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ECSIN's methodological approach for hazard evaluation of engineered nanomaterials

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Abstract. The increasing production volumes and commercialization of engineered nanomaterials (ENM), together with data on their higher biological reactivity when compared to bulk counterpart and ability to cross biological barriers, have caused concerns about their potential impacts on the health and safety of both humans and the environment. A multidisciplinary component of the scientific community has been called to evaluate the real risks associated with the use of products containing ENM, and is today in the process of developing specific definitions and testing strategies for nanomaterials. At ECSIN we are developing an integrated multidisciplinary methodological approach for the evaluation of the biological effects of ENM on the environment and human health. While our testing strategy agrees with the most widely advanced line of work at the European level, the choice of methods and optimization of protocols is made with an extended treatment of details. Our attention to the methodological and technical details is based on the acknowledgment that the innovative characteristics of matter at the nano-size range may influence the existing testing methods in a partially unpredictable manner, an aspect which is frequently recognized at the discussion level but oftentimes disregarded at the laboratory bench level. This work outlines the most important steps of our testing approach. In particular, each step will be briefly discussed in terms of potential technical and methodological pitfalls that we have encountered, and which are often ignored in nanotoxicology research. The final aim is to draw attention to the need of preliminary studies in developing reliable tests, a crucial aspect to confirm the suitability of the chosen analytical and toxicological methods to be used for the specific tested nanoparticle, and to express the idea that in nanotoxicology, "devil is in the detail".

1. Introduction

ECSIN – European Centre for the Sustainable Impact of Nanotechnology - is a research center part of Veneto Nanotech, located in the city of Rovigo, Veneto Region of Italy. ECSIN's most relevant activities are in the fields of nanotoxicology and risk assessment. In particular, the main objective of the facility is to apply the most recent and innovative scientific findings to the study of the impact of nanomaterials on the environment and human health as well as to investigate ethical and social aspects of this emerging technology. Final aim is to support companies in the production of non-toxic products, and to provide support for survey systems and measurement standards required by companies, public authorities and investors.

As standard protocols for nanotoxicity assessment are still lacking, we have optimized some of the most frequently used toxicology protocols, adapting them for the use with nanoparticles (NPs). In this report we review the most important issues that should be addressed in the optimization of nanotoxicity testing. We highlight some of the most important technical considerations that should drive the development of standardized protocols for nanotoxicology, suggesting control experiments and protocol modifications to avoid artifacts.

2. Development and optimization of nanotoxicity *in vitro* tests

In vitro testing represents a first common step for assessing ENM related health hazards. The most commonly used vitality and functional assays (e.g. MTT, WST-1, XTT, MTS, LDH, DCFH₂ and caspase 3/7) rely on absorbance or fluorescence measurements for quantification. These *in vitro* assays have been established for small molecules of drugs and chemicals. Their application to Engineered Nanomaterials (ENM) opens new questions regarding interferences with the expected response of the assays because of the unique physico-chemical properties. Potential interferences are summarized in Table 1.

Table 1

	Artifact
Optical interferences	NPs absorbance adds to the assay absorbance
	NPs absorbance decreases the excitation light and the emission spectrum of fluorescent probes
Physical interferences: adsorption on NPs surface	Reduction of end products and/or enzymes decreases absorbance or fluorescence and prevents enzymatic reactions
Physico-chemical interferences: NPs reactivity	NPs or their ions catalyze or inhibit <i>in vitro</i> assays
Interferences by systems used	Culture medium and serum may catalyze oxidation of several substrates used in the assays

2.1. Optical interferences

Most metal NPs, carbonaceous and some metal oxides NPs absorb and scatter light over the spectral regions used in the assay systems [1–3]. Therefore, *in vitro* assays could be affected by the presence of NPs interacting with visible light. Data generated using colorimetric *in vitro* assays may derive by a linear combination of chromophore and NPs contribution, resulting in false positives. NPs may also affect data obtained with fluorescence-based assays, reducing the incident light absorbed by fluorophores and generating false negatives.

