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A guide to nanosafety testing: considerations on cytotoxicity testing in different cell models

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Abstract

Safety testing of nanoparticles (NPs) relies on robust, predictive, and reproducible methods. Integrated test strategies usually start with the assessment of *in vitro* cytotoxicity. Methods for this purpose are only partially established for NPs. The aim of this study was to evaluate three cytotoxicity assays for their applicability to NPs in several human cell models representing the most important NP target organs and to compare cell responses.

The assays covered different biological principles, *i.e.* ATP content, redox metabolism, and membrane permeability, as well as different read-out principles, *i.e.* luminescence, colorimetry, and fluorescence. For all assays standard operating procedures were developed. A549 and NCI-H441 lung epithelial cells in single and in co-culture with THP-1 derived macrophages served as lung epithelial models. Normal human epidermal keratinocytes and *in vitro* reconstituted human epidermis were employed as skin models. Naïve and differentiated Caco-2 gut epithelial cells were applied as gut epithelial models. Primary CD34-derived dendritic cells (CD34-DCs) served as an immune model. We used well-characterized model NPs, *i.e.* 50 nm amine-modified and 40 nm carboxyl-modified polystyrene NPs (PS-NH_2 and PS-COOH, respectively).

PS-COOH NPs showed no effect in any test while PS-NH_2 displayed cytotoxicity in most cell models. CD34-DCs was the most sensitive cell model tested. The tetrazolium-based MTS assay was the most robust assay in our study, applicable to all cell models investigated. Therefore, this assay could become an integral part of a NP testing strategy. Other assays might be also useful, depending on the cell model or the type of investigation.

Keywords: Nanoparticles, cytotoxicity, cell viability, reproducibility

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1. Introduction

The development of *in vitro* methods for toxicological testing of nanoparticles (NPs) is a major research endeavor. NPs can be synthesized in many different variants by varying size, shape or by applying chemical surface modifications. Therefore in order to save costs and time and to reduce animal testing reliable *in vitro* methods are urgently needed to screen for hazardous NPs, to prioritize NPs for further testing and also to obtain initial data for risk assessment. Most toxicological endpoints require prior knowledge of the acute toxicity of the test compound. Many pathways of (regulated) cell death have evolved in multicellular organisms. Some can be specific for certain cell types and/or stimuli, including several types of regulated necrosis and apoptosis (Galluzzi et al. 2014; Green et al. 2014). While mechanistic insight is important in some cases, a mechanism-independent measurement often gives an initial starting point for more elaborate testing. *In vivo,* many forms of cell death (e.g. apoptosis) lead to early removal of the dying cells by macrophages. However, *in vitro*, in the absence of macrophages, all forms of cell death will eventually cause loss of integrity of the cellular membrane. Certain dyes such as trypan blue and propidium iodide will cross only compromised membranes and can be used to specifically stain dead cells. In addition, the concomitant release of cytosolic enzymes into the cell culture medium can be used as a read-out since the released enzyme activity correlates with the extent of membrane rupture and therefore is a measure for cell death. Most often lactate dehydrogenase (LDH) activity is measured in cell culture medium using the NADH-dependent conversion of a dye (Korzeniewski and Callewaert 1983). Another approach is the measurement of the number of viable cells after treatment. Arguably the most popular method is based on the reduction of a tetrazolium salt to a colored formazan salt derivative by cellular NAD(P)H-dependent oxidoreductase enzymes, introduced by Mosmann (1983). The reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) represents a measure for the cellular metabolic capacity and correlates with the number of cells (Mosmann 1983). Oxidoreductases responsible for the dye reduction are mainly located in the cytoplasm, *i.e.* in the endosomal/ lysosomal compartment and at the plasma

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membrane, and are also partly associated with oxidative phosphorylation in mitochondria (Stepanenko and Dmitrenko 2015). Meanwhile a couple of assays using similar dyes such as MTS, WST or XTT are available as well. In some forms of cell death such as apoptosis mitochondria play an important role. During apoptosis cellular proteins involving the pro-apoptotic bcl-2 family disrupt mitochondrial function (Galluzzi et al. 2014; Green et al. 2014). In other forms of cell death such as necrosis, loss of mitochondrial function and of oxidoreductases is a later event coinciding with plasma membrane rupture (Aki et al. 2015; Green et al. 2014). Thus, dyes, that accumulate in mitochondria to test their function can be used as well to assess cell viability (Petronilli et al. 2001). Finally, metabolic capacity can also be measured by determination of the intracellular ATP content using luciferase or other ATP consuming reactions (Kangas et al. 1984). Viable cells under normal culture conditions maintain a relatively constant amount of ATP. It is assumed that the capacity for ATP synthesis is lost early during cell death and remaining ATP is rapidly used up or eliminated by ATPases.

For cytotoxicity measurements it would be optimal to use non-dividing cells as otherwise it may become difficult to distinguish effects on cell viability from effects on cell proliferation. However, often this is not possible. Thus, it is very important to understand and to precisely describe how a particular assay is performed, as this affects the interpretation of the results. Furthermore, cytotoxicity should be assessed under similar conditions (*i.e.* concentrations, time points) as other toxicological endpoints of interest, which might be exploited later on in the test strategy.

It should be noted that specific issues arise when testing cytotoxic effects of NPs *in vitro*. In particular, enzyme- and dye-based assays need to be carefully checked for possible interferences from NPs. Assay reagents might react or be immobilized on the NP's surface. Especially assays using spectrophotometric read-out might be impaired as NPs can scatter or absorb light or exhibit interfering plasmon resonance (Bonvin et al. 2017; Kroll et al. 2012; Monteiro-Riviere et al. 2009; Stone et al. 2009; Tournebize et al. 2013). Several consortia have already worked on evaluation and harmonization of cytotoxicity assays for NPs (Elliott

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et al. 2017; Piret et al. 2017; Rösslein et al. 2015; Xia et al. 2013). Moreover, a standard assay specifically dedicated to NPs is currently in preparation using the MTT variant 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay as an *in vitro* cytotoxicity assay (ISO/AWI 19007 Modified MTS assay for measuring the effect of NPs on cell viability, ISO, Geneva, Switzerland).

In this study we decided to further explore three assay types. As a late response assay the LDH assay was chosen. For an earlier response assay the MTS assay was chosen. Finally, ATP level measurements were selected as an early marker of cellular stress.

In toxicology, automated testing based on high-throughput screening (HTS) or high-content screening (HCS) is becoming increasingly important (Hartung 2008, Judson et al. 2013). It may be considered that in the future HTS/ HCS might be an integral part of the decision-making tools used routinely for the toxicity screening of chemicals, as well as NPs (Judson et al. 2013). Thus, another goal of this study was to identify assays which can be used in HCS approaches to investigate the cytotoxicity of NPs. The cell membrane impermeable TOPRO nucleic acid stain may be considered as an LDH equivalent assay for an HCS approach (Anguissola et al. 2014). As an HCS equivalent for the MTS assay cyanine monomers as nucleic acid counterstains such as TO-PRO-1/3/5 and similar dyes may be considered (Anguissola et al. 2014). For the ATP assay we have not identified an HCS equivalent.

