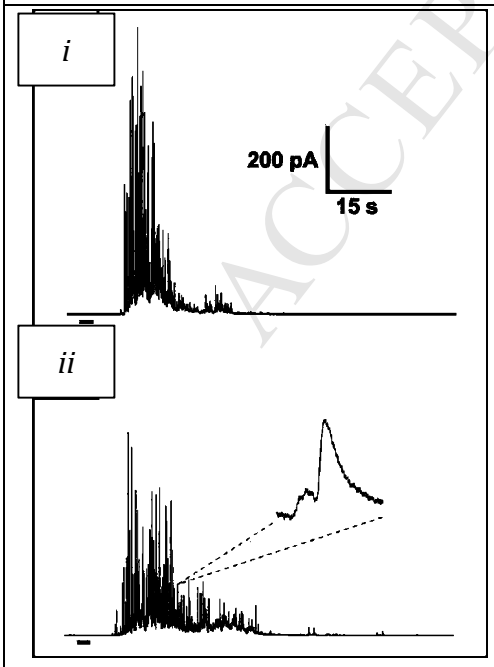

0,5 pA
10 s
Control
Magn
Magn-Plu

ii)

B)


Vesicle
SNAREs assemblies
Chemical messengers
Intercellular medium
Extracellular medium

C)


i
200 pA
15 s
ii

D)

Figure 4

Figure 4: Schematic of DNA damage evaluation by an electrochemical DNA-sensor. The current signal are reprinted with permission from ref. [77]

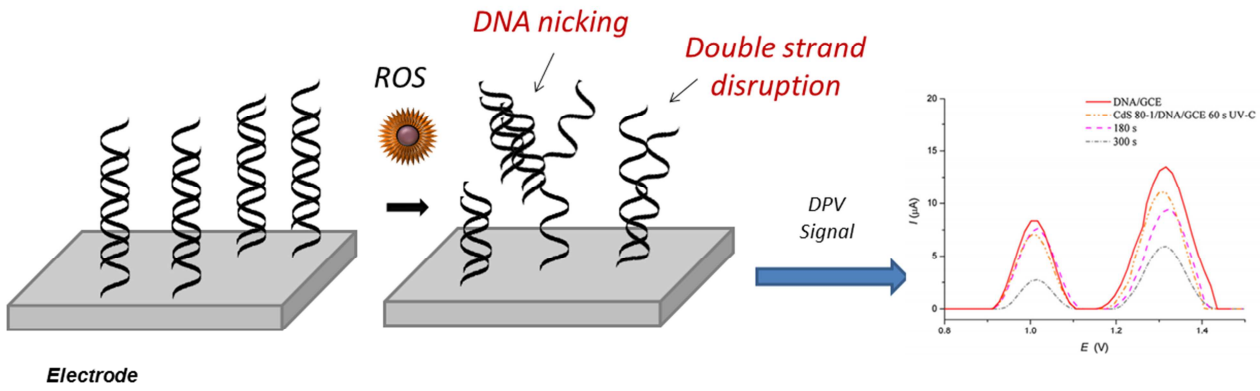
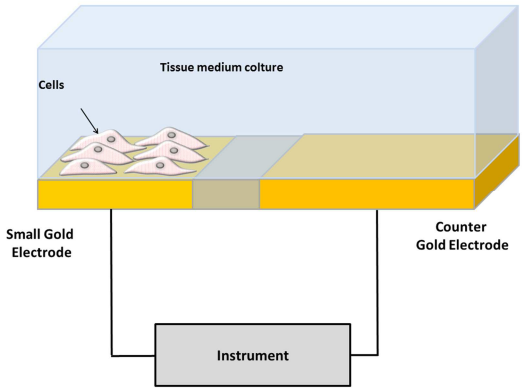
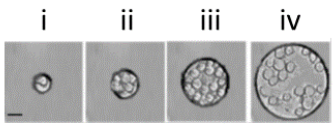