In vitro assays used for evaluating metabolic activity are based on tetrazolium salts reduction and purple formazan formation. Kroll et al (2011) investigated the optical interferences of twenty-three ENM – CeO₂, TiO₂, Ti-Zr, ZrO₂, SrCO₃, carbon black, AlOOH and Al-Ti-Zr – on three standardized *in vitro* assays: MTT, LDH and DCFH₂ [4]. They found interferences of the nanomaterials with all the *in vitro* toxicity test systems. These interferences were abolished removing NPs from formazan solution, at least at low NPs concentrations used.

Optical interferences may also affect quantification of lactate dehydrogenase (LDH) release. In the assay, levels of extracellular LDH released from damaged cells is measured as an indicator of cytotoxicity. The assay is based on the ability of LDH to convert lactate into pyruvate with the subsequent reduction of NAD to NADH. Although quantification of LDH can be measured recording absorbance induced by NADH at 340 nm, several commercial kits have been developed. These kits are based on the reduction of iodotetrazolium chloride to formazan with an absorption peak between 500 nm and 600 nm. Wilhelmi and colleagues reported alterations in LDH quantification due to the NPs used [2]. In presence of fine and ultrafine TiO₂, zinc oxide and SiO₂ NPs, an increase in absorption and then an overestimation of LDH release occurred. Also in this case, interferences were abolished removing NPs from formazan containing solution.

Caspase 3/7 assay represents the most commonly used test to investigate the molecular mechanisms involved in cell death induced by NPs, as it indicates apoptosis induction. It is based on the cleavage of non-fluorescent caspase substrate by caspase 3/7 to create a fluorescent molecule. Since excitation wavelengths of cleaved substrate overlap with NPs absorption, the resulting emission spectrum decreases in the presence of NPs. An example is reported in Figure 1 where fluorescence of cleaved substrate decreases in the presence of silver NPs, while it returns to the positive control values after NPs removal.

In conclusion, artifacts due to optical interferences can be solved removing NPs from the chromophore/fluorophore containing solutions. In the case of colorimetric assays, NPs contribution can be conveniently subtracted by sample absorbance.

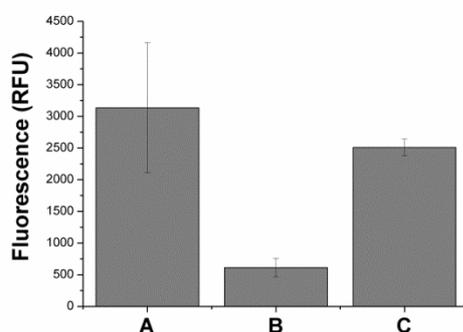


Figure 1. Caspase 3/7 assay on A549 cells treated with staurosporine 0.5 μM (Positive control) (A), after addition of silver NPs (B) and after their removal (C).

2.2. Physical interferences: adsorption on NPs surface

Some NPs may adsorb formazan or other end products as well as LDH or other enzymes reducing the assay absorbance or preventing enzymes from being measured, respectively [5]. Moreover, they can carry pyrogenic contaminations, difficult to detect but biologically strongly active [6]. If this interference occurs, alternative methods to test NPs cytotoxicity need to be sought.