To generate data relevant to toxicological problems, the route of exposure should be taken into account. In terms of potential NP hazards, inhalation has been identified as the most important exposure route. Experimental studies suggest that NPs can cross from the lung into the bloodstream (Balasubramanian et al. 2013; Kendall and Holgate 2012; Kreyling et al. 2014). Cutaneous and oral exposure to NPs, which may be a fraction of bulk materials and not necessarily specifically engineered NPs, are already commonplace for some materials such as silica and titanium dioxide (Peters et al. 2012; Weir et al. 2012). Historical data for oral intake of these materials show no adverse effects (Lewinson et al. 1994; Martin 2007; Skocaj et al. 2011). Dermal penetration is considered negligible at least for healthy skin and for the most commonly used metal and metal oxide NPs (Labouta and Schneider 2013;

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Nohynek and Dufour 2012; Piret et al. 2014). In the case that NPs breach the barriers of the first-line exposure organs, blood and lymphatic system are usually the first compartments reached, prior to secondary organs such as liver and kidney. In order to take into account different possible routes of exposure we tested several widely used cell lines representing first-line exposed organs, skin, intestinal tract and respiratory tract, and also the immune system. A549 and NCI-H411 are epithelial cells derived from human, male, lung carcinomas, and were used to represent lung epithelial models. A549 cells have an alveolar type II-like phenotype (Foster et al. 1998) and NCI-H411 exhibit both alveolar (i.e. type II cell) and bronchiolar (i.e. club cell) epithelial phenotype (Hermanns et al. 2004; Salomon et al. 2014). Caco-2 cells are epithelial cells derived from human, male, colorectal adenocarcinoma. These cells can undergo enterocytic differentiation (Jumarie and Malo 1991; Piret et al. 2012) and represent the gastro-intestinal tract in this study. THP-1 cells are monocytes derived from human, male, acute monocytic leukaemia, and can be differentiated into macrophage-like cells (Chanput et al. 2014).

Generally, primary cells are considered to closer resemble the *in vivo* phenotype while cell lines are mostly of tumor origin and/or virus-transformed. Therefore, in this study we also included two primary cell types. Normal human epidermal keratinocytes (NHEK) from a pool of three donors were used as skin *in vitro* model. CD34 dendritic cells (CD34-DCs) were derived through differentiation of progenitor cells from different human cord blood donors. Dendritic cells are part of the immune system; they process antigen material and present it on the cell surface to the T cells of the immune system. However, when using primary cells in toxicology one needs to consider that access to them is often limited and variability in test results will be higher due to donor variability.

In addition, we included a few more complex models of the target organs such as 3D models and co-cultures. The use of complex cellular models has been discussed for some time as superior to the single layer dish culture (Guguen-Guillouzo et al. 1983). For their use in toxicology it is expected that such models could yield more relevant data requiring less modelling or extrapolation (Nam et al. 2015). Thereby, they are assumed to more reliably

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identifying a human hazard (Grainger 2014). As a more complex model we employed a commercially available reconstituted human epidermis (RHE) produced from a pool of human keratinocytes from different donors. These RHE are characterized by the presence of the 4 typical epidermis layers (basal, spinous, granular, and topical cornified layer) observed in *in vivo* epidermis and express specific differentiation markers such as keratin 14, keratin 10 or involucrin (Poumay et al. 2004). RHE were treated at the air-liquid interface. Furthermore, Caco-2 cells were differentiated on filters forming continuous polarized monolayers, and acquire several of the characteristics of normal enterocytes (Hillgren et al. 1995; Volpe 2008). Co-cultures of aforementioned A549 and NCI-H411 lung epithelial cells with THP-1 cells differentiated into macrophage-like cells were used to represent a situation more close to the lung surface (Hjort et al. 2003; Wottrich et al. 2004).

All cell models employed in our study are listed in Supplemental Table 1. The selection of cell models was based on a literature survey, initial experiments, as well as on availability and experience in the participating laboratories. The aim of our study was to investigate the applicability of three cytotoxicity assays in a variety of different cell models representing the most important target organs for NPs. Amine-modified polystyrene NPs (PS-NH_2) and carboxyl-modified polystyrene NPs (PS-COOH) were used as 'model' NPs as they exhibit narrow size ranges and low agglomeration (Bexiga et al. 2014; Kim et al. 2013; Wang et al. 2013). Moreover, the cationic PS-NH_2 have been demonstrated to induce cytotoxicity at doses below 50 μg/ml (Anguissola et al. 2014; Bexiga et al. 2011; Ruenraroengsak et al. 2012; Wang et al. 2013; Xia et al. 2008). We have developed standard operation procedures (SOPs) and discussed some limitations and pitfalls. All SOPs are included in the Supplementary Information.

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2. Material and methods

2.1. Chemicals and nanoparticles

Benzalkonium chloride (BC, Sigma-Aldrich), known to induce cell death in multi-layered engineered tissues like RHE and in differentiated Caco-2 monolayers was used as positive control (Supplemental Table 2) (Coquette et al. 2003; Piret et al. 2012; Vankoningsloo et al. 2010). Zinc sulphate (Sigma-Aldrich) and Triton X-100 were used as positive chemical control for CD34-DCs or A549/THP-1 cells respectively (Supplemental Table 2). Monodisperse aqueous dispersions of polystyrene (PS) NPs with amine (PS-NH_2) and carboxyl (PS-COOH) surface functionalization (nominal size of 50 nm diameter) were obtained from Bangs Laboratories (catalog number PA02N) and Polysciences, Inc. (catalog number 15913-10), respectively. These NPs can be easily dispersed by simple vortexing as has been assured in an accompanying round robin using Dynamic Light Scattering (DLS) and Differential Centrifugal Sedimentation (DCS) (Langevin et al., under review). NPs were dispersed in cell culture media by vortexing for 30 sec immediately prior to experiments. Particle size distributions in water and in cell culture medium with FCS were determined using Dynamic Light Scattering (Langevin et al., under review).

2.2. Cell culture

2.2.1. A549 cell line

Human lung epithelial A549 cells (obtained from LGC Standards, catalog number: ATCC® CRM-CCL-185™) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v) heat-inactivated (20 min, 56°C) fetal calf serum (FCS) (Sigma-Aldrich), 1% (v/v) penicillin-streptomycin (Invitrogen). Cells were seeded at 10^4 cells/well in 96-well plates 42 h prior to exposure.

2.2.2. NCI-H411 cell line

Human lung epithelial NCI-H411 (catalog number: ATCC® HTB-174™) cells obtained from LGC Standards were cultured in RPMI 1640 medium (Gibco) supplemented with 10% (v/v)

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heat-inactivated FCS (Sigma-Aldrich), 1% (v/v) penicillin-streptomycin (Invitrogen). Cells were seeded at 10^4 cells/well in 96-well plates 42 h prior to exposure.

2.2.3. Naïve Caco-2 cell line and differentiated monolayers

Human colon carcinoma Caco-2 cells were purchased from European Collection of Cell Cultures (ECACC, Salisbury, UK, catalog number: ECACC 86010202). Caco-2 cells were maintained in culture in 75-cm^2 polystyrene flasks (Costar) with DMEM High Glucose (Gibco) containing 100 U/ml penicillin, 100 µg/ml streptomycin (BioWhittaker), 1% (v/v) non-essential amino-acids (Gibco) and 10% FCS (Gibco).

To obtain a tight intestinal epithelium, Caco-2 cells were differentiated as described previously (Piret et al. 2012). Briefly, 50,000 Caco-2 cells were grown on transwell polycarbonate filters (12 wells, pore diameter of 3 µm, 1.12 cm^2 growth area, Corning Costar) for 16 days. Medium (500 µL in the insert -apical side-, and 1.5 mL in the well -basolateral side) was renewed every 48 h. The tightness of the intestinal monolayers was controlled by transepithelial electrical resistance (TEER) measurement, performed with probes connected to a Millicell-ERS Volt-Ohm meter (Millipore). TEER data were calculated by subtracting the value of blank inserts and normalized for growth area ($\Omega.cm^2$). For toxicity assays, intestinal epithelia were incubated for 24 h with 500 µL of complete DMEM containing increasing concentrations of PS-NH_2.