Figure 5

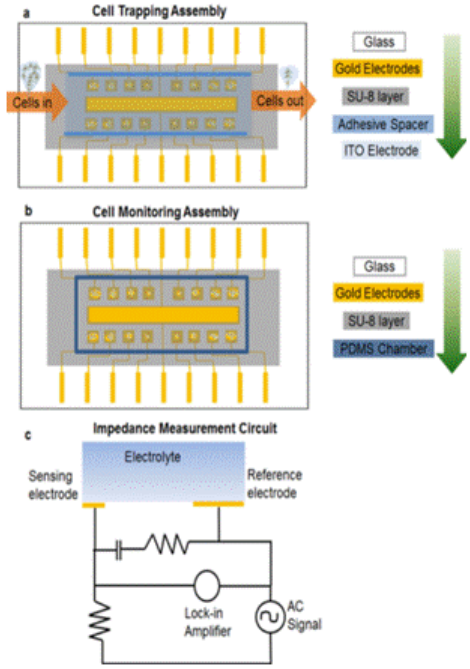
Figure 5: Examples of the impedimetric approach for evaluating cell toxicity. A) Schematic of cell analysis trough electrochemical impedance; B) Microwells image captured with an upright optical microscope after controlled cell trapping. The numbers of cells captured in a microwell are controlled by the size of the microwells. A 20 μm microwell (i) host 1 cell, a 30 μm microwell (ii) hosts 4 cells, a 50 μm microwell (iv) hosts about 10 cells, and a 80 μm microwell (v) hosts about 18 cells. The scale bar is 20 μm. Reprinted with permission from ref. [94] (Copyright (2016) American Chemical Society); C) Cells-on-chip-assembly: a) MEMS device assembly for dielectrophoresis trapping of cells; glass wafer is the base, gold sensing electrodes are under the 8-microwell pattern, spacer is used to hold the top ITO electrode that acted as one of the two electrodes (the others are gold microwell electrodes) to generate a non uniform dielectric field; b) MEMS device assembly when top ITO electrode is removed and PMDS chamber is placed to contain cell growth solution while monitoring cell impedance; c) electronic cell impedance sensing circuit representing a single electrode for simplicity. Reprinted with permission from ref. [94] (Copyright (2016) American Chemical Society).



A)



B)



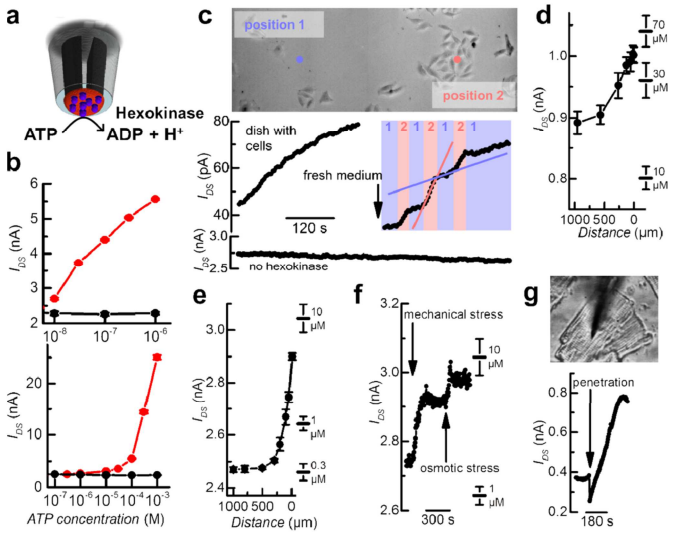
C)

MEMS: MicroElectroMechanical System; SU-8: photoresist used to create the microwell; ITO: Indium Tin Oxide; PMDS: Polydimethylsiloxane