2.3. Physico-chemical interferences: NPs reactivity

Metal NPs dissolution may interfere with *in vitro* assays either directly or indirectly. Oxidative dissolution of metal NPs can generate formazan increasing the assay absorbance. Holder et al (2012) analyzed the potential interference of several carbonaceous and one TiO_2 NPs on formazan formation [5]. Activated carbon did not react with MTT at any concentration, while soot, oxidized soot and TiO_2 produce formazan only at the highest concentration used (50 $\mu\text{g}/\text{mL}$). Diesel particles generated formazan at all concentration tested. Reduction of tetrazolium salts by TiO_2 was also demonstrated by Wang et al (2011) [7]. Authors reported that in comparison with trypan blue exclusion assay, MTT and XTT predict inaccurately cell toxicity or overestimate cell viability, respectively. The superoxide produced by TiO_2 [8,9] resulted in the conversion of MTT and XTT to formazan causing misleading results. Ion released from oxidative dissolution of metal NPs may inactivate enzymes involved in formazan production denaturing them or blocking their active site [10]. Han et al (2011) investigated the effects of copper NPs, silver NPs and TiO_2 NPs on LDH activity [11] as a function of their dissolution rates. All the NPs inhibited LDH activity, although in a different way. Because of the high dissolution rate, copper NPs could interfere with the LDH assay by inactivating the enzyme. Conversely, TiO_2 NPs were found to adsorb LDH molecules. LDH inhibition by silver NPs was attributed to the carbon matrix used to cage the particles during the synthesis.

2.4. Ion release: methods and limitations

As described above, ions released from NPs may inhibit enzymatic assays. Therefore, it is important to establish the dissolution rate of NPs in working conditions. This data is also important to disclose molecular mechanisms underlying NPs cytotoxicity. Several methods are currently used, though they provide different types of information: ultracentrifugation [12] or ultrafiltration [13] coupled with ICP-MS, fluorescent probes [14,15] and ion-selective electrode potentiometry [16]. Although it has been calculated that ultracentrifugation at 100000g for 60 minutes is sufficient to move particles with diameter larger than 4 nm at least 2.5 cm, NPs can be present in the supernatant causing an overestimation of the total ion released. On the other side, ultrafiltration permits to measure only ions bound to small molecules, while those bound to large molecules or proteins such as, for instance, serum are retained. In the presence of complex media, ultrafiltration does not provide the total amount of released ions but only free-ions or ions chelated with small molecules. The information obtained using molecular probes depends on their affinity constants. Molecular probes with low affinity constants detect only high concentrations of free-ions, while they are not able to chelated ions bound to other molecules. The use of ion-selective electrode potentiometry can be invalidate by interferences with complex matrix such as culture media. In this case, it is very important to perform titration curves in the complex matrix in order to avoid interferences.

2.5. Culture medium and serum interferences

Evaluation of NPs-generated oxidative stress is based on oxidation of dichlorofluorescein (DCFH₂) or its derivatives. Several critical issues may occur: DCFH₂ oxidation by culture medium during loading into cells or leakage from cells; DCFH₂ adsorption on NPs surface; DCFH₂ oxidation by NPs and optical interferences. DCFH₂ can be deacetylated and subsequently oxidized by culture medium as reported in Figure 2. Therefore, increased fluorescence intensity may depend on aspecific oxidation by medium or NPs occurring during DCFH₂ loading or its leakage. To avoid aspecific DCFH₂ oxidation, it is important to evaluate culture medium and serum quality and, eventually, load it into cells in a very short-time using phosphate buffer and before NPs addition. NPs ability to oxidize DCFH₂ has to be tested. Contribution of oxidized DCFH₂ as a function of leakage can be avoided by rinsing cells with phosphate buffer before the assay.

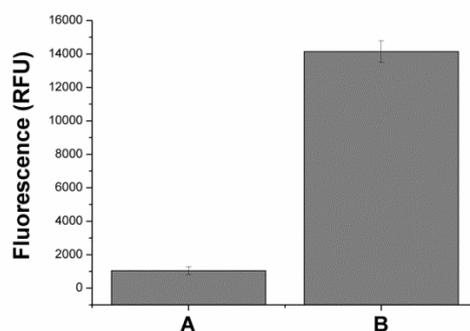


Figure 2. Aspecific oxidation of DCFH₂ in phosphate buffer (A) and cell culture medium F12 (B).

3. Cell models and optimization of culturing procedures

3.1. Primary cells versus cell lines

Primary cells are considered to be the closest *in vitro* cellular model for human organs, and the use of cell lines as surrogates of primary cells has been questioned by different authors. Cell lines often derive from transformed or transfected cells which have lost their basic proliferation control mechanisms, and show a great number of genetic and chromosomal aberrations which potentially increase at each passage. Their phenotypical difference compared to the primary cells results for

example in differences in the basal levels of enzymes, as in the case of hepatocytes [17]. Also the response to cytotoxic exogenous stimuli can be different between primary cells and cell lines. In the case of hematopoietic cells, we have shown that the level of cytotoxicity induced by antimony trioxide nanoparticles exposure can be very different between primary cultures of bone marrow hematopoietic progenitors and seven human cell lines of hematopoietic origin: K562, HL-60, CEM, CEM-R, Thp-1, Jurkat, and Molt-4 [18].

Nevertheless, nano-cytotoxicity assays need to be standardized and cells need to be as stable as possible for use in different laboratories all over the globe. For this purpose, the use of primary cells present strong limitations such as limited life span, phenotypical instability, large variability between different human donors, limited availability and high costs. These limitations have brought to extensive use of cell lines for nanotoxicity screenings, mainly due to the fact that cell lines are easy to maintain and can be sub-cultured for many passages, batch-to-batch variability is supposed to be low and their cost is affordable. In a recent report, Kermanizadeh et al (2012) have compared primary human hepatocytes with the C3A cell line in respect to their response to a panel of nanomaterials [19]. The cell line was very comparable with the primary hepatocytes with regards to their cytotoxic response to ZnO, MWCNTs, Ag and positively functionalized TiO₂ NPs. This confirms that some cell lines can be used as a model in place of primary cells for cytotoxicity screenings. Taken together, these studies bring to the consideration that confirmation analysis should ideally be done for each cell line and relative to each cell assay, keeping in mind that it is not possible to predict what the interaction of NPs with cells in living organisms will be, solely by extrapolating from nanotoxicology studies on cell models.

3.2. Choice of cell type

The choice of the cell model is based on the evaluation of the potential exposure route to individual nanoparticle, depending on the specific application. Table 2 shows the cell lines we have chosen as representative of cellular models in reference to different nanoparticles applications and routes of exposure.

Table 2

Examples of NP applications	Exposure Route	Primary target organs	Cell lines used at ECSIN
Paintings and any type of surface coatings, air treatment and purification, textiles, worker exposure to dry nanoparticles	Inhalation	Respiratory tracts	A549 (lung) NR8383 (rat alveolar macrophages)
Food industry and processing, food contact material and kitchen utensils, dental care products	Ingestion	Digestive tract	Caco2 (colon)
Cosmetics, textiles, bandages and wound care, sunscreen lotions, surface coatings	Dermal	Skin	L929 (fibroblasts from subcutaneous connective tissue) HaCaT (keratinocytes)
Nanomedicine, secondary organ exposure following uptake and biodistribution	Blood Circulation	Blood cells, endothelial cells, internal organs	HepG2 (liver) HEK293 (kidney) MDCK (dog kidney) MG63 (osteoblasts) Thp1(monocytes)

3.3. Proliferating versus differentiating cells

One issue that we would like to bring to light here regards the choice of cell cycle state at which cells are exposed to NPs for cytotoxicity assays. In a previous study we have treated primary hematopoietic progenitor cells during proliferation and during differentiation state with Sb_2O_3 NPs at 5 ppm. When NPs were added during the proliferation phase, either at start of culture or at later point, cells looked morphologically altered within 6 hours, were mostly dead at 24 hours and completely dead within 96 hours after start of treatment. When, instead, NPs were added during the differentiation phase we observed no effect on differentiation as confirmed with flow cytometry analysis and real-time PCR analysis of differentiation markers. We then performed the exposure experiment on Thp-1 cell line. Sb_2O_3 NPs had no significant toxic effect on proliferating Thp-1 but inhibited the PMA-induced differentiation of Thp-1 cells into macrophages, driving them to death. These results demonstrate that the choice of cell proliferating state may greatly influence the cytotoxicity response, as the cytotoxic effect can be very different if cells are in highly proliferating versus differentiating states.

This is not surprising, as it is well known that cells in different states of their life express different biomolecules, and may respond in different manners to exogenous stimuli. Nevertheless, if we consider some of the recently published studies where A549 cells are used for nanotoxicity assays, we see that the same cell line is not always used at the same cell cycle state [4, 20-24]. Cells of the A549 human lung adenocarcinoma cell line have an epithelial morphology and are widely used as an *in vitro* model for NPs cytotoxicity assay due to inhalation exposure. This cell line is hypotriploid with a modal chromosome number of 66 which occurs in 24% of cells, with higher ploidies occurring at an infrequent rate (0.4%). This brings to the possibility to select clones if sub-culturing is not performed when cells are below confluency. When these cells are kept growing to confluency, they form a monolayers with Type II characteristic morphology and tannic acid staining for typical lamellar bodies [25], indicating differentiation.

For nanotoxicity screening we noticed heterogeneity of the experimental procedures in regard to the number of cells/cm² at time of plating. Some researchers plate these cells at low concentration, so that cells are not confluent at time of NPs exposure [20-22]. In other words, cells are in their proliferating phase during exposure to NPs. Other authors, instead, indicate plating of cells at high concentration in order to reach confluency at time of exposure [4,23,24], so that cells have formed monolayers and are starting to differentiate. These two different plating protocols for nanotoxicity screenings on the same cell line may allow to different results due to the different physiological state of cells at time of treatment.

3.4. Optimization of assays with cells

In our laboratory, we have chosen to run the cytotoxicity assays MTS and LDH on A549, MDCK and HepG2 cells after 6, 24 and 48 hours of exposure to NPs, according to the ASTM standard protocol E2526-08 (Evaluation of Cytotoxicity of Nanoparticulate Materials in Porcine Kidney Cells and Human Hepatocarcinoma Cells) with modifications.

Our first step of the optimization phase of the procedure has been focused on the selection of the best cell plating concentrations for each of the 3 cell lines, so that we can perform both MTS and LDH assays on the same cultures. Exposure is preceded by a 24 hours incubation to allow cell attachment and start of proliferation. In order to select a plating concentration that would allow us to expose NPs to cells that are proliferating during the whole incubation period, we have run preliminary experiments. Figure 3 shows an MTS assay performed with A549 cells that were plated at 3,000, 5,000 and 8,000 cells/well in 96 well plates (surface area of approximately 0,35 cm² each well), in a total volume of 200 μl . After 24 hours, medium was changed to mimic start of exposure, and cells were incubated for the additional indicated time (6, 24 and 48 hours after medium change). At the end of incubation, supernatant was removed and assayed for LDH activity (Biovision kit). MTS assay was performed following providers instructions (Promega Cell Titer Kit).

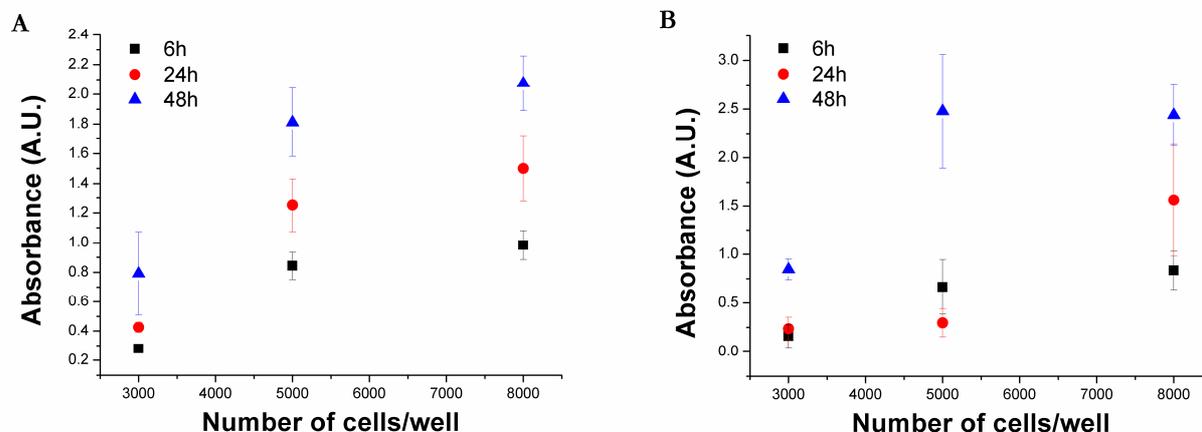


Figure 3. MTS (A) and LDH (B) assays on A549 cells at different incubation times after medium change: 6 hours (black squares), 24 hours (red dots) and 48 hours (blue triangles). X axis: number of cells. Y axis: absorbance values at 510 nm (MTS assay) or 500 nm (LDH assay), after subtraction of blank (cell free well). Values represent the mean of 3 replicates. Error bars represent standard deviation.

From results shown in Figure 3, we have selected 5,000 cells/well as the best starting concentration of cells for these assays, as at this concentration we can see a linearity both in terms of absorbance reading and in terms of incubation time. A density of 3,000 cells/well was not chosen because absorbance values are too low to allow a detectable difference in case of toxic stimulus, especially for samples at 6 hours incubation. On the other hand, 8,000 cells/well is too high because cells are already starting to enter the stationary phase, where alterations of metabolic activities would not be detectable properly.

Following the same procedure, we have performed the optimization test to select the best cell concentration on dog renal MDCK and human hepatocellular carcinoma HepG2 cell lines. Figures 4 and 5 show their results.

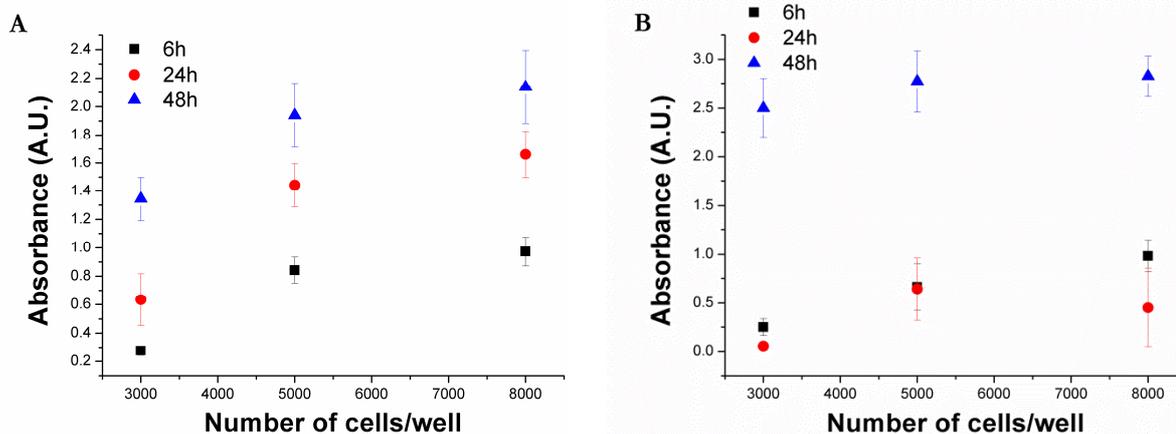


Figure 4. MTS (A) and LDH (B) assays on MDCK cells at different incubation times after medium change: 6 hours (black squares), 24 hours (red dots) and 48 hours (blue triangles). X axis: number of cells. Y axis: absorbance values at 510 nm (MTS assay) or 500 nm (LDH assay), after subtraction of blank (cell free well). Values represent the mean of 3 replicates. Error bars represent standard deviation.

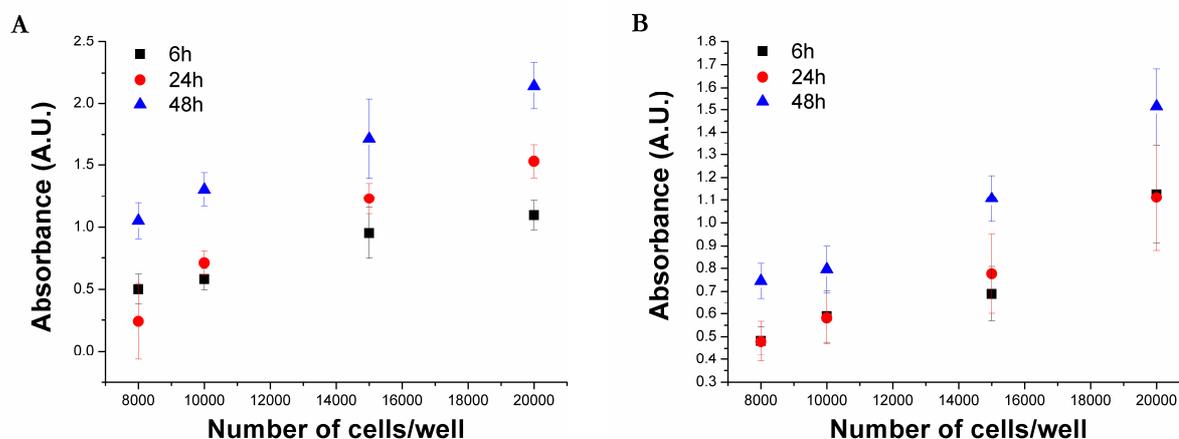


Figure 5. MTS (A) assay and LDH (B) on HepG2 cells at different incubation times after medium change: 6 hours (black squares), 24 hours (red dots) and 48 hours (blue triangles). X axis: number of cells. Y axis: absorbance values at 510 nm (MTS assay) or 500 nm (LDH assay), after subtraction of blank (cell free well). Values represent the mean of 3 replicates. Error bars represent standard deviation.

For MDCK cells, instead, we have selected 3,000 cells/well as these cells have a higher metabolic activity than A549 cells. For HepG2 cells we have chosen 15,000 cells/well as starting cell concentration for plating, in fact these cells are smaller and have a lower metabolic activity compared to A549 cells.

This first step of optimization is performed only once for each cell type and assay type. On the contrary, the second step of the optimization procedure is repeated for each NP type, as it is focused on the interaction of NPs to the assay. For this purpose, we add the NP being tested into the cell-free medium in the presence or absence of a positive control and run the assay. If the assay results show that the positive control is not influenced by the presence of the NPs at different concentrations, then we conclude that the NPs do not interfere with the assay. If, on the contrary, we observe an interference, then we consider all the possible interference mechanisms that have been described above. For the optimization of assays that require the reading of a fluorescent dye which is trapped inside live cells (such as microplate DCFH₂ assay for ROS determination), we apply a few additional technical considerations. In particular, as proliferating cells do not cover the whole well substrate surface, it is necessary that the reading occurs at different points of the well, to avoid reading only at one site where cells are not representative of the whole well. To solve this problem, it is necessary to use a reader that allows to perform an area scan.

4. Conclusions

In line with the actual multi-disciplinary efforts to develop and validate standard nanotoxicity assay protocols, our work at ECSIN has been focused on optimization of a few frequently used protocols. These assays have been borrowed from classical toxicity testing but require a careful consideration of all components and steps, in order to be adapted to the use with NPs. This is due to the peculiar physico-chemical characteristics of NPs, which often cause problems at different steps of the tests, such as optical interferences with the readout, adsorption of assay components onto NPs surface, NPs reactivity with assay molecules. Control experiments have to be performed for each type of NP being tested, in order to determine whether any of these interferences could be induced. Some of these issues can be overcome by removing NPs from the readout solutions, subtracting appropriate blanks or reducing the concentrations of NPs. When these modifications are not sufficient, for example in case of interference due to the released ions, then the assay should be abandoned and the same molecular endpoint should be sought using different methods. The choice of the cell model and cell density has to be carefully evaluated. The former is based on the evaluation of the potential exposure route to individual nanoparticle, while cell density at time of plating is important to compare results obtained from different laboratories.

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