2.2.4. THP-1 cell line-derived macrophages and co-culture with lung epithelial cells

Human acute monocytic leukaemia THP-1 cells (obtained from LGC Standards, catalog number: ATCC® TIB-202™) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FCS (Sigma-Aldrich), 1% L-glutamine, 1% penicillin/streptomycin (Invitrogen), 1% Hepes. THP-1 cells (non-adherent monocytic cells) were seeded at 8×10^4 cells/well in a 96-well plates and differentiated in macrophage-like adherent cells according to the method previously described (Lanone et al. 2009) with 30 ng/mL of phorbol-12-myristate 13-acetate (Sigma-Aldrich) in RPMI medium 40 h prior to exposure. For co-culture, differentiated THP-1 cells were detached with 0.25% trypsin and seeded for 2 h on top of epithelial cells at a ratio of 1 macrophage for 2 epithelial cells on the day of exposure.

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2.2.5. Normal human epidermal keratinocytes (NHEK)

Neonatal NHEK (pool of three donors) were purchased from Lonza (catalog number: 192906). NHEK were grown in 75 cm^2 flasks (Corning) to 80% confluence in presence of keratinocytes SFM medium supplemented with bovine pituitary extract and recombinant human epidermal growth factor (Gibco). Importantly, all steps were performed at 4°C when splitting NHEK in order to prevent differentiation.

2.2.6. Reconstituted human epidermis (RHE)

RHE (EPI/001) differentiated for 14 days and the related culture media were purchased from StratiCELL s.a. (Les Isnes, Belgium). RHE were produced from a pool of human keratinocytes from different donors seeded on a 0.63 cm^2 polycarbonate filters and grown at the air-liquid interface. RHE were maintained in culture according to the manufacturer's recommendations.

2.2.7. Primary CD34-derived dendritic cells (CD34-DCs)

$CD34^+$-progenitor-cell isolation and culture procedures have been described previously (Nelissen et al. 2009). Briefly, human cord blood samples were collected from umbilical blood vessels of placentas of full-term infants, born at the Heilig Hart hospital in Mol and the St. Dimpna hospital in Geel, Belgium. Informed consent was given by the mothers and the study was approved by the ethical commission of both hospitals. Mononuclear cells were separated from the cord blood by density gradient centrifugation (Ficoll-Paque™ plus, GE Healthcare). Subsequently, $CD34^+$ progenitor cells were extracted using positive immunomagnetic selection (EasySep® human CD34 positive selection kit, Stemcell Technologies, Grenoble, France) according to the manufacturer's guidelines. The progenitor cells were cultured for 12 days in Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 10% fetal bovine serum (PAA laboratories, Pasching, Austria), 2% penicillin/streptomycin (Gibco) and 1% bovine serum albumin (Sigma-Aldrich) in the presence of TNF-α (250 U/ml, Roche applied science), granulocyte macrophage colony-stimulating factor (5000 U/ml; Gentaur, Brussels, Belgium), stem cell factor (5 U/ml,

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Biosource, Nivelles, Belgium) and interleukin 4 (1000 U/ml, Biosource) to induce proliferation and differentiation towards immature CD34-DCs.

All cell lines and complex *in vitro* models were maintained at 37°C in a 5% CO_2 incubator with humidified air.

2.3. Cytotoxicity testing

The detailed SOPs for MTS, LDH and ATP assays are included in supplemental files.. Cells were seeded as described above and treated with NPs in concentrations ranging from 1-100 µg/ml, corresponding solvents or control chemicals, or left untreated for a period of 24 h. Afterwards the different assays were applied. We have carefully checked for possible interferences for each of the assays, instructions are included in the SOPs.

2.3.1. MTS assay

For the MTS assay CellTiter 96® AQueous One Solution Reagent from Promega was used. After 24 h treatment with NPs, the medium was removed from each well and replaced by MTS solution, which has been prepared according to manufacturer instructions. The plate was then incubated at 37°C in a cell incubator for another 1 h. The absorbance was read at 490 nm. This SOP has been tested in an inter-laboratory comparison study (Nelissen et al, under review).

2.3.2. LDH assay

For the LDH assay, CytoTox-ONE™ Homogeneous Membrane Integrity Assay from Promega was used. After 24 h treatment, the medium was collected from each well and centrifuged at 15,700×*g* for 30 min to remove potentially interfering NPs and also cell debris. Afterwards 100 µl cell supernatant was mixed with 100 µl CytoTox-ONE™ Reagent, incubated for 10 min at room temperature. Then, 50 µl stop solution was added and fluorescence was recorded within one hour with an excitation wavelength between 530-570 nm and an emission wavelength between 580-620 nm.

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2.3.3. ATP assay

For this purpose, ATPlite™ kit (PerkinElmer) was used according to the manufacturer's instructions. Additionally, total cellular protein content was assessed according to the Bradford method (BioRad) (Bradford 1976), and the ATP content normalized to it.

2.4. Data analysis

Half maximal effective doses (ED_{50} values) were determined by curve fitting performed in the R statistical computing environment (R Development Core Team, 2009) in combination with the drc package (Ritz and Streibig, 2005) using the five parametric logistic function. Mean ED_{50} values were calculated from the averages of the logarithm of the ED_{50} of independent biological experiments. Upper and lower bounds correspond to the standard deviation of the logarithmic mean. Student's *t*-test with a significance cut-off level *p* of 0.05 was used to compare mean values of NP-treated with those of untreated cells.

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3. Results

In this study we evaluated the suitability of three different cytotoxicity assays for NP testing. We included six different single cell models and four complex cell models (Supplemental Table 1).

3.1. Skin cell models

For the skin models we used NHEK and RHE and applied three different assays (*i.e.* MTS, LDH and ATP assay). The quaternary ammonium compound and surfactant BC was used at 0.005 mg/mL (NHEK, MTS and LDH assays; 0.0025°mg/mL ATP assay) or 1 mg/mL (RHE) as a positive control and quality control to verify the performance of the assays (Supplemental Table 2).

In NHEK cells, we detected a clear dose dependent effect of PS-NH_2 on cell viability using the ATP and MTS assays (Figure 1 A and B), with ED_{50} values of 37 µg/mL and 25 µg/mL, respectively (Table 1). For PS-COOH we could not observe any effect for all the tested concentrations. Please note, that although the manufacturer of the ATP assay recommended the normalization of the results using the sample's protein content, in our experience normalization introduced high variation in the results as seen for NHEK (Supplemental Figure 1A). Therefore, and because plated cell numbers were tightly controlled in the experiments, the ATP assay results are reported without such normalization. However, reduced ATP levels and reduced cell numbers cannot be distinguished in our results. The LDH assay proved to be less sensitive and a cytotoxic effect was only detected for PS-NH_2 at a dose of 100 µg/mL (Figure 1C).

In parallel, *in vitro* RHE were treated with PS-NH_2 or BC at the air-liquid interface. As the use of RHE was cost intense and all previous tests using PS-COOH on NHEK did not show an effect on viability, only PS-NH_2 at a dose of 31.25 µg/cm^2 (equivalent to 100 µg/mL inducing the highest toxic effect on NHEK, Figure 1) was tested. NPs were added topically as a liquid dispersion on the cornified layer and incubated for 24 h. Phosphate Buffered Saline (PBS) solution was used to evaluate the potential effect of a buffered liquid on RHE viability. No toxic effect of PS-NH_2 on epidermises was detected using either the MTS or LDH assay

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(Figure 2), while the positive control BC caused a dramatic loss of epidermis viability measured with both assays (Supplemental Table 2).

3.2. Lung cell models

As a model for lung exposure we used two lung related cells lines (A549 cells or NCI-H411 cells) in monoculture as well as in co-culture with THP-1-differentiated macrophages. Triton X-100 was used as a positive control in the lung cell experiments (Supplemental Table 2). The lung cells models alone (*i.e.* A549 or NCI-H441 cells) reached ED_{50} values of 117 µg/mL and 77 µg/mL in the MTS assay, respectively (Figure 3, Supplemental Figure 2, and Table 1). In contrast, monocultures of THP-1 cells were observed to be more sensitive with an ED_{50} of 54 µg/mL in the MTS assay (Table 1). Co-cultures showed similar effects compared to the lung cell models (i.e. A549 and NCI-H441) alone with ED_{50} values of 112 µg/mL and 64 µg/mL in the MTS assay, respectively (Table 1). Similar results were derived in the ATP and the LDH assays (Figure 3, Supplemental Figure 2, and Table 1). In all assays THP-1 cells alone were the most sensitive cells. In addition, PS-COOH were tested in the three assays and the five airway cell models, and no cytotoxicity was detected (Figure 3, Supplemental Figure 2).

3.3. Intestinal cell models

The response of Caco-2 cells to NPs was tested using undifferentiated and differentiated cells as intestinal model, representing oral intake. BC was used at as positive control (Piret et al. 2012) to verify the performance of the assays (Supplemental Table 2). In addition, TEER was measured on differentiated Caco-2 cells to evaluate the effect of NPs on the intestinal epithelium integrity.

Undifferentiated Caco-2 cells exhibited a dose dependent loss of viability after PS-NH_2 exposure with an ED_{50} of 73 µg/mL in the MTS assay and a somewhat higher ED_{50} of 103 µg/mL in the ATP assay (Figure 4A and B, Table 1). PS-COOH did not show any cytotoxicity in both assays (Figure 4A and B). Using the LDH assay, low levels of fluorescence signals and high background were encountered with this cell line.

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Recommended changes to the protocol to overcome these issues such as reduction of serum (that may contain LDH) to reduce high background and the use of pyruvate-free medium together with an increase of incubation time and temperature to reduce low overall fluorescence signals were unsuccessful. Therefore, no LDH assay results were obtained for this cell line.

The impact of PS-NH_2 on viability and integrity of intestinal Caco-2 monolayers obtained after differentiation was evaluated by the MTS assay and measurement of the TEER, respectively. In order to respect the same NP doses/cm² used with undifferentiated Caco-2 cells during viability assays (using 96-well plates), intestinal monolayers (using transwells) were exposed to PS-NH_2 concentrations between 70 µg/mL (*i.e.* 100 µg/mL on undifferentiated Caco-2 cells, corresponding to 31.25 µg/cm² in both the cases) and 0.7 µg/mL (*i.e.* 1 µg/mL on undifferentiated Caco-2 cells, corresponding to 3.125 µg/cm² in both the cases). No effect on intestinal epithelium viability and integrity was observed with PS-NH_2 independently of the concentration (Figure 4B and C, respectively), while BC reduced viability to 20% and caused complete breakdown of the electrical resistance (Supplemental Table 2). PS-COOH were not tested since no effect was seen on naïve Caco-2 cells (Figure 4A and B).

3.4. Immune cell models

In addition to the THP-1 cells discussed earlier (Figure 3, Supplemental Figure 2), CD34-DCs prepared from different human cord blood donors were investigated as models of the immune system. As expected, donor variability had a strong impact on the quality of the assay results. However, similar trends to THP-1 cells were observed as PS-NH_2 induced cytotoxicity at low doses for some donors (Figure 5). Zinc sulphate (144 nM) was used as a positive control showing less than 20% cell viability or of ATP content compared to untreated controls cells in all donors (Supplemental Table 2).

The average ED_{50} for PS-NH_2 in the ATP assay was 26 µg/mL (Figure 5A and Table 1), and for the MTS assay the ED_{50} was 82 µg/ml (Figure 5B and Table 1). LDH release was observed only at doses of 100 µg/mL (Figure 5C and Table 1), which was not significant. In all cases, PS-COOH did not display any cytotoxic effect to the cells (Figure 5).

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4. Discussion

We compared three cytotoxicity assays (MTS, ATP, and LDH assays) that cover different biological principles (redox metabolism, ATP content, membrane permeability) and different read-out principles (absorption, fluorescence and luminescence) on different cell models (Supplemental Table 1). The cell models covered the main routes of exposure to NPs (cutaneous exposure, ingestion, inhalation, and *i.v.* injection) and therefore represented the most important target organs. PS-NH_2 and PS-COOH were used as an example of ideal NPs as they were available with a perfect spherical shape with a very narrow size distribution and were easy to disperse by vortexing yielding stable dispersions. Moreover, previous studies already suggested that PS-NH_2 induce cytotoxicity and PS-COOH are of low or no cytotoxicity (Anguissola et al. 2014; Bexiga et al. 2011; Ruenraroengsak et al. 2012; Wang et al. 2013; Xia et al. 2008). Indeed, in this study all assays showed that PS-COOH had no effect on the cell viability within the tested dose range up to 100 µg/mL in none of the cell models. In contrast, PS-NH_2 induced a dose-dependent cytotoxicity in most cell models tested in all assays. We thoroughly tested for possible interference of the NPs in all the test assays and we could confirm absence of interferences. Therefore, our data confirm that PS-COOH and PS-NH_2 are very good candidates for negative and positive control nanomaterials, respectively, for *in vitro* cytotoxicity measurements.

The ATP assay was the most sensitive assay, yielding the lowest ED_{50} values in most cell models. However, ED_{50} values of the MTS assay were often in the same range as those of the ATP assay. This might be related to the fact that ATP results were not normalized by the protein content. Importantly, for a conclusion on general cytotoxicity such a conflation is unproblematic provided that cells were not significantly proliferating during the treatment period. Therefore, the MTS and the ATP assays both appear suitable for cytotoxicity testing of NPs as previously described (Piret et al. 2017). While the LDH assay also indicated cytotoxicity in most cell models it proved to be the least sensitive assay. Often ED_{50} values were not reached within the tested dose range. These results are in line with the biological basis of the methods which suggests ATP levels to be affected earliest, NAD(P)H

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oxidoreductases somewhat later and loss of membrane integrity as a late effect. However, some issues of high background and low overall levels of fluorescence signals in the LDH assay were encountered with certain cell models, *i.e*. Caco-2 cells and CD34-derived dendritic cells. These render this assay not recommendable for those cell models.

The two tissue models involving monocultures of differentiated cells, *i.e.* reconstituted epidermis and differentiated (intestinal) Caco-2 cell monolayer were unaffected by PS-NH_2 in the tested dose range regardless of the type of viability assay. This was in contrast to the results obtained in the respective cell models that were used to grow these more complex models, *i.e.* NHEK and Caco-2 cells. *In vitro* reconstituted human epidermises exhibit the barrier function of cornified cells, emphasizing the excluding/protective role of the upper layer of the epidermis in case of acute exposure to PS-NH_2 The susceptibility of NHEK cells indicated a potential toxic effect in case of a breach of the barrier function. In the case of differentiated Caco-2 cells (Jumarie and Malo 1991), the change of cellular features such as cell polarity, microvilli formation, tight junctions, domes and increased brush-border membrane enzymatic activities, and the secretion and assembly of an extracellular matrix because of the long undisturbed culture period might be responsible for protecting them from the cytotoxic effect of PS-NH_2 that was observed in the undifferentiated counterparts. In addition, the integrity of the intestinal epithelia generated by the Caco-2 differentiation was not affected by the NPs as confirmed by TEER measurements. All together, these data underline the importance of considering more complex cell models in the evaluation of NP toxicity as differences were observed depending on the differentiation status and complexity of cell models.

Cells of the immune system are expected to be among the first cells to encounter NPs after they breach barriers and become systemically available. Here we tested two types of models for immune cells, phorbol ester-primed THP-1 monocytes and CD34-DCs. Both cell types showed a higher sensitivity towards PS-NH_2 compared to the epithelial cell types. However, in a co-culture of THP-1 cells with lung cells the co-cultures exhibited ED_{50} values more comparable to the lung epithelial cells alone, arguing that within the incubation time the effect

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of the immune cells is rather low (Loret et al. 2016). However, immune cells, especially macrophages, can elicit a so-called respiratory burst during which they release reactive oxygen species and cytokines into the surrounding tissue (Forman and Torres 2002). The respiratory burst has been observed in response to NP treatment (Cruz et al. 1997; Prietl et al. 2014). Prolonged exposure and hence release of reactive oxygen species and cytokines can lead to inflammation. Upon chronic stimulation this might lead to tissue scarring and reduced organ function and possibly tumorigenesis (Babior 2000). This argues, that macrophages should always be included when testing NPs because of the necessity to record possible respiratory burst reactions at least for lung epithelial models.

Table 2 summarizes the findings of this study with respect to the different cell models. Overall, we achieved to develop robust SOPs for all three assays, *i.e.* MTS, ATP and LDH assays, which have been tested for a variety of important cell models. However, several possible improvements of these SOPs can be considered in future. Improvements could concern the exposure of the NPs, such as pre-incubation of the NPs with a more realistic biofluid prior to cell treatments. For instance, surfactant can be used in the case of lung models (McKenzie et al. 2015; Raesch et al. 2015) and for oral uptake the different consecutive pH values encountered along the stages of the gastro-intestinal tract can be simulated (Peters et al. 2012). Furthermore, skin and lung models may be exposed at the air-liquid interface (Stoehr et al. 2015) while ingestion models could include a more realistic digestion fluid on one side. Finally, cell models are being refined, and new developments such as microfluidic systems and organoids promise to allow more realistic models and to eliminate the species differences in toxicological data by using human cells (Esch et al. 2014; Tralau et al. 2015).

5. Conclusions

All assays investigated here should be applicable for all NPs when known issues of interference and artefacts have been controlled for (Bonvin et al. 2017; Kroll et al. 2012; Monteiro-Riviere et al. 2009; Stone et al. 2009; Tournebize et al. 2013). In summary, the MTS assay was applicable for all tested cell models, including primary cells and more

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complex models and therefore represents the most robust and reproducible assay out of the three tested assays. Thus, our data confirm previous findings that the MTS assay should be integrated in an *in vitro* NP test strategy (Piret et al. 2017). However, other assays can be useful as well, depending on the NP type and the cell model. The ATP assay proved to be more sensitive in several cell models investigated here and thus could be useful in a test strategy too, provided that the issues with normalization can be overcome. Our data also show that combining different assays, which represent different biological read-out gives a more complete picture especially on the chronological sequence of different events and allows already first conclusions on possible underlying mechanisms of cytotoxicity. Furthermore, our data suggest that PS-COOH and PS-NH_2 may be used as negative and positive control NPs, respectively, for NP cytotoxicity testing.

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8. Declaration of Interest

The authors declare no competing interests.

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9. List of abbreviations

BC:	benzalkonium chloride
CD34-DCs:	CD34-derived dendritic cells
FCS:	fetal calf serum
HTS:	high-throughput screening
HCS:	high-content screening
ISO:	International Organization for Standardization
LDH:	lactate dehydrogenase
MTS:	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NP:	nanoparticle
NHEK:	normal human epidermal keratinocytes
PS-COOH	40 nm carboxyl-modified polystyrene nanoparticles
PS-NH_2	50 nm amine-modified polystyrene nanoparticles
RHE	reconstituted human epidermis
SOP	standard operation procedure
TEER	transepithelial electrical resistance

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11. Tables

Table 1: Cytotoxicity of PS-NH_2 in different cell models. Average ED_{50} values and lower and upper bounds in µg/mL.

	ATP			MTS			LDH		
	avg	lower	upper	avg	lower	upper	avg	lower	upper
NHEK	37	27	52	25	23	28			
A549	40	22	71	117	92	149	155	143	167
A549 + THP-1	47	24	90	112	95	133	191	165	220
NCI-H411	65	58	71	77	73	81	104	96	113
NCI-H411 + THP-1	52	46	59	64	58	71	120	94	153
Caco-2	103	101	106	73	62	85			
CD34-DCs	26	13	53	82	38	174			
THP-1	23	12	42	54	43	68	96	41	218

Table 2: Cytotoxicity of PS-COOH and PS-NH_2 in the different cell models. Negative: no cytotoxicity observed up to 100 µg/mL; positive: average ED_{50} determined (see Table 1), n.d.: not determined.

	PS-COOH			PS-NH_2		
	ATP	MTS	LDH	ATP	MTS	LDH
NHEK	negative	negative	negative	positive	positive	positive
RHE	n.d.	n.d.	n.d.	n.d.	negative	negative
A549	negative	negative	negative	positive	positive	positive
A549 + THP-1	negative	negative	negative	positive	positive	positive
NCI-H411	negative	negative	negative	positive	positive	positive
NCI-H411 + THP-1	negative	negative	negative	positive	positive	positive
Caco-2	negative	negative	n.d.	positive	positive	n.d.
Differentiated Caco-2	n.d.	n.d.	n.d.	n.d.	negative	n.d.
CD34-DCs	negative	negative	negative	positive	positive	n.d.
THP-1	negative	negative	negative	positive	positive	positive

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12. Figure legends

Figure 1

Cytotoxicity of surface-modified polystyrene nanoparticles in normal human epidermal keratinocytes.

Cells were exposed to increasing concentrations (0, 1, 10, 25, 50 and 100 µg/ml) of PS-COOH (filled symbols) or PS-NH_2 (open symbols) for 24 h. Effects on cells were assessed using the (A) ATP assay, (B) MTS assay, and (C) LDH release assay. Results were expressed as percentages (ATP –A- and MTS –B- assays) or as fold induction levels of control cells (LDH assay). For the ATP assay and the LDH release assay, 2 independent experiments were performed each (diamonds and triangles, respectively); individual data points are shown. The MTS assay was performed in 3 independent experiments; mean values with standard deviation are shown (circles). Curves are regressions over mean values.

Figure 2

Cytotoxicity of amine-modified polystyrene nanoparticles in reconstituted human epidermis (RHE).

RHE were incubated for 24h with an equivalent volume of vehicle (phosphate buffered saline, PBS), 1 mg/ml benzalkonium chloride (BC, positive control), or 31.25 µg/cm^2 PS-NH_2 for 24 h. Effects on cells were assessed using the (A) MTS assay, and (B) LDH release assay. Results expressed as percentages (MTS assay) of untreated control RHE or as fold induction levels of control RHE (LDH assay). For the MTS assay and the LDH release assay one experiment was performed each, error bars represent the standard deviation of technical replicates.

Figure 3

Cytotoxicity of surface-modified polystyrene nanoparticles in A549 cells, THP-1 cells and A549/THP-1 co-cultures.

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Cells were exposed to increasing concentrations (0, 1, 10, 25, 50 and 100 µg/ml) of PS-COOH (filled symbols) or PS-NH_2 (open symbols) for 24 h. Effects on cells were assessed using the (A) ATP assay, (B) MTS assay, and (C) LDH release assay. Results of the ATP and MTS assays expressed as percentages of untreated control; LDH release was normalized to maximum activity after Triton X-100 treatment and expressed as percentages of untreated control. Mean values of 3 independent experiments each with standard deviation are shown. Curves are regressions over mean values.

Figure 4

Cytotoxicity of surface-modified polystyrene nanoparticles in Caco-2 cells and differentiated Caco-2 cells.

Cells were exposed to increasing concentrations (0, 1, 10, 25, 50 and 100 µg/ml) of PS-COOH (filled symbols) or PS-NH_2 (open symbols) for 24 h. Effects on cells were assessed using the (A) ATP assay, (B) MTS assay, and (C) TEER assay. Results were expressed as percentages of untreated control. For the naïve Caco-2 cells (diamonds) 3 independent experiments were performed each; mean values with standard deviation are shown. For the MTS assay and TEER measurement on differentiated Caco-2 cells (triangles) one experiment of each is shown. Curves are regressions over mean values.

Figure 5

Cytotoxicity of surface-modified polystyrene nanoparticles in CD34-derived dendritic cells (CD34-DCs).

Cells were exposed to increasing concentrations (0, 1, 10, 25, 50 and 100 µg/ml) of PS-COOH (filled symbols) or PS-NH_2 (open symbols) for 24 h. Effects on cells were assessed using the (A) ATP assay, (B) MTS assay, and (C) LDH release assay. Results were expressed as percentages (ATP –A- and MTS –B- assays) of non-treated cells or as fold induction levels of control cells (LDH assay). Mean values of 3 independent experiments each with standard deviation are shown. Curves are regressions over mean values.

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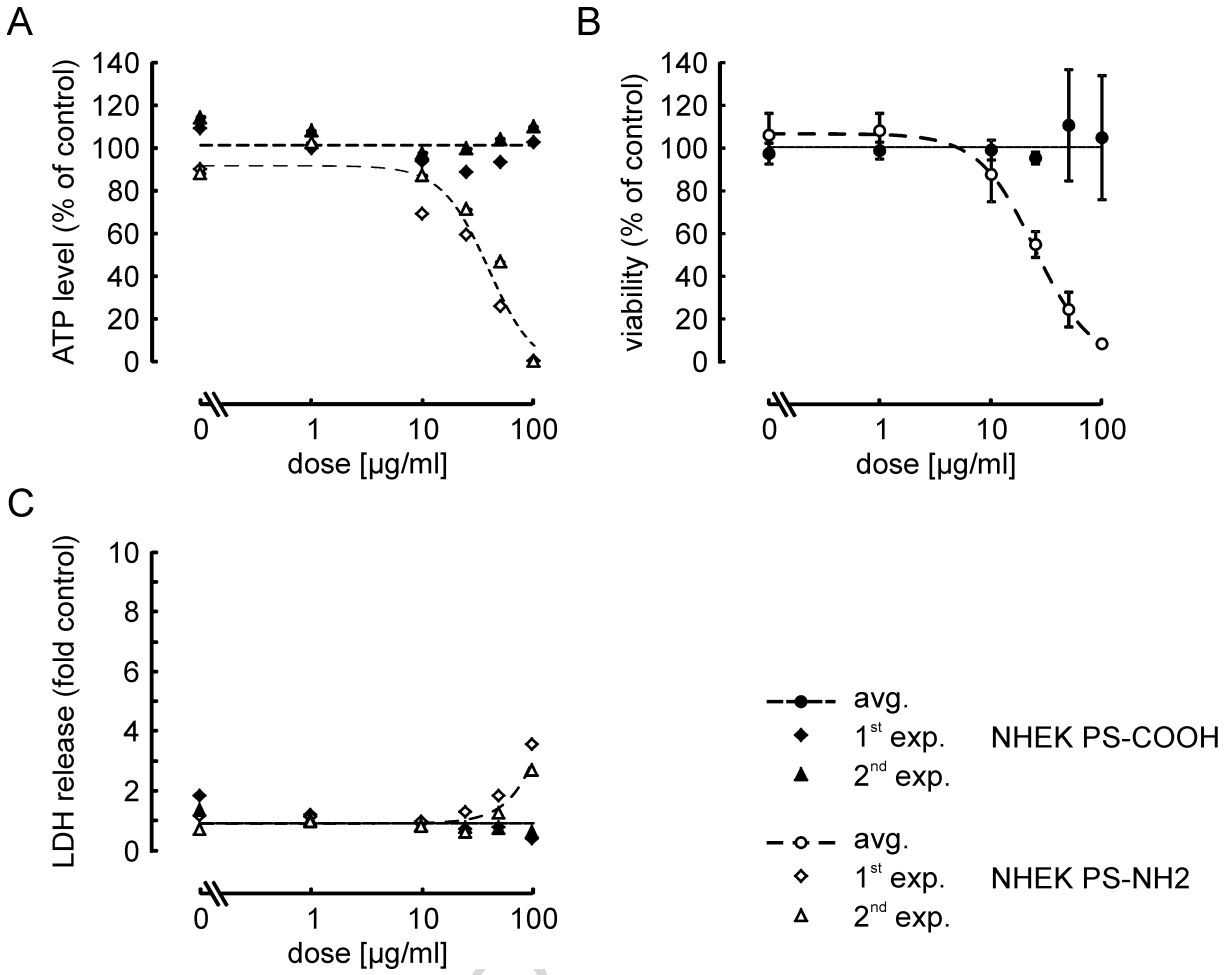

Fig. 1

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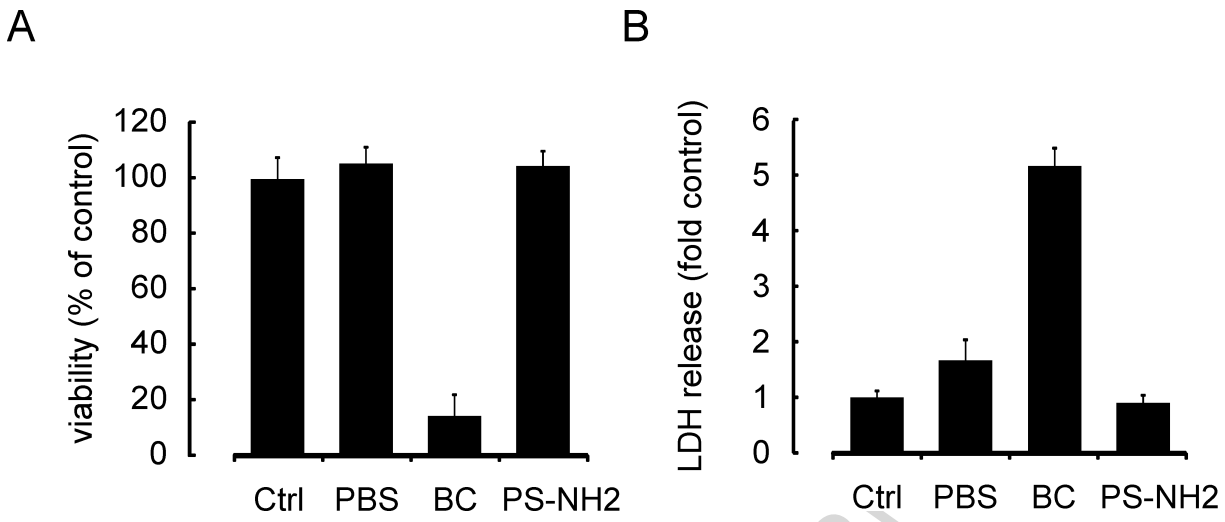

Fig. 2

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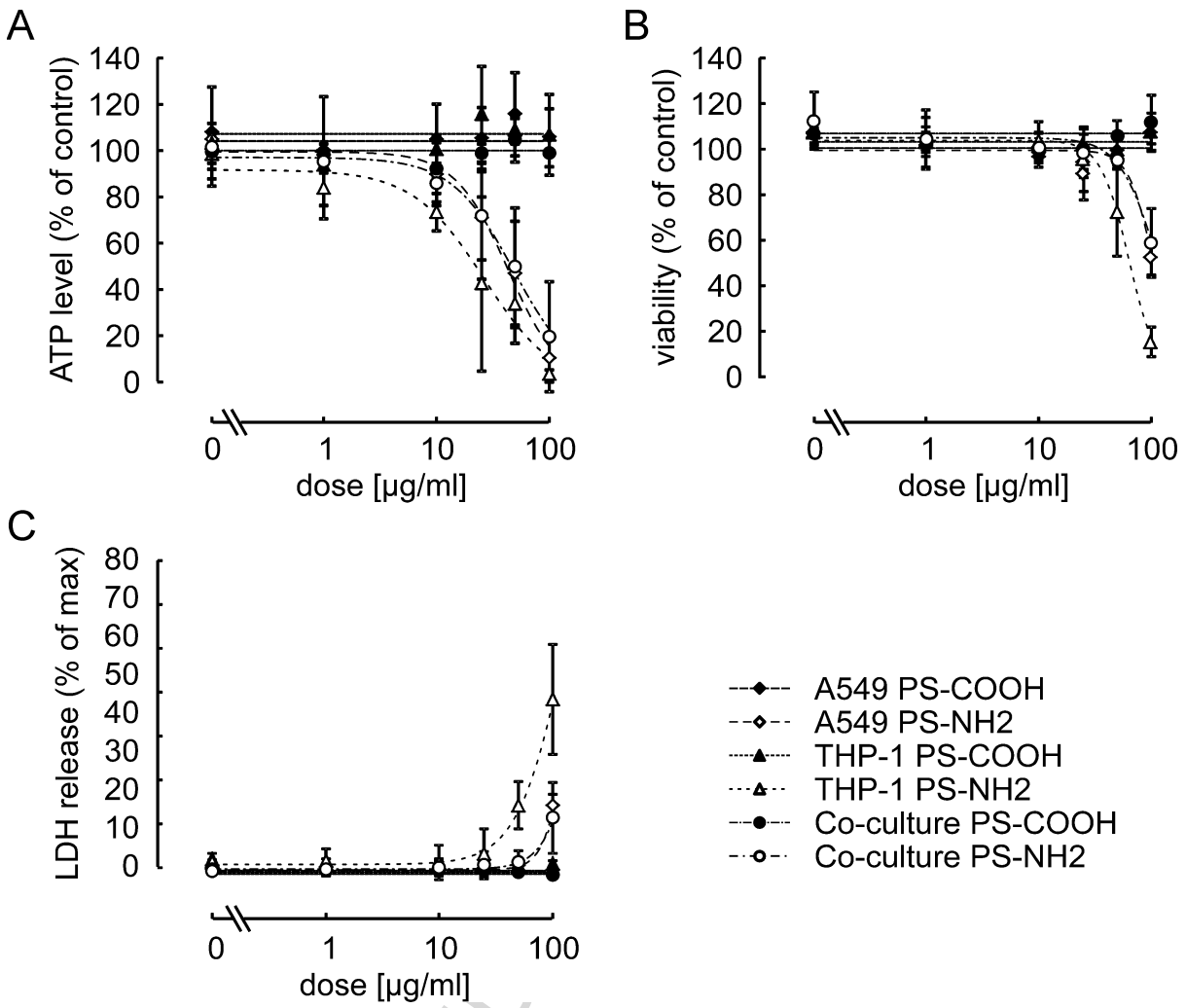

Fig. 3

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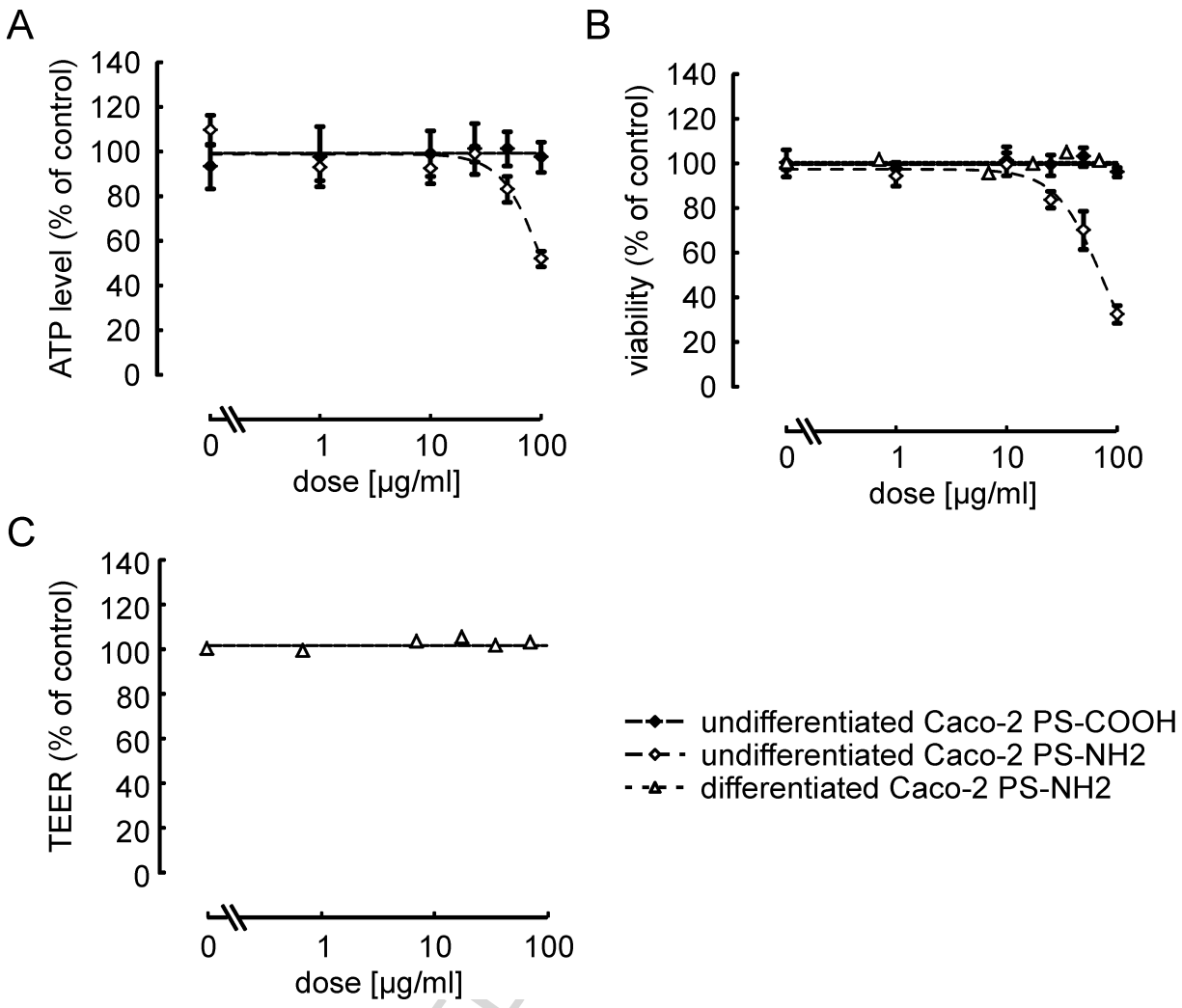

Fig. 4

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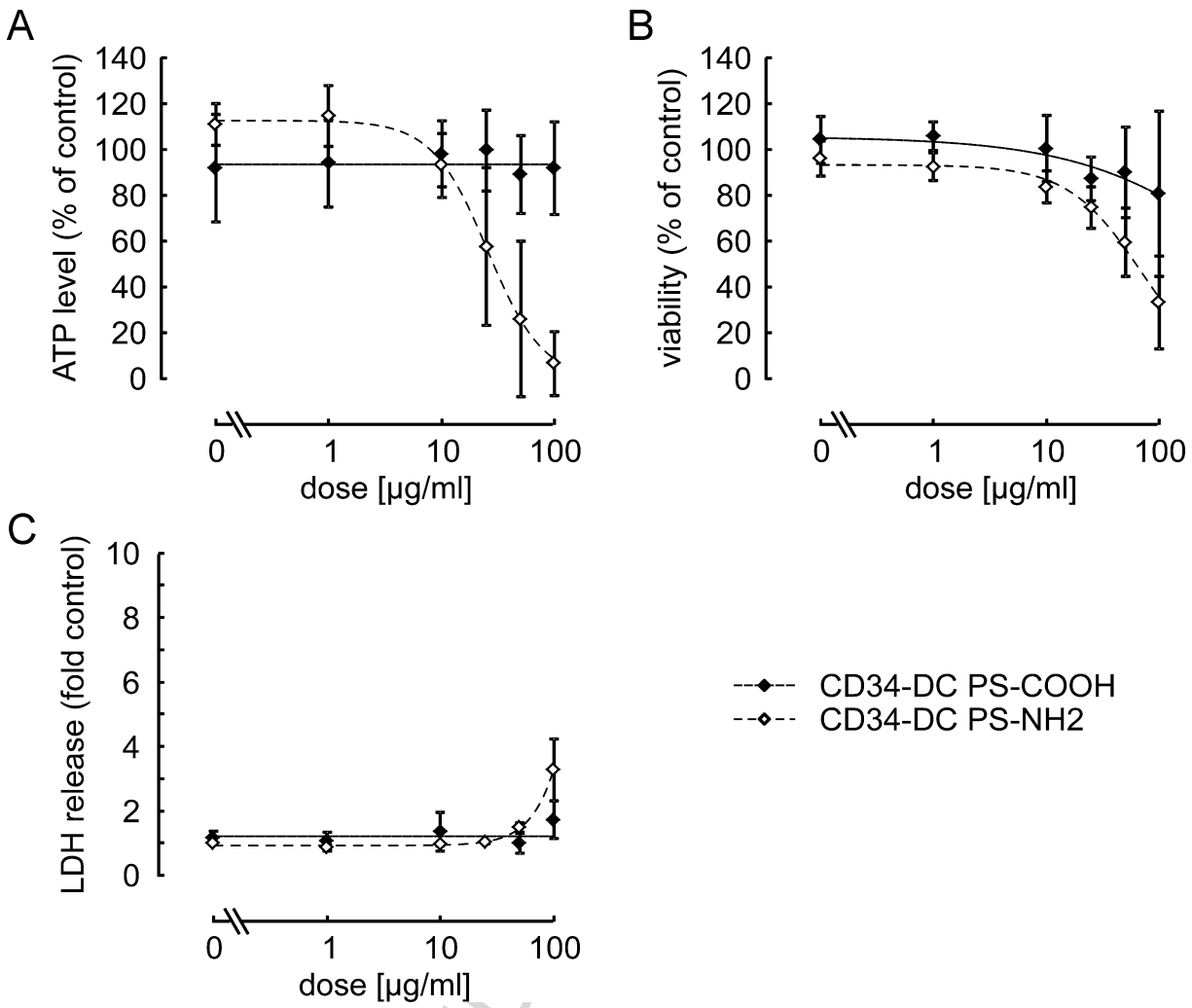

Fig. 5

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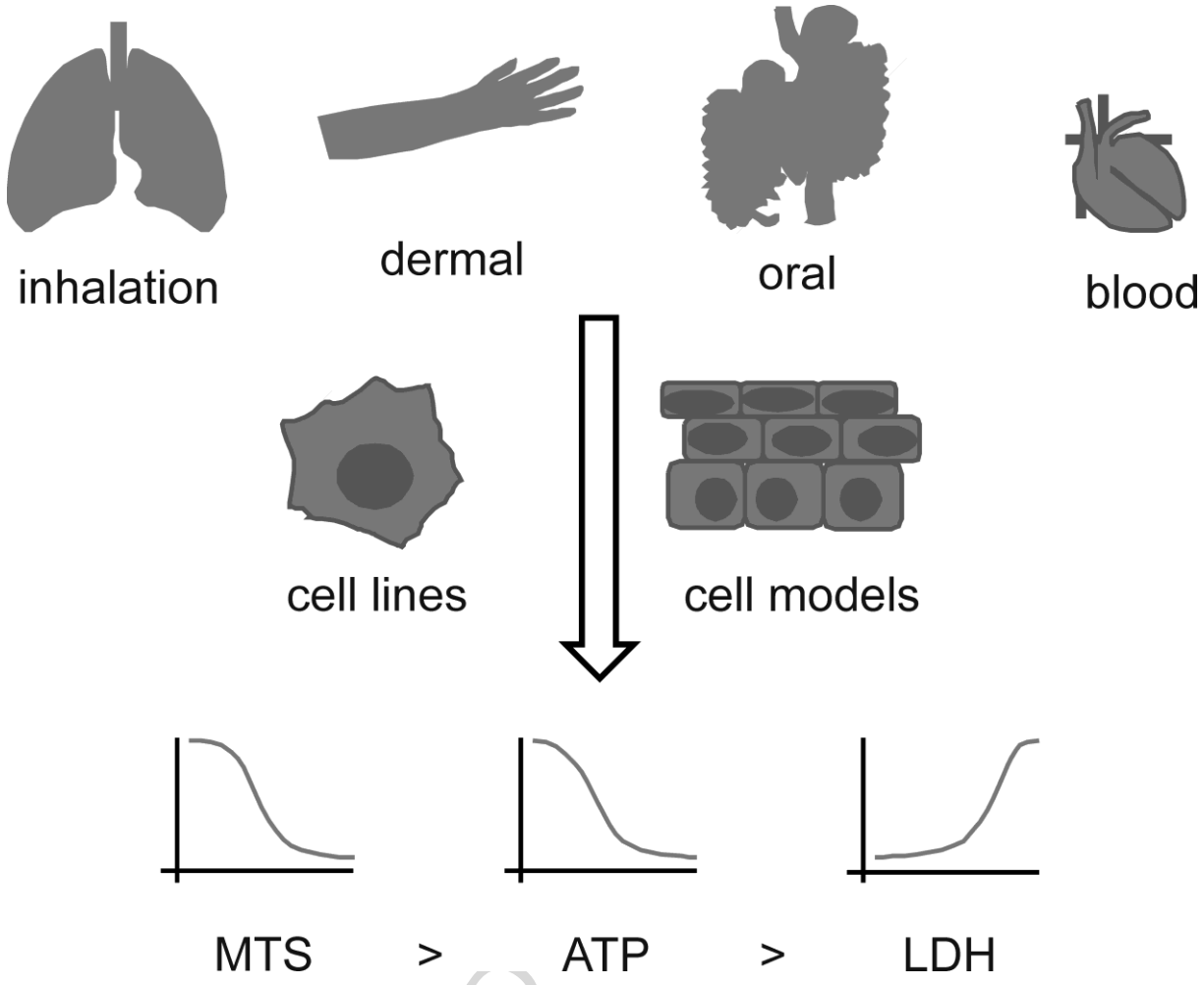

Graphical abstract

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A guide to nanosafety testing: considerations on cytotoxicity testing in different cell models

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Highlights

1) Comparison of three cytotoxicity assays covering different biological principles
2) Comparison of ten cell models, *i.e.* cell lines, primary and complex cell models
3) Cell models are representative of the main routes of exposure to NPs
4) MTS assay was applicable to all, and was the most robust and reproducible assay
5) PS-COOH and PS-NH2 are useful negative and positive controls for cytotoxicity testing

"include 3 to 5 bullet points (maximum 85 characters, including spaces, per bullet point)"

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