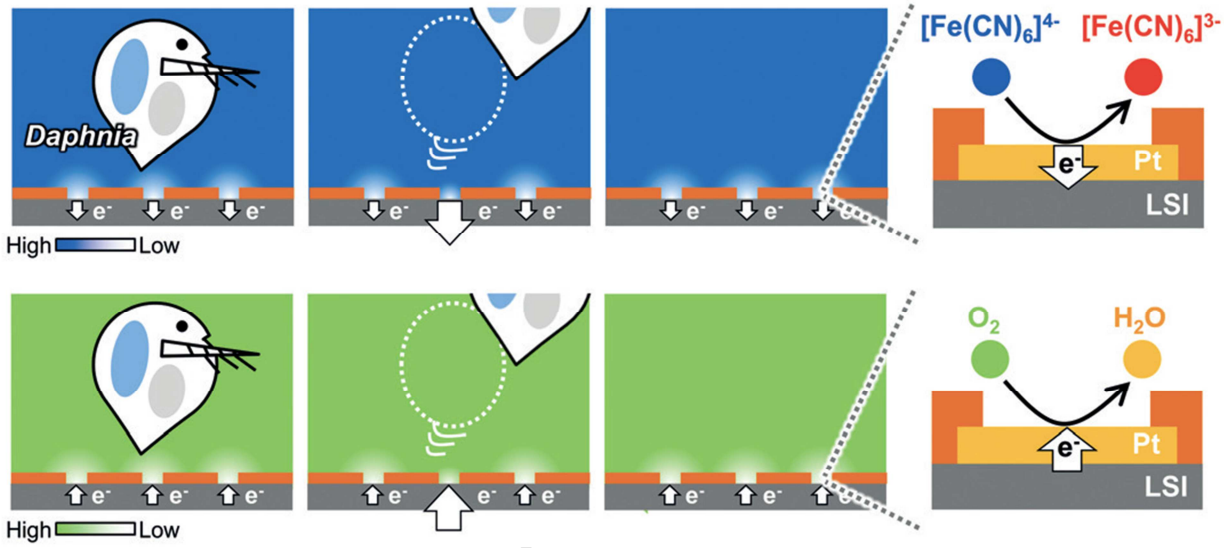
Figure 6

Figure 6: Examples of innovative technologies: A): PPy nano-FET probe to perform highly localized ATP measurements for cell analysis. The probe detects pH changes caused by hexokinase. Hexokinase is immobilized onto the tip of the probe (a). Calibration curves are shown for two individual sensors, black-dot traces show the control experiment of a nano-FET not modified with hexokinase (b). Real-time ATP measurements over a nonpopulated spot and a group of melanoma cells demonstrate the high rate of ATP release from the cells (c). ATP gradients released from an isolated melanoma cell are measured by vertical approach of the sensor (d). Localized ATP measurements are also performed on a single cell (cardiomyocyte) (e−g). The cell produces a vertical gradient from its proximate microenvironment into the bulk solution (e). Additional ATP release is induced by touching the cell with the sensor and adding water to the solution to create osmotic stress (f). The probe allows for penetration into the cell (g). Reprinted with permission from ref [104]. B): Electrochemical imaging of the motion of a *Daphnia magna* microorganism. $[Fe(CN)_6]^{4-}$ or O_2 can be used as redox species. $[Fe(CN)_6]^{4-}$ is oxidized at + 0.60 V, while O_2 is reduced at -0.50 V. Different colors represent the concentrations of $[Fe(CN)_6]^{4-}$ and O_2, respectively. When the microorganism moves, redox compounds are supplied to electrodes near it, increasing the redox currents. C: Electrochemicolor imaging of the respiration and alkaline phosphatase (ALP) activities of embryoid bodies (EBs). (A, B) Detection schemes for the respiration and ALP activities, respectively. The blue and orange colors indicate the O_2 and paraminophenylphosphate (PAP) concentrations, respectively. (C−F) Optical and electrochemical images of the undifferentiated (C, E) and differentiated EBs (D, F). These EBs were cultured for four (C, D) or eight (E, F) days. The electrochemical images consist of signals at −0.5 and 0.3 V for depicting the reduction currents of O_2 and the oxidation currents of PAP, respectively. These images were acquired after 180 s of the potential steps. (G) ΔCurrents of the O_2 reduction currents in the EBs. (H) Oxidation currents of PAP in the EBs. Au sensor electrodes were utilized for detection. Reprinted with permission from ref.[122]

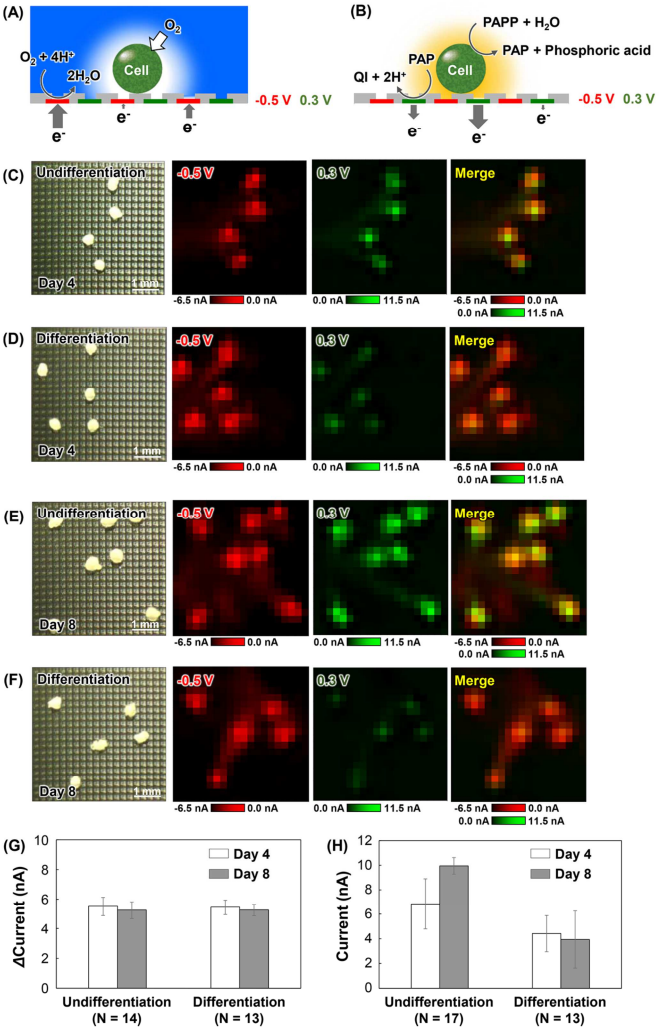
A)


a
Hexokinase
ATP
ADP + H+
b
ATP concentration (M)
c
position 1
position 2
dish with cells
fresh medium
120 s
no hexokinase
d
Distance (µm)
e
Distance (µm)
f
mechanical stress
osmotic stress
300 s
g
penetration
180 s

B)


Daphnia
High
Low
[Fe(CN)6]4-
[Fe(CN)6]3-
Pt
LSI
O2
H2O

C)



Declaration of interests

☐X The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: