Assessing nanoparticle toxicity in cell-based assays: Influence of cell culture parameters and optimized models for bridging the *in vitro-in vivo* gap.

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ABSTRACT

The number of newly engineered nanomaterials is vastly increasing and so are their applications. Even though a lot of interest and effort are being put into the development of nano-based biomedical applications, the level of translational clinical output remains limited due to uncertainty on the toxicological profiles of the nanoparticles (NP). As NP used in biomedicines are likely to directly interact with cells and biomolecules, it is imperative to rule out any adverse effect before they can be safely applied. Nanotoxicity is preferably evaluated *in vitro*, but extrapolating the obtained data to a realistic *in vivo* scenario remains challenging. In addition, generated data are often conflicting, which consolidates the *in vitro*-

in vivo gap and impedes the formulation of unambiguous conclusions on NP toxicity. To bridge this gap, more consistent and relevant *in vitro* and *in vivo* data need to be acquired, which is in conflict with the incentive to reduce the number of animals used for *in vivo* toxicity testing. Therefore the need for more reliable *in vitro* models with a higher predictive power, mimicking the *in vivo* environment more closely, becomes more prominent. In this review we will discuss the current paradigm for nanotoxicity evaluation and summarize the principles of current routine methods for nanotoxicity assessment. We will also give an overview of adjustments that can be made to the cultivation systems to optimise current *in vitro* models, thereby bridging *the in vitro-in vivo* gap as well as describe various novel model systems and highlight future prospects.

1. Introduction

Since the 1980's the field of nanotechnology has increasingly gained importance, leading to a boom in applications since the 1990's. In our current society, inorganic nanoparticles (NP) are being applied in many different ways. ZnONP, for example, are used in sunscreens and toothpastes and AgNP can be found in food packages, deodorants and are applied as a preservative in cosmetics [1-3].

Given the ever increasing use of NP in technological applications and everyday consumer goods and the high interest of exploiting the exceptional features of the NP in biomedical applications, it is expected that both intentional and unintentional exposure will become more frequent [4, 5]. Because of this, the increase in nanotechnology implementation in our daily lives is joined with the raising of concerns on potential adverse effects towards human health [6]. It is therefore recommended that the safety of these products, towards consumers and especially towards workers at the production site, is carefully evaluated before its introduction to the market [4, 5, 7]. However, there are currently only very limited regulations on the use and the safety criteria for nanomaterials in industrial applications or consumer goods. Major obstacles on the route to an appropriate legislation are the broad nature of nanotechnology, the incredible pace at which the field keeps advancing and the enormous variety in types of nanomaterials, each with different physicochemical properties and specific applications [7]. An appropriate legislation should cover all aspects of nanotechnology without any material or application being left out, which from a practical point of view, is extremely hard to obtain. In order to try and overcome this predicament, the European Commission launched a recommendation on a definition of nanomaterials in 2011 that states that a nanomaterial is: "A naturel, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of

the particles in the number size distribution, one or more external dimensions is in the size range from 1 - 100 nm [8]." As every definition has its limitations and introduces technical challenges, regulatory bodies have not yet come to a global agreement of the correct definition, but the most used criterion is the size limitation [9-11]. Nanotechnology itself can subsequently be described as the manipulation and application of particles and systems with at least one dimension below 100 nm [10]. For nanomedicine purposes these technologies are being used to develop applications for diagnosis [12, 13], imaging [14, 15], treatment [16, 17] and prevention of diseases [18-20]. One of the latest developments in nanomedicine are theranostic particles, which combine imaging or diagnostic and therapeutic features in a single construct [21, 22]. Wang *et al.* for example, developed magnetic micelles for gene delivery, enabling the monitoring of the delivery efficiency after administration and Kirui *et al*. created an immunotargeted gold-coated iron oxide NP (IONP) to visualise colorectal tumours by magnetic resonance imaging followed by treatment with hyperthermia [23,24].

It is due to the minute dimensions of the NP that they exhibit many unique properties (e.g. IONP are superparamagnetic [25] and AuNP have a localised surface plasmon resonance [26]) because of which they can be implemented in novel innovative applications [27]. These dimensions are on the other hand also often the cause of adverse health effects through the higher surface to volume ratio and enhanced surface reactivity [28, 29]. The fact that both the great potential and the high risk lie in the miniature dimensions of the materials is refered to as the nanomaterial-paradox and underscores the importance of a thorough toxicological analysis [9]. Even though nanotechnology has been evolving since the 1980's, it was only in 2004 that Donaldson *et al.* mentioned the importance of nanotoxicology - as a subcategory of toxicology - to enable the further development of safe and sustainable nanotechnology [30]. Nanotoxicology itself refers to the study on interactions between NP and biological systems

with an emphasis on establishing a relationship, if any, between the physicochemical properties of the NP and the toxicological responses [31]. It is crucial to view nanotoxicology as a distinct category of toxicology since it has been observed that standard toxicity assays, which were initially developed for the evaluation of pharmacological agents, are often inadequate for nanotoxicity assessment. This can be attributed to the different mechanisms through which NP may evoke toxic effects, the specific behaviour of the NP in culture media and the possible interference of NP with various *in vitro* and *in vivo* toxicity assays [9, 32-34]. Therefore the classic toxicity-testing paradigm needs to be optimised to be applicable for nanosafety evaluation. Not only the assays but also the cultivation systems have to be optimised, as it has been shown in literature that the experimental design has the potential to influence the uptake and/or the observed toxicological effects especially since many NP tend to agglomerate and sediment on top of cells cultured in classical 2D cultures [35-38]. This review provides an overview of current methods used for nanotoxicity evaluation and factors related to the cultivation system that are likely to influence the outcome of the experiments. Furthermore we will propose adjustments that can be made to the cultivation system to optimise the current *in vitro* models in order to minimize artefacts and more closely resemble the *in vivo* situation, thereby bridging the *in vitro-in vivo* gap. These propositions will be illustrated with recent findings from literature.

2. Nanotoxicology

2.1. Common mechanisms causing nanotoxicity

As mentioned above, the field of nanotoxicology is a specific subcategory of toxicology. This subdivision is an absolute necessity, as general toxicology paradigms cannot completely cover the toxicity induced by NP [30]. Generally higher levels of toxicity are observed for NP in

comparison to the bulk material, which can be attributed to their minute dimensions, as they can reach intracellular compartments and show altered *in vivo* biodistribution patterns [20, 31]. Important factors in this respect are the higher surface to volume ratio, higher surface reactivity and susceptibility to degradation and ionleaching [28, 39]. Furthermore NP tend to behave differently in dispersion in comparison to chemicals as some are prone to degradation, agglomeration or aggregation and sedimentation, influencing NP uptake and subsequently NP toxicity [35, 40, 41]. Since these effects, as well as other adverse effects, are highly dependent on the physicochemical properties of the NP a thorough characterisation in both the dry state and in suspension is an absolute necessity to interpret results and formulate conclusions on the correlations between the NP's properties and the observed effects [29, 42]. From these issues it can be deducted that nanotoxicity assessment can often be more complex than toxicity testing of chemical substances. It is known that most chemicals induce toxicological responses through interaction with specific biomolecules whereas a single type of NP may cause toxicity via a combination of different mechanisms like the induction of reactive oxygen species (ROS), genotoxicity, morphological modifications, NP degradation and immunological effects [31, 40, 43].

Nel *et al.* have put ROS induction forth as one of the main common effects following NP exposure and therefore one of the main mechanisms through which inorganic NP induce toxicity as it has been observed in a multitude of *in vivo* and *in vitro* studies [44-48]. For instance, Wang *et al.* observed a significant decrease in GSH/GSSH ratio in the olfactory bulb and hippocampus in mice after intranasal exposure to IONP [49]. Soenen *et al.* obtained comparable results in an *in vitro* setting where C17.2 neuronal progenitor cells showed a highly significant increase in ROS levels after 4 or 24h IONP exposure [50]. ROS can be induced by several mechanisms, including (i) the interfernce with redox active proteins, (ii) interaction

with oxidative organelles such as the mitochondria, (iii) chemical reactions of the coating, reactive surface groups or ions leached from the NP surface in the acidic environment of endo- or lysosomes and (iv) by activation of several signalling pathways through interaction with cell surface receptors [28, 40]. This paradigm does however not account for all NP: CeO_2NP, for example, were found not to cause ROS induction but on the contrary even showed a protective effect against ROS damage *in vivo* as well as *in vitro* [51, 52]. Furthermore, Gao *et al.* observed an intrinsic peroxidase-like activity for unimpaired IONP in a cell free environment and Huang *et al.* saw the same effect in mesenchymal stem cells, , where IONPs were found to promote cell proliferation [53, 54]. Unlike for ROS induction the mechanism behind this protective effect has not yet been unravelled. But these data underscore the complexity of nanotoxicology and the importance of a obtaining a toxicological profile that covers as many facets as possible.

Persistent ROS induction at high levels, leading to oxidative stress, can have very grave consequences as it can cause many secondary effects like protein denaturation, modulation of specific signal transduction pathways, inflammation, (mitochondrial) membrane damage and DNA damage [28, 55]. The ability of NP to induce DNA damage through ROS is however not the only route to genotoxicity as it is known that NP can also alter gene expression via interactions with signal transduction pathways or with the transcriptional and translational machinery through perinuclear localisation of the NP [56, 57]. Finally, very small NP with a diameter below 5 nm may directly interact with DNA resulting in DNA damage [58]. ROS can furthermore cause actin stress fibre formation and therefore alter the cell's morphology, motility and adhesion [59, 60]. In turn can morphological changes cause mitochondrial membrane depolarization, leading to higher levels of ROS induction [61].

Degradation of NP in a physiological environment might also lead to adverse effects. After endocytosic uptake, the NP are exposed to an oxidative environment in late endosomes or lysosomes. Here they face degrading enzymes like cathepsin L and a pH of 5.9 in late endosomes or 4.5 in lysosomes [40, 62]. This might cause degradation or dissolution of the NP resulting in the leaching of free ions [40]. The effect on cell wellbeing depends on the chemical composition of the NP. For Cd-containing QD, for example, the leaching of highly toxic Cd^{2+}-ions is considered to be the main cause of any observed toxicity [63, 64].

Finally, it is known that NP can interact with their environment and in most cases avidly bind serum proteins to their surface to form a protein corona [65]. The nature of this corona depends on the physicochemical properties of the NP and on the composition of the microenvironment (e.g. cell culture media) surrounding the NP [31, 66]. The binding of serum proteins to the NP is an important determinant in how the cells 'see' the NP and therefore influences NP uptake and toxicity [67-69]. The proteins forming the corona might furthermore undergo conformational changes, because of which the cell may see them as an antigen and initiate an immune response [20, 30]. The protein corona can also stimulate opsonisation by macrophages *in vivo*, thereby activating the complement system and evoking an immune reaction [70]. Another plausible pathway to immunotoxicity is the enhancement of an allergic immune response, which by Nygaard *et al.* observed for single- and multi-walled carbonnotubes (CNT) in mice [71, 72].

In the current nanotoxicity paradigm, ROS induction has thus been set forth as the main toxic effect caused by NP, which may (when sustained) lead to secondary effects. Other often observed toxicological responses are morphological alterations, genotoxicity, immunotoxicity and effects caused by leached ions. Each of these potential effects should consequently be addressed when evaluating nanotoxicity, implying the need to evaluate

multiple parameters via a multiparametric method. Here we will discuss the principles for *in vitro* and *in vivo* routine methods as well as the main shortcomings to these methods. Subsequently novel principles, methods and future prospects will be discussed.

2.2. Routine methods for nanotoxicity testing

In order to obtain a complete toxicological profile, NP toxicity should be evaluated *in vitro* as well as *in vivo* [73, 74]. The *in vitro* methods are most commonly used and typically precede any *in vivo* work. It is essential to note however, that prior to any toxicity testing, the NP must be thoroughly characterized with respect to their purity (chemical and biological contaminants) and physicochemical properties both in a dry and wet state [42, 74]. More detailed information on characterisation of NP will not be provided here as this falls beyond the scope of the current review but can be found in overviews elsewhere [42, 75-77]. In this section the main methodological principles for in *in vitro* and *in vivo* nanotoxicity evaluation will be reviewed as well as the most important shortcomings of these methods.

a. Routine in vitro methods

In vitro assays are mainly the first to be conducted during a toxicological evaluation [78]. Most *in vitro* studies are conducted in classical 2D monocultures of cancer or long-lived cell lines although the use of stem cells or primary cells is steadily increasing. The selection of a relevant cell type and source generally depends on the expected *in vivo* target organ and application of the NP [79]. In the vast majority of the studies the cells are exposed to the NP dispersion during a single incubation period ranging from 3 up to 48 hours [78, 80, 81]. Afterwards, the induced toxicity is evaluated with methods that were initially optimised for toxicity assessment of chemical substances [82]. Biochemical assays with a fluorometric or

spectrophotometric read-out, including enzymatic and (enzyme-linked) immunoassays, are used to the utmost extend due to the relatively short duration of most assays, the uncomplicated detection principle and straight forward data processing [83]. Furthermore the possibility of upscaling and automation of the execution, detection and data processing make these assays highly convenient with regard to a future high content screening approach for every newly synthetized NP [83, 84]. Another popular approach is staining the entire cell or specific cellular components with cellular dyes, fluorescently labeled antibodies (AB) or molecular probes, which will interact with a specific biomolecule [40, 78, 85]. The samples can on the one hand be analyzed using a plate reader, but if information is requested on the variation between different cell types or status (e.g. apoptotic or not), flow cytometry is a more commonly used technique [86-89]. On the other hand microscopic analysis can also be performed. This is an important tool for evaluating morphological features like cell spreading but will also increasingly be applied to evaluate cellular processes in the context of high content screening [90, 91]. The use of such stainings in combination with microscopy however mostly generates qualitative or semi-quantitative data. In order to generate quantitative data, other easily quantifiable assays are required as for example the comet assay, PCR or microarrays to evaluate DNA damage [78].

Finally specialized techniques are used to evaluate specific parameters. For example, ion leaching can either be detected in cell-free conditions using specialized buffers (pH 7.4; 5.5 and 4.5) and the intactness of stem cell functionality can be evaluated by observing the efficiency of cellular differentiation induced by specific protocols [40, 92].

b. Routine in vivo methods

Subsequently *in vivo* studies can be performed testing a dose range derived from *in vitro*

experiments or realistic exposure doses [82]. Most studies, certainly preliminary studies, are conducted on rodents as costs are lower, animals easier to access and infrastructural requirements less elaborate [73]. Official instancies have determined the species and number of animals and that should be used for a specific tests, to meet the incentive to reduce the number of animals used as urged by the 3R concept by Russel and Burch, ethical criticism and the pressure to develop a more cost-effective toxicity assessment protocol [93, 94]. The European Commission has bundled it's guidelines in the REACH (Registration, Evaluation and Administration of Chemicals) regulation. Even though this regulation was initially designed for chemical substances it was plainly adopted to nanotoxicity evaluation [95]. The approach of simply adopting REACH regulations to NP has however been put to question as classical toxicity assays show major shortcomings when they are applied to nanotoxicity assessment, as will be discussed in section 2.4. For assay protocols REACH in turn refers to the guidelines drafted by the Organisation of Economic Co-operation and Development (OECD), which will not be discussed in detail but can be found in the 'Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials' [94].

When assessing nanotoxicity *in vivo*, the following aspects ought to be evaluated according to REACH guidelines: acute, subchronic and chronic toxicity, skin and eye irritation or corrosion and skin sensitisation, genotoxicity, reproductive toxicity, carcinogenicity and the NP's toxicokinetics [94]. An overview of the parameters that are evaluated in an *in vitro* or *in vivo* setting is given in table 1.

A commonly used technique for *in vivo* studies is the histopathological examination of selected organs and tissues from a sacrificed animal, often in combination with an appropriate staining [94]. This technique is used for all required aspects except for the skin and eye irritation or corrosion and skin sensitisation and genotoxicity experiments [94, 96-98].

Examination of the organs includes an evaluation of its morphology in term of length, width and shape. Any changes in tissue colour must also be documented [94]. Not only whole organs are analysed as any assay performed in an *in vitro* setting can be performed on cells from a (sacrificed) animal. This way, more detailed information on ROS induction, inflammation or activation of the immune system *in vivo* can be obtained. General *in vivo* observed effects can then be clarified by the molecular mechanism found in *in vitro* experiments. REACH also requires blood and urine samples to be taken at regular time points, the weight of the animal to be documented as well as its behaviour and food and water consumption [94]. When performing carcinogenicity experiments the endpoint of the study is the appearance of tumours. These can subsequently be resected and analysed in terms of number, size, shape etc. [99]. These experiments are often combined with long term in vivo studies as proposed by the OECD to further reduce the number of animals needed [100].

	In vitro	***In vivo***
Acute toxicity	X	X
Subchronic toxicity		X
Chronic toxicity		X
Oxidative stress	X	
Morphology	X	X
Skin irritation/corrosion and eye irritation	X	*
Immunotoxicity and sensitization		X
Genotoxicity	X	X
Carcinogenicity		X

Reproductive toxicity		X
Toxicokinetics		X

Table 1: assays performed in an *in vitro* and *in vivo* setting.

*As validated alternative methods are available, these must be used according to REACH regulation.

c. Issues with routine methods

Currently, methods applied for evaluating NP toxicity, such as biochemical cell viability assays and the use of fluorescent markers, are adopted from routine methods for toxicity assessment of chemical substances. The evaluation of NP toxicity using these common assays has however resulted in conflicting data as can be found in reviews listing data on the toxicity of a specific NP or the correlation between the NP's physicochemical properties and the toxic effects it evokes [29, 101-103]. This led to an increasing awareness that these methods, and especially the *in vitro* methods, are not as appropriate and well-suited for nanotoxicology purposes as was previously assumed [82, 93]. Nel *et al.* have first raised this thought in 2006, emphasizing on the necessity to optimize the classical *in vitro* toxicity assays as they show several shortcomings when they are being applied for NP toxicity evaluation [28]. We believe that the major issues with the current *in vitro* methods are (i) the lack of a complete characterization of the particles, (ii) the lack of standardisation and guidelines on how to perform a toxicological evaluation *in vitro*, (iii) the possibility of NP interfering with the assay and therefore lack of appropriate methods to evaluate nanotoxicity and (iv) the shortcomings inherent to the most used classical 2D monocultures [11, 82, 93, 104, 105]. It is therefore clear that further research on the optimization of methods is highly recommended in order to obtain reproducible data that would allow drawing firm conclusions regarding NP toxicity. This

section provides an overview of issues with routine methods, which are not correlated to the cultivation system, as these will be the topic of section 3 until 6.

Issues with routine *in vitro* methods

A first shortcoming to the current nanotoxicity testing strategy is the lack of a complete characterisation of the particles, as many parameters such as NP size, charge, shape etc. often differ between studies rendering it nearly impossible to retrieve reliable conclusions on the effect of a specific parameter. This originates from the fact that altering one physicochemical parameter, for example surface charge, without affecting any other (hydrodynamic size, colloidal stability, nature of the coating…) is not an easy task [105]. Therefore a multitude of parameters, that might potentially influence the outcome, must be optimised and standardised as far as possible. Therefore consensus should be reached on which parameters must be characterised as well as on the methods applied for this purpose.

Besides the NP's physicochemical parameters, NP concentration is one of the parameters that could be worked on. Here consensus is needed on methods to determine the concentration as well as on the unit, in which this parameter should be expressed [106, 107]. Expressing the concentration in terms of mass/volume is the easiest option, but is however not always the most relevant [105]. Which was pointed out in a review by Johnston *et al.*, as they often encountered a stronger toxic response for the smaller NP in comparison to their larger counterparts at similar mass/volume doses [38]. It was suggested by Wittmaack that particle number/volume might be the best dose metric [108]. Other groups however suggested to express the concentration in terms of surface area/volume as it is known that toxicological responses depend on the NP's surface properties and that the surface area exponentially increases with a decrease in NP size [45, 106, 109]. The applicability of this unit was demonstrated by Rushton *et al.* who found a significant correlation between the oxidative response

observed *in vitro* and the *in vivo* inflammatory response for a group of nine different NP, with distinct physicochemical properties, when the concentration was expressed in surface area/volume and the steepest slope method was applied [110]. When the same group applied this method to results from Sayes *et al.*, who could not obtain an in *vitro-in vivo* correlation back in 2007, a clear correlation was now established[110, 111]. Han *et al.* also found a clear correlation when comparing the oxidative stress *in vitro* and the *in vivo* inflammatory response after TiO_2NP exposure, confirming the applicability of the steepest slope method using surface area/volume as dose metric [112].

Subsequently attention should go to standardisation of nanotoxicity methods in terms of incubation conditions like NP concentration and incubation time as overexposure conditions should be avoided [113]. The importance of avoiding overexposure conditions becomes clear when evaluating genotoxicity for example, as acute toxicity at overexposure levels can mistakenly be interpreted for genotoxicity since apoptosis itself induces DNA fragmentation [114, 115]. This implies the need to screen for genotoxicity at sublethal levels. The determination of relevant dose ranges is however severely hampered by the lack of exposure data and required doses of NP for specific applications. Therefore *in vitro* (and *in vivo*) toxicity testing currently focuses on determination of the No Observed Adverse Effect Level (NOAEL) values instead of evaluating realistic exposure scenarios or dosages [38]. This may lead to an overestimation of nanotoxicity as according to Paracelsus' principle every type of material will evoke toxic effects at sufficiently high concentrations.

Another important factor inducing variation in nanotoxicity data is the medium in which the NP are dispersed. This can affect the agglomeration or aggregation state of the NP, which determines its behaviour in dispersion and subsequently the uptake and toxicity induced by the NP either in a positive or negative way [116, 117]. Therefore some groups have focussed on

the development of methods to create a stable and uniform dispersion via surface modification, addition of surfactants etc. [118-120]. But opposing opinions exist on whether NP agglomerates must be redispersed before addition to the cells or not since results from a study by Oberdörster *et al.* in which surfactant stabilised dispersions were used, has been put to question as the observed toxicity might have been caused by surfactant residuals [121]. It is therefore presumed that it is best not to alter the dispersion state before adding the dispersion to the cell culture but to strive for a medium resembling the *in vivo* situation as close as possible [122, 123].

A fourth major issue with *in vitro* toxicity assays is the potential of NP to interfere with the assay in various ways [82]. Obviously NP with optical properties might alter the outcome of an assay based on a spectrophotometric or fluorometric read-out [33]. Other possible interference mechanisms are interactions with enzymes or substrates because of the high absorbance capacity and/or catalytic activity of the NP [82, 104]. Han *et al.* saw this for AgNP and TiO_2NP, which respectively inactivated and adsorbed to LDH leading to an underestimation of the acute toxicity [124]. Kroll *et al.* looked into the interference of 24 well-characterised NP with four frequently used *in vitro* assays and observed concentration, NP and assay-dependent interferences [32]. These data underscore the importance of validating the assays for each specific type of NP. Until then, researchers must assure that the measured toxicity or the lack of toxicity is indeed caused by the NP and is not merely a consequence of interference with the assay, which can be done by introducing appropriate controls [125, 126]. Besides a negative (no treatment) and positive control (maximum effect), which should be included in every assay when possible, the positive control can also be tested in combination with the NP. The reagents should also be incubated with the NP to rule out any possible interaction [127]. Damoiseaux *et al.* further suggest the use of multiple assays for a single parameter to validate

the obtained results. Ideally the assays must supply complementary information and have a different assay- and detection principle [128].

Issues with routine *in vivo* methods

The standard methods for *in vivo* toxicity evaluation seem to be more applicable for nanotoxicity assessment: NP do not seem to influence the obtained results as gravely since there are no present reports on NP interacting with the assays, although scepticisms has been raised on the subject. In this context the fewer number of *in vivo* studies must be considered, as interactions may not have been uncovered yet.

The major issue for *in vivo* nanotoxicity testing is the set up of a relevant dose range that mimics actual human exposure to NP [82]. This issue is very complex since total exposure includes intentional and unintentional exposure for consumers as well as for workers at the production site via different exposure routes with the respiratory system, the GI tract and the skin being the main portals of entry [11, 74, 115]. Additionally exposure may occur during every stage of the NP's lifecycle, being the development, manufacturing, use and disposal of the NP [123]. The extent and complexity of this problem thus requires a case-by-case division into multiple scenarios so total exposure for a specific group can be reconstructed from the categorical exposure doses. The determination of these doses is however highly impeded by the lack of validated methods, which allow measuring exposure doses in a standardised manner in different settings.

Inhalation studies are regarded as the 'golden standard' for *in vivo* nanotoxicity studies as exposure via this route is expected to be the most abundant and exposure to airborne NP has already been associated with adverse health effects [129]. Therefore most of the relatively low number of conducted *in vivo* studies considered toxicity after pulmonary exposure. But as currently no standard protocols are available for this type of study, optimization is still

required. Several groups have therefore made efforts to optimise various aspects of the protocol with the major hurdle being the preparation of the aerosols, as NP tend to aggregate because of their strong hydrophobic properties [84, 130]. The same issue of sample preparation also occurs for studies exploring other portals of entry. Therefore a number of groups have focused on developing methods to create stable and uniform dispersions in a controlled manner. But similar as for *in vitro* experiments it has been put to question whether the agglomerates should be redispersed prior to administration and so far no consensus has been reached on the subject [130]. Another shortcoming parallel to *in vitro* studies is the lack of a complete characterization of the evaluated NP so no clear correlations between the observed effect and the physicochemical properties of the NP can be formulated.

Subsequently, detection strategies are correlated to the type of NP tested and may therefore differ for different types of NP. Fluorescent NP can for example easily be detected but other NP will need to be linked with a fluorescent probe to allow detection by the same methods. This strategy might however not be the most suitable, as the formation of the protein corona and interaction with biomolecules might be altered because of this probe and therefore alter its *in vivo* behaviour and toxicological profile [74]. Finally, methods should be developed to obtain information on the stability of the NP *in vivo* to see whether NP remain unimpaired or undergo degradation as this will influence the observed toxicity and may elucidate toxicity mechanisms [31].

Overviewing these findings, it can be concluded that routine *in vitro* and *in vivo* methods suffer from the same artefacts when they are applied for nanotoxicity evaluation, without any modifications to the protocols to fit this new purpose. One of the most important issues is the setup of a representative dose range based on realistic exposure scenarios in both the *in vitro* and *in vivo* setting. The issue of selecting the most suitable dose metric is only mentioned for

the *in vitro* experiments but is likely to also account for the *in vivo* situation. The same is true for the issue of NP characterisation as thorough characterisation is always inevitable in order to allow drawing firm conclusions. Aggregation of NP in dispersion media is another issue that also occurs on both levels of toxicity testing and so does the possible interferences with the assays used. *In vivo* methods show an additional shortcoming, as detection strategies are not always the most suites so the need for novel methodologies becomes more prominent. Thus, it can be stated that both *in vitro* and *in vivo* methods cannot be plainly applied to nanotoxicity studies and that the entire nanotoxicity testing paradigm should be subject to thorough optimization, which has been stimulated by the combined efforts of various research groups.

2.3. Novel methods for toxicity testing

Recently, a number of groups have pointed out several shortcomings to the current nanotoxicity methods and the corresponding need for optimization (see 2.2.c). Especially the optimization of *in vitro* methods receives a lot of attention since they will be the basis of a future screening approach, which is highly needed to keep up with the rapid pace at which the entire field of nanotechnology keeps evolving. This section provides a number of new insights and methods for nanotoxicity assessment, guided by examples from recent literature.

a. Particokinetics

As mentioned in section 2.2.c. NP dosimetry poses several challenges. To meet this challenge Teeguarden *et al.* proposed the concept of particokinetics, representing the solution dynamics of the NP [116]. They marked diffusion, sedimentation and aggregation as predominant processes determining the NP's faith in dispersion. Subsequently they suggested a distinction between the administered, delivered and cellular dose respectively being the dose added to the cell culture, the dose reaching the cell surface and the dose actually reaching the interior of the cell (Figure 1). The latter dose is the most interesting for nanotoxicity and cell labelling studies but is also the hardest to determine. Until methods become available to determine the cellular dose *in vitro*, preferably in real-time, they proposed the application of a model that enables the calculation of the delivered or cellular dose starting from the administrated dose and the behaviour of the NP in dispersion [116]. This model was recently optimized by Hinderliter *et al.* and is since then referred to as the *In vitro* Sedimentation, Diffusion and Dosimetry model or ISDD [131]. Lison *et al.* however believe that the hereby-calculated doses will be underestimated for monodisperse NP suspensions since this model does not take convectional forces into account, which mostly develop in solutions [132].

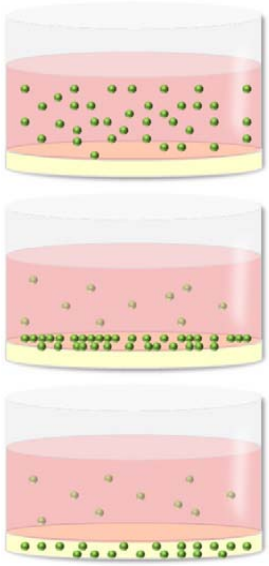


Figure 1: Schematic representation of the concept of administered dose (a), delivered dose (b) and cellular dose (c).

b. Multiparametric nanotoxicity evaluation

Since it was observed that NP can cause multiple effects via different mechanisms, awareness has risen on the necessity to evaluate nanotoxicity *in vitro* by a multiparametric method [43, 83]. This method should include the evaluation of different endpoints via multiple assays preferably in multiple cell types from different organisms to increase the predictive power [128, 133, 134]. Endpoints like acute toxicity, ROS induction, morphological alterations, genotoxicity and NP degradation have been put forward as important parameters [40, 128, 133] . Consensus on the optimal design of this multiparametric method has however not yet been reached, but several examples have been proposed recently, including one from our group that is displayed in figure 2 [43, 127]. Methods to evaluate the different parameters are preferably biochemical or microscopy-based assays as they are easily amendable for a future screening approach [128]. Some groups also suggest the implementation of omic-techniques to screen for genotoxicity, alterations in protein expression or biomarkers related to cellular pathways [84, 135]. Any multiparametric method shall require a high content screening (HCS) setup to enable simultaneous testing of multiple doses in different cell types by using various assays in a reasonable amount of time [128, 133]. This screening approach appears to be very promising as O'Brien *et al.* found a sensitivity and specificity of respectively 93% and 98% for a five-parameter HCS method in comparison to a single parameter approach that showed a sensitivity of 25% [136]. Jan *et al*. furthermore demonstrated the applicability of HCS in establishing nanotoxicity profiles, underscoring the potential of this approach [137].

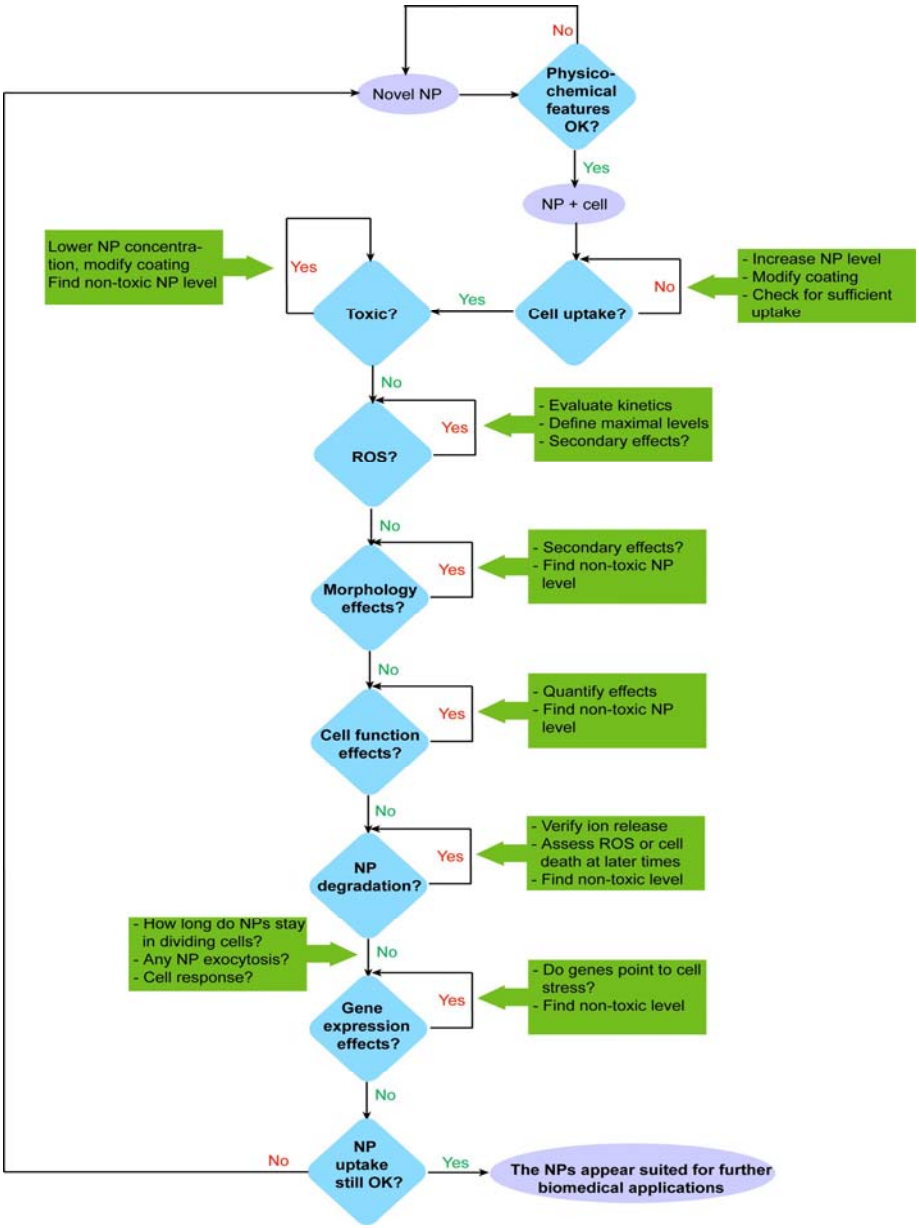


Figure 2: Schematic overview of the multiparametric method developed by Soenen et al.

c. QSAR and in silico models

These screening approaches will generate a vast amount of data at a high pace, causing the bottleneck of nanotoxicity testing to shift from the assay execution to data processing. Therefore bio-informatics are gaining importance as an automated data analysis will be necessary to be able to keep up with the rapidly evolving field of nanotechnology [134].

A popular subject in bio-informatics is the set up of an *in silico* approach to nanotoxicity with the development of quantitative structure-activity relationships (QSAR) as the ultimate goal.

These QSARs will in turn allow the prediction of the toxicity of newly engineered NP based on their physicochemical properties enabling an even faster evolution of nanotechnology through a safety-by-design approach [134]. The development of *in silico* models and HCS go hand in hand as on the one hand the development of a reliable *in silico* model requires a large amount of data, which can be provided by HCS, and on the other hand bio-informatic tools are indispensible for an efficient processing of HCS data [134]. Recently efforts from Puzyn *et al.* have resulted in the development of a QSAR predicting the toxicity of metal oxide NP in E. Coli based on the effect of 17 different metal oxide NP [138]. However, many obstacles still need to be overcome before the first QSAR can be implemented that allows the prediction of the adverse effects of any NP towards human health. Therefore, clear correlations between the NP's physicochemical properties and observed effect must be found, large-scale comparative studies should be performed and the QSARs must be validated, which requires the indentification of reference nanomaterials with well-known effects [40, 83].

d. Novel in vivo methods

Novelties to *in vivo* methods are not as numerous as for their *in vitro* counterparts, but recently some research has been performed on the use of zebrafish embryos for an *in vivo* screening approach. Similar as for most *in vitro* methods used in nanotoxicity assessment, Hill *et al.* initially developed this method for the evaluation of the toxicity of chemical substances [139]. Usenko *et al.* subsequently demonstrated the suitability of this model for nanotoxicity evaluation and follow-up studies examining AuNP, AgNP and QD toxicity underscored the potential of this model [140, 141, 142]. The main advantages to other *in vivo*

models are the reductions in cost, labour, time and infrastructural requirements as zebrafish are small and have a short life cycle [140]. Another important breakthrough is the use of an integrated screening approach using the zebrafish model and HCS to obtain a hazard ranking of different NP, which was recently proposed by George *et al.* [143].

As the field of nanotechnology is rapidly evolving, nanotoxicology could not fall behind. The most important innovations for *in vitro* methods in the recent years are the concept of particokinetics and the development of mutiparametric methods. The latter initiated the development of a HCS approach and *in silico* models with QSARS and a safety-by-design approach as the ultimate goal. Innovation to *in vivo* experiments can be found in the screening approach using the zebrafish embryomodel, which can subsequently be combined with *in vitro* HCS. Innovation off course does not stop here, as these methods require optimisation and standardisation and many obstacles still need to be overcome.

3. The nature of cell-type dependent effects

In vitro studies are mostly performed on cancer cell lines or long-lived cell lines as these are readily available, relatively inexpensive and easy to cultivate as they exhibit an enhanced proliferative capacity. Yet, it is known that cancer cells show a disturbed apoptotic balance and a higher metabolic activity to sustain their high proliferation rate [144]. Long lived cell lines in turn express a phenotype that is not entirely stable, as changes may have been induced unintentionally during the long cultivation time and extensive *in vitro* manipulation or intentionally during their immortalization [145, 146]. Alterations may include changes in cellular homeostasis, growth potential, biological responses, signal transduction etc. Hence, doubts have been raised on whether these cell lines are a reliable representation of the *in vivo* situation and on their usefulness in NP hazard assessment. Subsequently the use of primary

cells or stem cells has been put forward as an alternative as it is assumed that these cells stand closer to the *in vivo* cellular situation as they undergo minimal manipulation *in vitro* in comparison to cell lines [146, 147]. But, since these cells are not always easily obtained and require specific handling, this assumption first needs to be proven by comparative studies in order to revise the current *in vitro* testing strategy. Primary cells may in turn suffer from batch-to-batch differences, which could affect the outcome of performed experiments and thereby reduce the reproducibility of the experiments, which is not an issue with cell lines. The use of pooled stocks may be a good strategy to overcome these interbatch differences and allows taking interindividual variations into account, which is in turn not possible when using cell lines. A summary of properties of cell lines and primary cells or stem cells can be found in table 2.

To verify whether various cell types differ in their way of handling administered NP several studies were undertaken comparing NP uptake and cytotoxicity in different cell types of which the results will be summarized in this section.

Cell lines	**Primary or stem cells**
Readily available	Not always readily available
Less expensive	More expensive
Easy to cultivate	Require specific handling
No interbach differences	Interbach differences
No interindividual differences	Interindividual differences
Much in vitro manipulation	Minimal in vitro manipulation
Disturbed apoptotic balance	Normal
Altered metabolism	Normal

Altered phenotype	Normal
Enhanced proliferative capacity	Normal

Table 2: Comparison of features of cell lines and primary or stem cells with regard to their properties important for nanotoxicity studies.

3.1. Effect of the cell type on nanoparticle uptake and processing

It has been shown in many studies that most NPs enter the cells through the process of endocytosis. Some exceptions can be found as for phagocytotic cells phagocytosis remains the most important mechanism [148]. Wang *et al.* have furthermore observed quantum dot (QD) uptake by passive diffusion in red blood cells. This must however be put into perspective as red blood cells are not capable of endocytosis [149]. The uptake mechanism is highly important as it determines the NP's intracellular location, as NP entering the cell by passive diffusion directly interact with the cytosol, while NP taken up by endo- or phagocytosis are retained in vesicles that are distributed widely in the cytoplasm or localized in a specific cellular region [149, 150]. Since the uptake kinetics and intracellular location are important determinants for the final toxic response and appear to differ between cell types, several studies have been conducted comparing these parameters in different cell types. For example, Sur *et al*. compared the uptake of AgNP modified with glucose, lactose or oligonucleotides in L929 and A549 cell lines, respectively a long-lived mouse fibroblast cell line and a human lung epithelium cancer cell line and observed a differential uptake rate in all cell lines with A549 cell showing the highest uptake of lactose modified NP [151]. Coulter *et al.* compared the uptake of 1.9 nm diameter AuNP in human prostate and breast cancer cell lines (DU154 and MDA-MB231) to a human lung epithelium long-lived cell line (L132), and observed the lowest uptake in the latter [152]. Diaz *et al.* compared the uptake of five different NP in normal human

monocytes, lymphocytes and erythrocytes, mouse macrophages and four human cancer cell lines: a myeloid-monocytic cell line (U937), a T-cell line (Jurkat), a B-cell line (HMY) and a prostatic cancer cell line (PC3). Human monocytes rapidly phagocytosed all NP tested whereas mouse macrophages showed an even higher uptake, which was comparable to the uptake in PC3 cells. The monocytic cell line however did not show NP internalisation, which conflicts with results from other studies showing a higher uptake capacity for cancer cell lines [151-153]. All other cell types tested did not show a significant uptake. The authors have therefore put the phagocytic machinery in combination with the cell type forward as predominant factors for NP uptake [154]. This was supported by findings from Greulich *et al*. as they observed avid uptake by human primary monocytes through phagocytosis but did not find any uptake in human primary lymphocytes [155].

Wang *et al.* recently evaluated the intracellular distribution for PEGylated micelles in a human lung cancer cell line and long-lived cell line (A549 and MRC-5) and a human kidney epithelium derived long-lived cell line (293T) which showed similar distributions [144]. Barua and Rege however observed significant variations in intracellular localisation of QD in three phenotypically closely related human prostate cancer cell lines, as can be deducted from Figure 3. It was observed that the QD were trapped in lysosomes scattered throughout the cytoplasm in PC3 cells, localized at a single juxtanuclear location in PC3-PSMA cells and a combination of both was found in PC3-flu cells [156]. They coupled these observations to the loss of polarity in malignant cells influencing the sorting and trafficking potency of the cells, the slight differences in receptor expression profiles and a disruption of the microtubule network in PC3 cells impeding further trafficking to the juxtanuclear region [156].

It can be concluded from these data that cellular uptake and distribution of NP depends on many factors like the difference in precise uptake mechanism, which is in turn cell type-

dependent. Furthermore uptake does not only significantly differ between human or rodent cells, but also between cancer or long-lived cell lines and primary cells with cancer cell lines generally showing the highest uptake. Besides uptake, cellular distribution of NP is cell type dependent as well and has even been found to differ substantially between phenotypically closely related cell lines. Therefore these findings underline the importance of selecting a representative cell type, which mimics the *in vivo* situation as closely as possible, as toxicity is logically related to the uptake determining the cellular dose of the NP.

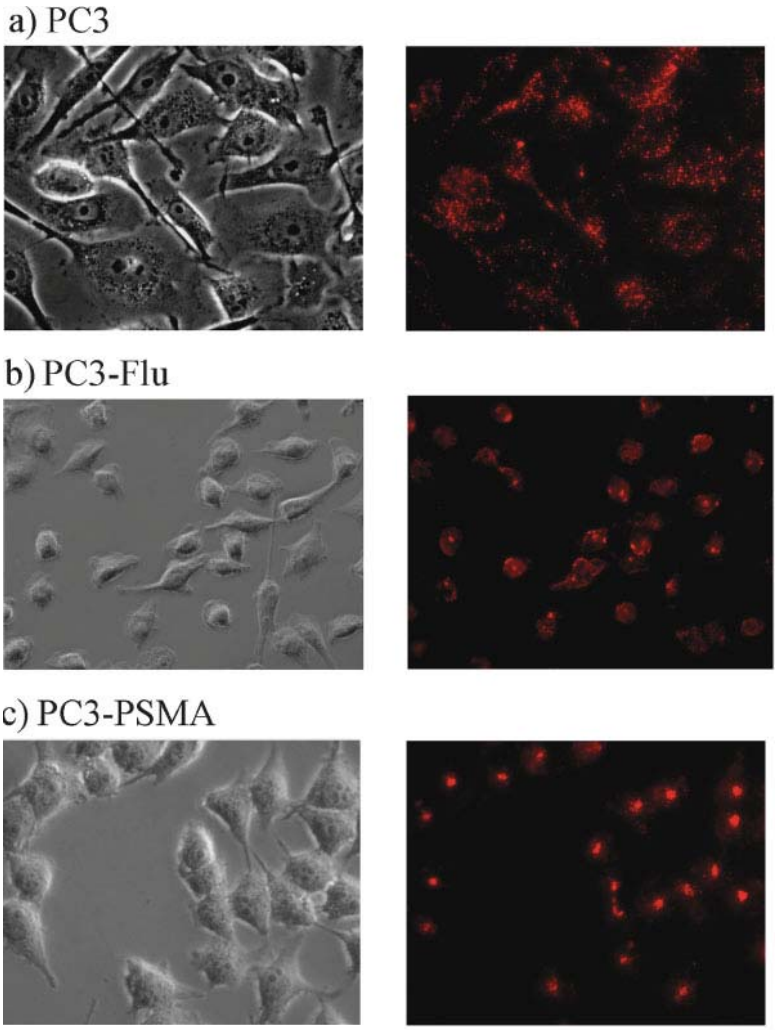


Figure 3: Differential intracellular localization of QDs in human PCa cells. a) PC3, b) PC3-flu, c) PC3-PSMA

3.2. Effect of the cell type on NP toxicity

Only recently more awareness was raised on cell type-dependent effects being one of the factors causing the generation of discordant results in *in vitro* nanotoxicity studies. Therefore the number of studies comparing NP effects in multiple cell types is relatively low. Similar as for uptake studies, some groups have focussed on comparing several cell lines. A recent study by Chowdbury *et al.* provides a comparison between two human breast cancer cell lines (SKBR3 and MCF7), a cervical cancer cell line (HeLa) and a mouse fibroblast long-lived cell line (NIH-3T3). HeLa cells appeared to be the most sensitive to toxicity induced by oxidized graphene nanoribbons, which could be linked to the higher uptake [157]. Xia *et al.* examined the toxicity of cationic polystyrene NP in 3 rodent cancer cell lines: RAW 264.7 (mouse leukemic monocyte macrophage), HEPA-1 (mouse hepatoma) and PC12 (rat pheochromocytoma); and 2 human long-lived cell lines: BEAS-2B (human bronchial epithelium) and HMEC (human micro-vascular endothelial). All cell lines showed NP uptake but only RAW 264.7 and BEAS-2B cell lines were prone to NP toxicity and respectively suffered apoptosis and necrosis while other cell lines were relatively resistant to particle injury [158].

As the use of cell lines for *in vitro* nanotoxicity assessment has been put to question, several groups rather focussed on comparing the effects of NP exposure in cell lines and primary or stem cells representing the same tissue. For instance, Albrecht *et al.* compared NP toxicity in primary rat alveolar macrophages to a rat alveolar macrophage long-lived cell line (NR8383) and found the latter to be more sensitive [145]. Wang *et al.* found primary catfish hepatocytes to be less sensitive to CuONP and ZnONP induced toxicity than cells from the human HepG2 hepatoma cell line [159]. When Wang *et al.* compared the toxicity of FITC-encapsulated SiO_2 core/ZnO shell NP in the Jurkat cell line and primary T-lymphocytes, which were less sensitive to nanotoxicity [160]. Hanley *et al.* compared ZnONP induced toxicity in human primary T-cells and two human T-cell lymphoma cell lines (Jurkat and Hut-78) and found the

two cell lines to be respectively 28- and 35-fold more sensitive to ZnONP exposure than normal T-cells (Figure 4a) [161]. In contrast to these studies Bregoli *et al.* observed an impaired proliferation in primary human hematopoietic progenitor cells after Sb_2O_3NP exposure while the proliferative capacity of none of the seven hematopoietic (cancer and long-lived) cell lines tested was affected [146].

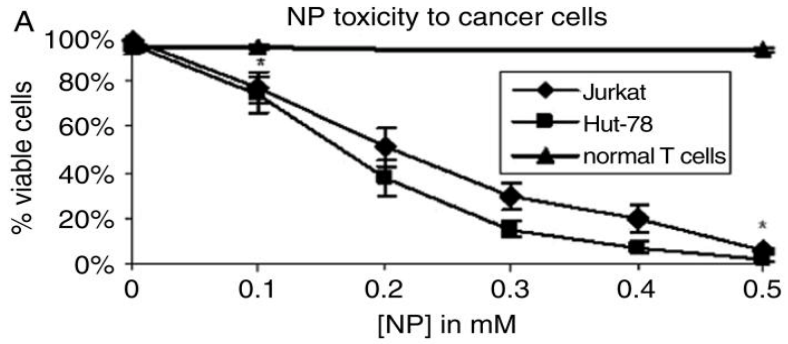
Several comparative studies have furthermore tried to elucidate the mechanisms behind the differential reactions to NP exposure. First, the degree of association between the NP and the cell membrane is known to be crucial as it influences NP uptake levels [161]. The physiological function of the cell also appears to be important with regard to NP uptake, as for example macrophages and monocytes mostly show higher uptake and are therefore more susceptible to nanotoxicity, which can be linked to the phagocytotic uptake mechanism and the capacity to clear xenobiotics from the body [154, 155, 162]. Another factor determining the differential sensitivity to nanotoxicity in various cell lines is the variation in proliferative capacity. Chang *et al.* compared three long-lived fibroblast cell lines (WS1, CCD-966sk and MRC-5) to three epithelial cancer cell lines (A549, MKN-28 and HT-29), with average doubling times of 128.4 and 23h respectively. All three epithelial cancer cell lines were less prone to silica NP induced injury, which may be due to the higher rate of cell division and the associated dilution of cellular NP levels [163]. Another study by Hanley *et al.* however showed a higher susceptibility to ZnONP injury for activated primary human T lymphocytes in comparison to resting human T lymphocytes, as can be seen in Figure 4b [161]. This preferential targeting of rapidly dividing cells can in turn be coupled to a higher uptake of the NPs in actively proliferating cells [164]. Furthermore, Mukherjee *et al.* have put the cells natural antioxidant levels forward as an important factor. They coupled the higher susceptibility of HeLa cells in comparison to HaCaT cells (a human dermal cancer cell line) towards AgNP toxicity to the lower natural

antioxidant capacity of HeLa cells, as AgNP caused stronger ROS induction and GSH depletion in the latter [165]. The natural antioxidant levels being a deciding factor in differential cellular responses to NP exposure is consistent with as ROS induction being the main common mechanism inducing nanotoxicity [28].

Since the focus of *in vitro* toxicity testing is shifting to primary cells, Zhang *et al.* evaluated whether differentiated or undifferentiated primary human bronchial epithelial cells showed different levels of toxicity following NP treatment. By evaluating toxicity via a multiparametric method they found the differentiated cells to be more sensitive to silica NP induced injury [166]. Haase *et al.* recently evaluated AuNP toxicity in co-cultures consisting of primary neurons and astrocytes and also found that more differentiated cultures were more sensitive to NP exposure [167]. Jan *et al.* obtained similar results studying QD toxicity in both differentiated and undifferentiated NG108-15 (mouse neuroblastoma x rat glioma hybrid) cells. They explained their findings by the fact that undifferentiated cells show a higher level of adaptability in comparison to the differentiated cells [137]. This is supported by findings from Saretzki *et al.* who found the stress defence mechanism (antioxidant capacity and DNA repair mechanism) in murine embryonic stem cell to be superior to that of differentiated murine cells (fibroblasts, hematopoietic progenitor cells and 3T3 fibroblast long-lived cell line) [168]. These results show higher sensitivity of differentiated cells to NP induced injury, probably due to the lower adaptability to their surroundings. Therefore not only the cell type but also the level of differentiation needs to be taken into account when evaluating nanotoxicity *in vitro*.

It is clear from these data that the response to NP exposure is highly cell type-dependent. Studies comparing sensitivity to nanotoxicity in primary cells and cell lines found significant differences with primary cells mostly being less sensitive to NP induced injury while cell lines

were expected to be more resilient. This can however not be generalised as conflicting results are published on the subject. The level of cellular differentiation also appears to have an influence since more differentiated primary cells were found to be more susceptible to nanotoxicity in several studies. These differences in sensitivity may largely be explained by variations in cell function influencing NP uptake, metabolic activity, natural antioxidant activity and proliferative capacity. These data underscore the importance of selecting a relevant cell system for hazard assessment, which is a balancing act since both primary cells and cell lines show specific advantages and major shortcomings. As for example the phenotype of differentiated primary cells resembles the *in vivo* situation more closely but keeping differentiated cells in culture remains a great challenge. Therefore further research is needed on finding the right cell system as well as optimization of cell culture protocols.



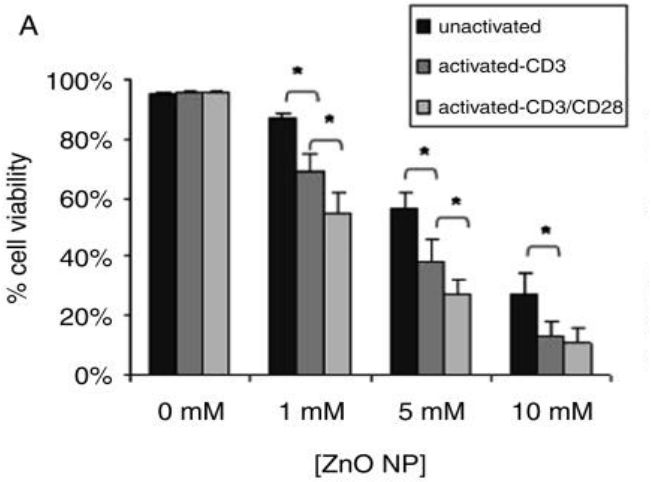


Figure 4: (a) differential viability after NP exposure for Jurkat and Hut-78 cancer cell lines and normal human T lymphocytes. (b) differential viability after NP exposure of unactivated and activated human T lymphocytes.

4. The effect of particle agglomeration and sedimentation

In a similar setup as for chemicals, NP hazard is mostly evaluated in classical 2D monocultures. As chemical substances typically dissolve in the cell medium, the dose to which the cells are exposed to is accurately represented by the concentration of the chemical in the solution. In first instance it was assumed that this also applied to NP, as they were thought to be evenly dispersed by Brownian motion [35]. But this appeared not to be true since NP in dispersion are not only subject to diffusion but also to sedimentation and agglomeration [116]. Therefore the concept of dose is more complex and dynamic for NP as it is for chemicals. To address this issue Teeguarden *et al.* developed the concept of particokinetics to model the NP behaviour in dispersion based on its likelihood to diffuse, sediment and agglomerate in dispersion [116, 131]. Since both diffusion and sedimentation are influenced by agglomeration, the latter is expected to have a major impact on the NP uptake and toxicity [169]. It is important to note the difference between agglomeration and aggregation since both terms are often mixed up: aggregates are formed by covalent bonds and are therefore not as easy to break up as agglomerates, which are held together by van der Waals forces, hydrophobic interactions and/or hydrogen-bonds [170]. The formation of agglomerates originates from the pursuit of a state with a lower free surface energy and is strongly promoted by the hydrophobic nature of most NP [154, 170]. Thus, agglomeration occurs in media when attractive forces overpower the electrostatic or steric repulsion between NP [171].

Several NP- and medium-related factors have been shown to influence NP agglomeration. Parameters in the first category are NP surface charge, size and shape. Brown *et al.* for example found that nanorods and –fibres agglomerate more easily than spheres and Limbach

et al. saw a higher agglomeration rate for smaller NP in comparison to their larger counterparts at similar mass doses, which was explained by the higher number density [172, 173]. This is of course also related to the concentration, which is another important factor as it is known that NP tend to agglomerate at higher concentrations [116]. Allouni *et al.* have put pH forward as one of the medium-related parameters influencing NP agglomeration [41]. Other parameters such as salt composition, ion concentration and ionic strength were also mentioned by a number of groups [41, 171, 174]. Albanese *et al.* have furthermore introduced the presence of proteins, in particular serum proteins, as a very important factor [171]. Its effect on NP agglomeration is however not yet fully understood as several groups found conflicting data on the subject, showing either less or more agglomeration in media containing serum [175, 176]. Still, it can be concluded that NP agglomeration can either be promoted or mitigated depending on the medium composition [176].

Since the majority of the NP are unstable in biological fluids, agglomeration is believed to be inevitable *in vivo* [171]. Consequently it is crucial to take the influence of NP agglomeration on diffusion and sedimentation and subsequently on NP uptake and toxicity into account when evaluating nanotoxicity *in vivo* as well as *in vitro*. Certainly since Albanese *et al.* observed that aggregates show altered kinetics in comparison to their single NP counterparts with the same size [171]. It is therefore crucial to evaluate NP agglomeration in the medium in which the NP will be added to the cells. Dynamic light scattering (DLS) is a successful technique but is limited to samples in simple or diluted media as other light scattering components, such as serum proteins, can interfere with the measurements [177]. It would however be more convenient if NP size and agglomeration could be studied in undiluted biological fluids. Therefore Braeckmans *et al.* developed a novel promising method, combining maximum entropy analysis and fluorescent single particle tracking (fSPT), which allows the accurate

and precise determination of the size distribution of fluorescent NP in undiluted biological fluids [177].

This section will provide an overview of studies evaluating the influence of NP sedimentation on uptake and toxicity and innovative setups to avoid the effect of sedimentation when assessing NP effects on cells.

4.1. Effect of nanoparticle agglomeration and sedimentation on cellular uptake

Since agglomeration influences the size, surface area and number/volume dose, which are all factors known to affect NP uptake, it was expected that agglomeration influences NP uptake. Limbach *et al.* therefore compared the uptake of 20-50 nm diameter single CeO_2NP and 250-400 nm diameter agglomerates in a human long-lived fibroblast cell line (MRC-9). They observed a higher uptake for the agglomerates as they reached the cells more rapidly by sedimentation than single NP, whose transport rate was limited by diffusion [173]. Brown *et al*. however observed a decrease in uptake when the agglomerate's size is similar to, or larger than the cell itself due to physical restrictions to the uptake processes [172]. This is in line with observations from Drescher *et al.* who found higher uptake for the single NP and small agglomerates in comparison to their larger counterparts in 3T3 fibroblasts [175]. These observations fit the assumption that larger aggregates do not enter the cell via the same mechanism as single NP or small agglomerates since most common endocytosis routes like clathrin- or caveolin-mediated endocytosis are limited to the uptake of materials with dimensions of maximally 80 nm [175]. Albanese *et al.* furthermore showed that the uptake mechanism not only depends on NP size but is also cell type-dependent. Since they observed a 25% reduction in uptake for AuNP agglomerates in HeLa cells and A549 cells but a 2-fold increase for the largest agglomerates in a human breast cancer cell line (MDA-MB435) [171].

Finally Lankoff *et al.* observed differential uptake of single AgNP, TiO_2NP and their agglomerates in HepG2, A549 and THP-1 cells, adding NP type-dependency to the picture [147]. From the available (conflicting) data it cannot be concluded whether single NP or small agglomerates are taken up to a higher or lesser extent by the cells. Reviewing these data, we do hypothesize that following factors are equally important; (i) the extent of agglomeration, (ii) the size of the agglomerates and (iii) the cellular uptake mechanism. The extent of agglomeration and the size of the agglomerates will not only determine the rate of sedimentation, and hereby the rate of NP transport towards the cells, but also the way in which the cells will handle these materials. Non-specialized cells will typically prefer smaller NPs while cells capable of ingesting larger materials will take up higher levels of agglomerates. Additional influencing factors are the cell type and type of NP being tested. As such, it is clear that agglomeration and sedimentation have an influence on NP uptake that cannot be neglected.

4.2. Effect of nanoparticle agglomeration and sedimentation on toxicity

Logically, when NP uptake is influenced by agglomeration and sedimentation, NP cytotoxicity might also be affected. Therefore Kirchner *et al.* compared toxicity for different concentrations of MPA- and PEG-coated QD in MDA-MB435 cells. At higher concentrations both NP types agglomerated, precipitated and caused injury, with the PEG-coated NP causing more severe cell damage [37]. Wick *et al.* also found agglomeration to increase particle toxicity as CNT agglomerates were more toxic to MSTO-211H cells than asbestos fibres and single CNT [178]. In contrast, some studies show that cytotoxicity is reduced for agglomerated NP, which is possibly due to the fact that smaller entities are in general taken up more avidly by non-specialized cells and are more able to reach intracellular structures such as the cell

nucleus or mitochondria, which are less accessible to larger particles or aggregates. [172, 179, 180]. Yoon *et al*. further hypothesized that adherent cells might be more affected by NP deposition through sedimentation than cells in suspension and therefore compared Al_2O_3NP and agglomerate toxicity in floating cells (THP-1) and three adherent cell lines (A549, 293T and J774A-1: a mouse macrophage cancer cell line). THP-1, A549 and 293T cell lines showed toxicity, but only A549 and 293T cell lines showed a time dependent toxicity that could be related to sedimentation of the agglomerates, which confirmed their hypothesis [181].

These data clearly show the influence of NP sedimentation on nanotoxicity and indicate that it can result in either underestimated or exaggerated toxicity estimations. Therefore, in order to reduce the *in vitro-in vivo* gap, there is a huge need for novel model systems in which the effect of NP sedimentation can be avoided and will hereby provide toxicity results that are more relevant to the applied dose of the NPs rather than reaching unrealistically high cell exposure levels due to NP sedimentation.

4.3. New model systems to minimize sedimentation effects

Following on from the sections above, it is clear that sedimentation is one of the major shortcomings to classical 2D cultures. Since sedimentation is not observed *in vivo* and was demonstrated to affect nanotoxicity *in vitro*, several groups have tried to develop novel model systems such as inverted cultures, flow models and microfluidic systems in which the influence of sedimentation can be reduced or even completely avoided.

a. Inverted models

Cho *et al.* evaluated the influence of sedimentation on uptake of Au nanospheres, -cages and -rods in human breast cancer cells (SK-BR-3) cultivated in an inverted cell model. This is an elegant and straightforward cell model in which sedimentation itself is not avoided but it will

not result in increased cellular exposure levels for cells that are cultured in the inverted configuration. The authors observed a much more avid uptake of NP in classical cell cultures(cells at the bottom) in comparison to cells cultured in the inverted configuration (cells grown on an insert, at the top of the medium, facing downwards) (Figure 5). However, no variations were observed in toxicity, as cell viability remained approximately 90% of the control values in all conditions tested, but this may be due to the type of material and the limited concentration-range that was tested in this study. The differential uptake in upright or inverted configuration depended on the physical properties of the NP and was most distinct for NP with a greatest propensity to sediment, underscoring the necessity to avoid the influence of sedimentation [35].

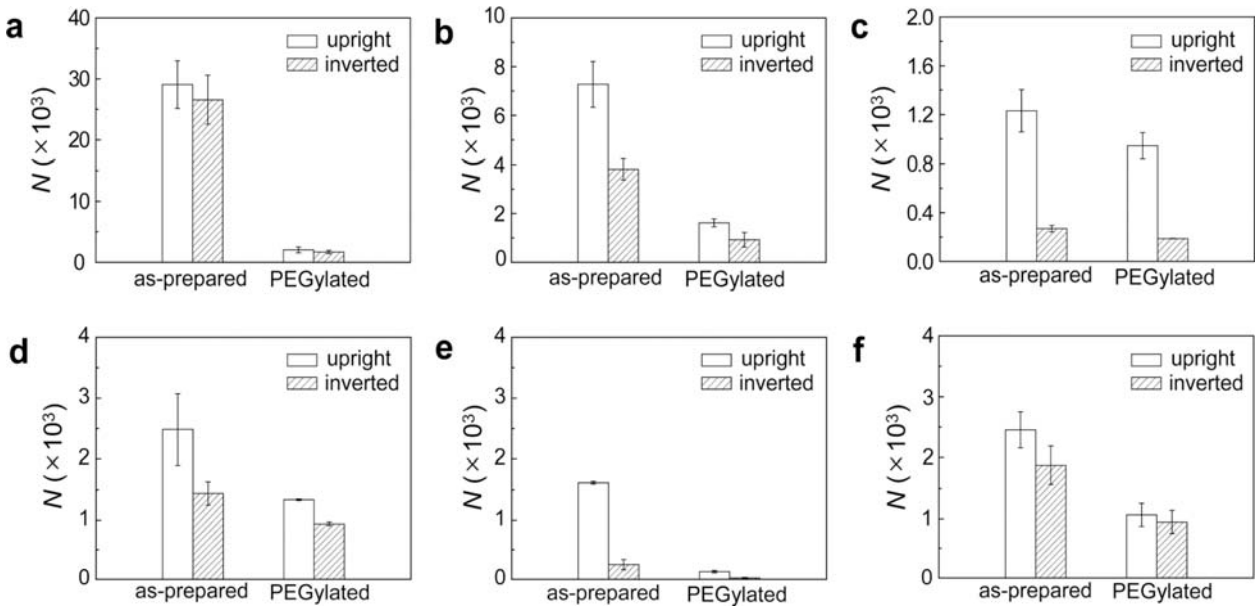


*Figure 5: Differential uptake of **a,** 15-nm nanospheres (120 pM); **b,** 54-nm nanospheres (20 pM); **c,** 100-nm nanospheres (2.8 pM); **d,** 62-nm nanocages (20 pM); **e,** 118-nm nanocages (2.6 pM); and **f,** nanorods (20 pM) in the upright and inverted configuration.*

b. Flow and microfluidic models

More complicated models are flow or microfluidic systems where gravitational setting is impeded or even completely avoided unlike for the inverted model. The applied flow also assists in acquiring a more homogeneous NP distribution and is believed to alter the cell-NP contact time [36, 214]. An additional advantage to applying a flow is the continuous renewal of medium, which ensures a sustained supply of nutrients and a constant pH [36].

It is expected that NP uptake might significantly vary under flow conditions in comparison to static conditions [36, 182]. These variations may also be contributed to altered cell morphology and function under the applied shear stress (SS). It is for instance known that SS induces elongation of endothelial cells in direction of the flow and the formation of actin stress fibres to protect the cells from hemodynamic damage *in vitro* as well as *in vivo* [183, 184]. Samuel *et al.* furthermore found the actin network to be more dense around the nucleus and observed membrane blebbing under flow conditions, where cells under static conditions showed a smooth and flattened morphology [185]. Flow can furthermore cause the activation of endothelial cells and a laminar flow has been shown to suppress proliferation, apoptosis and ROS induction [183]. Taking these aspects into account application of a flow provides a more dynamic and *in-vivo*-like model. Therefore several groups have compared NP uptake under flow and static conditions. Lin *et al*. for instance found a higher uptake of functionalised polystyrene NP in activated human aortic endothelial cells (HAEC) in the classical 2D setup than under flow conditions [186]. Bhowmick *et al.* confirmed these results as they observed 30% higher uptake under static condition for targeted NP in activated HUVECs and EAhy296 cells (a human umbilical vein long lived cell line) [184]. On the contrary Samuel *et al.* did not observe uptake in the static configuration in cells from a HUVEC cell line (CRL-1730) whereas these cells did take up untargeted NP under flow conditions [185].

Some groups have also investigated whether different levels of SS have an influence on NP uptake. For example Farokhzad *et al*. looked into the uptake of targeted NP by two human prostate cancer cell lines (LNCaP and PC3) under three different SS conditions (0.28, 1.1 and 4.5 dyn/cm^2) and observed the highest adherence and uptake under the lowest SS [187]. Kusunose *et al.* found similar results for VCAM-1 targeted liposomes in TNF$_\alpha$-activated HUVECs where the targeting efficiency was the highest under the lowest shear condition [188]. Bhowmick *et al.* observed the same effect for targeted NP *in vivo* in mice and attributed this effect to impaired endocytosis by the augmented SS due to of an increased stiffness of the cytoskeleton [184].

Samuel *et al*. furthermore looked into the influence of the activation of endothelial cells on NP uptake and observed none for untargeted NP in CRL-1730 cells [185]. Lin *et al.* did however find higher NP uptake levels in histamine-activated HAECs for P-selectin targeted polystyrene NP [186]. Bhowmick *et al.* once more supported these findings as they observed higher uptake of functionalized NP in activated HUVECs and EAhy296 cells [184]. These findings implicate that NP uptake under flow conditions does not only depend on the level of SS but also on the activation status of endothelial cells and on whether NP are functionalized or not. As uptake is significantly altered under flow conditions it is expected that the same will be true for nanotoxicity. On the one hand Mahto *et al.* observed significantly higher levels of QD toxicity in 3T3 fibroblasts in static configuration: cells in static condition showed approximately 30% cell viability whereas the percentage of living cells under SS remained 75% and a significantly higher number of deformed and detached cells was found under the static condition [36]. Kim *et al.* on the other hand found significant levels of toxicity under high SS (3.3 and 6.6 dyn/cm^2) whereas HUVECs under static conditions or low SS (0.5 dyn/cm^2)

did not show significant toxicity after 2h exposure to unmodified SiO_2NP. They accounted this difference to the possible activation of the HUVECs under higher SS [182].

It is clear from these data that toxicity levels found in classical 2D monocultures are either exaggerated or underestimated in comparison to levels obtained from *in vitro* experiments where (the effect of) sedimentation is avoided. When NP toxicity is evaluated in a flow system and an extra level of complexity is added as the induced SS influences cell morphology, stiffness, the endocytotic pathway and can activate HUVECs which are all factors influencing NP uptake and toxicity. It is observed that functionalised NP are taken up more avidly under higher SS conditions, which may be explained by the activation of the endothelial cells in this condition, while non targeted NP show higher uptake under static conditions. Results on nanotoxicity are on the contrary conflicting so no firm conclusions can be drawn on this subject yet. But, as toxicity levels measured in these novel model systems significantly differ from those obtained from classical static 2D monocultures and the former are believed to be more *in vivo* like, these models should be optimised and their use should be promoted. Certainly since many of the apparent discrepancies encountered in the available data can be attributed to the level of sedimentation of the NP. Therefore the level of sedimentation in complex biological media should also be analysed for which the described models are also good tools.

5. The effect of cell communication

In most *in vitro* experiments a single cell type, mostly the parenchymal cell type, from the target organ is used in an attempt to predict the *in vivo* effect [38]. These monocultures are static models with a very limited level of complexity while the *in vivo* environment is complex and dynamic [31, 182]. As organs consist of multiple types of differentiated cells, all with their

specific function, modelling the *in vivo* response by only using a single cell type is nearly impossible [189]. This is believed to be a major factor contributing to the large discrepancies that are often found between *in vitro* and *in vivo* data.

An important shortcoming of the monoculture model, partially causing the *in vitro-in vivo* gap, is the lack of intercellular communication. This crosstalk between different cell types is known to be vital in sustaining homeostasis at both the cellular and the organ level and in complicated processes requiring the interaction of multiple cell types such as the processing of xenobiotics, inflammation and immune responses. It is therefore a major challenge to accurately model these processes *in vitro*. Consequently, several processes like inflammation or immune responses cannot be accurately modelled in monocultures [190, 191]. In order to overcome this shortcoming several groups have focussed on establishing co-cultures to bridge the *in vitro-in vivo* gap. Multiple types of co-cultures can be set up as two or more cell types can be combined and cells can be cultured either in direct contact or be separated by culture inserts. Kasper *et al.* observed that cells co-cultured on culture inserts show a more differentiated phenotype and are polarised, implying that the cells have an apical and basolateral membrane with a distinct composition [192]. Furthermore co-cultured cells often display tight and adherent junctions [193]. Thus, these models more closely mimic the *in vivo* environment and are therefore assumed to have a higher predictive power. Since it was also expected that NP uptake and NP induced injury would significantly differ in co-cultures in comparison to monocultures, several groups evaluated NP uptake and toxicity in these novel model systems that were initially developed for hazard assessment of chemical substances. An overview of these data is provided in this section.

5.1. Effect of intercellular communication on NP uptake

Since it is well known that the majority of NP are rapidly taken up by the reticulo-endothelial system after intravenous administration of the NP *in vivo*, a number of groups investigated whether the same trend could be observed *in vitro* in co-cultures of epithelial and phagocytotic cells. For instance Rothen-Rutishauer *et al*. evaluated uptake of polystyrene, Au and TiO_2NP in a co-culture consisting of A549 cells, human monocyte derived macrophages (MDM) and human monocyte derived dendritic cells (MDDC). They observed preferential uptake of all NP in MDM and the least uptake in A549 cells (Figure 6). Compared to the MDDCs uptake in MDM was twice as high, which was explained by the 2-fold greater phagocytotic capacity of the latter [194]. Pinkernelle *et al.* looked into IONP uptake in mixed cultures of rat primary neuronal cells, a spinal chord/peripheral nerve graft co-culture and a Schwann cell/fibroblast co-culture. IONP were evenly taken up in both cell types in the latter co-culture, while in both the mixed culture of primary cells and the spinal chord/peripheral nerve graft co-culture, the highest uptake was observed in the microglia, which are the resident macrophages of the CNS [195]. Since both studies show a preferential uptake in macrophages due to their phagocytotic capacity, this effect should be taken into account when evaluating NP hazard on epithelial or endothelial cells as NP uptake can be overestimated in monocultures leading to exaggerated NP toxicity outcomes.

Kasper *et al.* compared silica NP uptake in H441 cells in a monoculture and H441/ISO-HAS-1 co-culture with H441 being a human lung adenocarcinoma epithelial cell line and ISO-HAS-1 a human microvascular endothelial cancer cell line. For the H441 cells, a higher NP uptake was observed in the monoculture experiments, which was explained by the fact that in co-culture H441 cells are further differentiated, polarised and show a more barrier-forming phenotype, impeding NP uptake [192].

It can be concluded from these data that NP uptake is significantly altered in co-cultures. When macrophages are added to the co-culture they will preferentially take up the NP. This might have a significant influence on the induced toxicity as the NP dose will not be equal in all cell types, but is likely to be higher in the macrophages and lower for other cell types in comparison to the intracellular dose in monocultures. Additionally, when macrophages take up high levels of these NP, this may result in strong reactions that naturally occur within these cells when dealing with pathogens, including the generation of a strong oxidative burst or secretion of pro-inflammatory cytokines, which may then affect any surrounding cells. This secondary damage, induced by the macrophages, would never be picked up in monoculture experiments underscoring the importance of performing co-culture experiments.

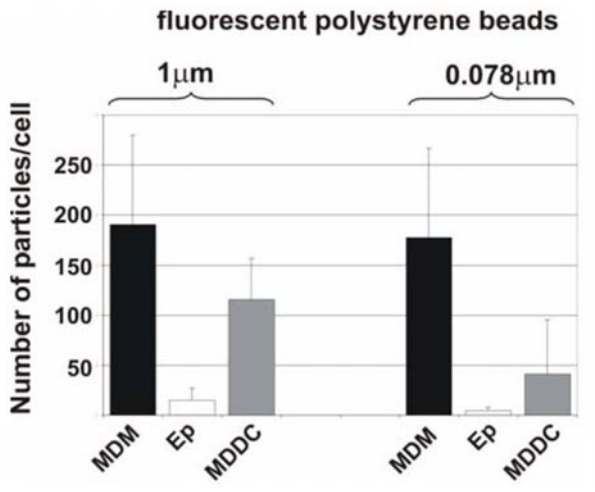


Figure 6: Preferential uptake of fluorescent polystyrene beads in human monocyte derived macrophages (MDM) in co-culture with A549 cells (EP) and human monocyte derived dendritic cells (MDDC).

5.2. Effect of intercellular communication on NP toxicity

It was hypothesized that intercellular communication in co-cultures could significantly alter NP toxicity, certainly since it was shown that NP are not taken up evenly in different cell types when phagocytotic cells are included in the co-culture. As such, activation of these cells and

the release of pro-inflammatory cytokines may have secondary effects on other cell types, the effect of which can only be studied using co-culture models.

Since the lung is expected to be one of the major target organs of NP toxicity via the inhalation of airborne NP, most studies are performed on co-cultures representing alveolar tissue. For instance Wottrich *et al.* compared the cytotoxicity induced by particulate matter (PM_{10}) in A549 and differentiated THP-1 monocultures and co-culture. They observed an increased cytokine release by the cells in co-culture in comparison to both monocultures [196]. Ishii *et al.* performed a similar study exposing a co-culture of human alveolar macrophages and human bronchial epithelial cells and the corresponding monocultures to PM_{10} and also found the cells in co-culture to secrete more cytokines than the sum of the cytokine secretion in the monocultures [197]. When Kim *et al.* evaluated the potential of 8 metal NP to induce apoptosis in a RAW264.7/MLE12 (murine alveolar long-lived cell line) co-culture and both monocultures they found the co-culture to be more susceptible to apoptotic damage, which was explained by the increased TNF_{α} release by the RAW264.7 cells and therefore to the intercellular communication [198].

Another popular combination consists of alveolar epithelial and microvascular endothelial cells to mimic the air-blood-barrier. Culture inserts separate the two cell types and only epithelial cells (representing the apical membrane) are exposed to NP. Using this model, the potential to activate endothelial cells, which can subsequently induce systemic effects, through intercellular communication can be evaluated. Kasper *et al.* evaluated the toxicity induced by 35 nm diameter SiO_2NP in the H441/ISO-HAS-1 co-culture and H441 monoculture. They found the co-culture to be more resilient to nanotoxicity in terms of acute toxicity but it showed an increased inflammatory response when compared to the H441 monoculture [193]. In a follow-up study similar results were obtained when 30 and 48.1 nm

diameter SiO_2NP were tested under the same circumstances [192]. Bengali *et al.* made similar observations when evaluating the effect of PM_{10}, CuO and TiO_2NP on a H441/HPMEC-ST1-6R (human pulmonary microvascular endothelial long-lived cell line) co-culture as apical exposure caused significant modulation of pro-inflammatory modulators in both cell lines without the endothelial cell being in direct contact with the NP [199]. Ramos-Godinez *et al.* on the contrary did not find any changes in cytokine release for a A549/HUVEC co-culture after TiO_2NP exposure. HUVECs from the co-culture did however show a significant increase in adhesion molecules, a 3 to 4-fold increase in monocyte adhesion and a 2-fold increase in nitric oxide (NO) production. These results were comparable to cells from the HUVEC monoculture, which were directly exposed to the NP, implying that endothelial cells were activated via intercellular communication in co-cultures [200]. In none of these studies NP were found to cross the culture insert, possibly due to the barrier formed by the epithelial cells or agglomeration of the NP. This supports the hypothesis that endothelial cells are activated through intercellular communication after exposure of epithelial cells to NP.

Since it was shown that macrophages can have an effect on the toxicity observed in epithelial cells, several groups investigated whether the activation status of macrophages has an influence. Soma *et al.* compared the effect of doxorubicin loaded PACA NP on a M5076/J774.A1 (mouse reticular cell sarcoma cell line/mouse monocyte-macrophage long-lived cell line) co-culture, with the latter cell line either being unactivated or activated via IFN-γ treatment. It was observed that non-activated macrophages acted as a reservoir, thus performing a protective function while activated macrophages elicited secondary toxicity in M5076 cells trough exocytosis of the drug and excretion of TNF_α and NO [201]. Al-Hallak *et al.* confirmed these findings as they obtained similar results for doxorubicin loaded NP in the MS-H/H460 co-culture (murine alveolar macrophages/human lung carcinoma cell line) [202].

van Berlo *et al.* furthermore found that the protective or aggravating effect on NP toxicity also depends on the combination of cell types as he evaluated DQ12 toxicity in co-cultures of A549 cells combined with either C57BL/6J mice neutrophils or C57BL/6J mice macrophages without culture inserts. The macrophages protected A549 cells against oxidative DNA damage whereas the neutrophils aggravated the effect [203].

Some groups added one or more additional cell types to create an even more complex environment. For example Alfaro-Moreno *et al.* evaluated the effects of PM_{10} in several combinations of cell lines: A549/HMC-1, THP-1/HMC-1, A549/THP-1/HMC-1 and A549/THP-1/HMC-1/EAhy296. All cultures had a distinct cytokine excretion profile with the co-culture of mast cells (HMC-1) and macrophages (THP-1) showing a synergistic increase in cytokine excretion in comparison to their monocultures. Which is interesting as it fits the observation that pollution aggravates asthma. They showed that cytokine production could be amplified or mitigated in co-culture, which was explained by differential expression of receptors at the cell surface depending on the cell type or changes in crosstalk between the cells by addition of an extra cell type [204]. Müller et al. compared nanotoxicity of PM_{10}, SWCNT and TiO_2NP in a co-culture consisting of A549, MDC and MDDC and the corresponding monocultures. They found that the interaction between the cells modulated the total antioxidant capacity and the cytokine excretion [205]. It can therefore be stated that the interplay between different cell types significantly modulates the oxidative stress levels and the inflammatory response, which are both known to be affected by NP exposure.

To summarize we can conclude from these data that cells react differently to NP exposure when they are cultured in mono- or co-cultures. Apart from some exceptions, co-cultures are mostly found to be more resilient to acute toxicity but show a significant increase in cytokine release, indicative of an inflammatory or immune response. The effect does however depend

on the combination of cell types that were co-cultured and can be altered by addition of an extra cell type. Furthermore, it has been shown that epithelial cells are capable of activating endothelial cells through intercellular communication after NP exposure. The activation of macrophages also appeared to be important as activated macrophages cause secondary toxicity whereas non-activated macrophages have a protective reservoir function. These data clearly indicate the importance of cell-cell communication and the differences in responses to NP exposure for cells in co-cultures. This is very important for NP hazard assessment since these models are more *in vivo* like and therefore more relevant and likely to have a higher predictive power.

6. The effect of a 3D environment

A final major shortcoming to classical 2D monocultures that will be discussed in this review is the loss of the specific 3D tissue architecture and associated with this, the loss of cell polarisation. 2D cultures therefore generally fail to reconstitute the *in vivo* microenvironment as they offer a reductionist approach with spatial limitations leading to expression of a different cellular phenotype and consequently to the vast *in vitro-in vivo* gap [206]. Not only cellular organisation and polarisation are lost in classical 2D monocultures, cells also fail to produce an *in vivo*-like extracellular matrix (ECM). Cells are still able to produce and secrete ECM proteins in a 2D environment but the extent of ECM production is altered and the barrier formed is less dense and incomplete in comparison to the *in vivo* ECM [207]. This ECM is a very important factor as it is a natural barrier with small pores and therefore limits NP diffusion into a tissue [208]. It is furthermore a key regulator in homeostasis and phenotype expression. Cells cultured in a 3D setup are consequently able to acquire tissue-like organization and differentiation, in part via the ECM stimulating the cells, to levels that have

thus far been impossible to reach in a classical 2D setting [206]. Since cells are more tightly packed in the 3D setup, these models also promote cell-cell and cell-matrix communication, necessary for maintaining homeostasis. In turn, the enhanced communication influences a number of important cellular functions such as migration, invasion, proliferation, apoptosis and differentiation [209]. It was, for example, shown by Kuo *et al.* that the epithelial-mesenchymal-transition, which is a hallmark in the metastatic process, is promoted for several cancer cell lines when they are cultured as spheres [210]. Thus, it can be concluded that the cultivation conditions have a major influence on the cellular phenotype and function and that cellular responses following NP exposure are therefore likely to be drastically altered in a 3D model when compared to a conventional monolayer [207].

A number of 3D systems have been developed of which the multicellular spheroid models, mimicking solid tumours, are most widely used. Another approach is the use of natural or synthetic hydrogels as a scaffold in which cells can be seeded [211]. Hydrogel-based scaffolds are however likely to be not very useful for NP studies as thick scaffolds will limit the diffusion of NP towards the cells, which will drastically alter the outcome of any uptake or toxicity studies. Initially many research groups applied these 3D models in *in vitro* cancer research as they tested multiple anti-cancer agents for their anti-proliferative capacity. Currently these models are also being used to evaluate the delivery and therapeutic efficiency of nanomedicines and the toxicity of engineered nanomaterials. The following section will provide an overview on recent data on NP uptake and toxicity in a 3D setup.

6.1. Effect of a 3D environment on NP uptake

As cells cultured in spheroids are known to produce a more dense and complex ECM, which is a natural barrier and hampers NP diffusion towards the cells, several groups compared NP

uptake in a 2D and 3D setting. For instance Mitra *et al.* found a slow diffusion and heterogeneous distribution of anti-cancer drug loaded NP in Y79 (retinoblastoma) spheres in comparison to an even distribution in classical monocultures of the same cells. The naïve drugs on the other hand were distributed homogeneously throughout the spheres [212]. Godugu *et al*. observed limited penetration of dox-loaded NP in comparison to free doxorubicin in three different human non small cell lung carcinoma spheres (H460, A549 and H1650). Consequently NP uptake was only observed in cells in the peripheral layers of the spheres [209]. Biondi *et al.* compared the uptake of dox-loaded NP in 3D-collagen matrices to 2D cultures of HeLa cells and also found the NP transport to be limited in a 3D environment and uptake to be higher in cells in a 2D culture [213]. Huang *et al.* furthermore compared the uptake of 2, 6 and 15 nm diameter AuNP in a MCF-7 monolayer, spheroid model and mouse breast tumours *in vivo*. In all experiments the same trend could be observed in that the 2nm NP were taken up more avidly than their larger counterparts. The spheroids and tumours showed the same trends in tissue penetration as 2 and 6 nm NP could penetrate the deeper regions of the cell mass, but 15 nm NP were only found in the periphery while all NP were evenly distributed in the monolayer [214].

Thus, it is generally seen that distribution of the NP is less homogeneous in 3D cultures when compared to the conventional monolayers. Furthermore, NP penetration into a 3D matrix is hampered by the ECM and uptake is consequently often limited to the cells in the peripheral layers of the 3D culture, especially for larger NP.

6.2. Effect of a 3D environment on NP toxicity

Since uptake is drastically altered in a 3D environment it was assumed that the same would be seen for cellular responses to NP exposure. Therefore several groups evaluated NP toxicity

simultaneously in 2D and 3D cultures. In the context of the initial application of spheroid models, Godugu *et al.* evaluated the anti-proliferative capacity of dox-loaded NP on three different human non small cell lung carcinoma spheres (H460, A549 and H1650) in comparison to the same cells cultured in a 2D monolayer. They found significantly altered toxicological profiles as IC_{50} values were elevated 5 to 20 times in the 3D setup, which is indicative of a lower toxicity in the latter [209]. Mitra *et al.* confirmed these findings as they found a significantly lower anti-proliferative capacity for anti-cancer agents enclosed in NP in Y79 retinoblastoma 3D cultures in comparison to both the naive agents or the NP used to label the cells in a 2D setting [212]. As mentioned before, this model was recently introduced to nanohazard assessment as to avoid shortcomings from the classical 2D monocultures. For example Lee *et al*. evaluated 2.9 nm diameter CdTe QD and 3.5 nm diameter AuNP toxicity in HepG2 2D and spheroid cultures and found a substantially lower toxicity in the spheroid cultures based on MTT, LDH and calcein AM assays and morphological analysis. The number of dead cells was significantly lower in the 3D setup and most dead cells were found in the periphery of the sphere creating a rugged surface (Figure 7). Longer exposure times induced more severe damage to the cells in periphery of the sphere while the interior remained unimpaired, while when the same incubation conditions were applied to 2D cultures more overall cell death was observed (Figure 8). They attributed these effects to the protective effect of the barrier consisting of the ECM layer secreted by the cells and the dead cells remaining on the exterior of the sphere, due to the tight packing of the cells, temporarily enhancing the efficiency of the barrier [215]. Luo *et al.* obtained similar results when evaluating CdSe/ZnS QD, IONP and SiNP toxicity in HeLa microspheres as they found nanotoxicity to be radial and lower in the 3D setup due to the protective effect of the surrounding cells [208]. Movia *et al*. compared SWCNT toxicity in THP-1 2D cultures and cell aggregates and found

the latter not to be affected while significant toxicity and elevated cytokine secretion were observed in the conventional monolayer [216]. On the contrary, Yu *et al.* found 5 and 30 nm diameter IONP to cause more severe toxicity starting at lower doses to porcine aortic endothelial cells in a 3D alginate matrix than for cells in a conventional monolayer. It must however be noted that cells were unable to divide once added to the matrix while cells in 2D culture retained their proliferative capacity. Since toxicity was only measured after 72h of exposure, cells in 2D cultures were likely to be exposed to lower doses of IONP as NP are diluted upon cell division. This protective effect was likely impeded in the 3D setup, which possibly caused the cells to undergo higher levels of stress as they faced higher doses of the IONP, resulting in elevated levels of toxicity [217].

An additional advantage to 3D models is the fact that cultivation times of most cells can be prolonged, enabling long(er)-term experiments *in vitro*. For instance Hackenberg *et al.* exposed 3D mini-organ cultures of human nasal mucosa on three subsequent days to ZnONP for 1h and did not find any acute toxicity or apoptosis but did observe DNA damage [218]. Thus, 3D models can be applied to assess cumulative effects while this is hampered in 2D cultures as cells do not survive longer cultivation periods, dedifferentiate or rapidly divide causing dilution of the NP and an associated dilution of any possible effects.

It can be concluded from these findings that culturing cells in a 3D setup has a major influence on the cellular phenotype and function and therefore causes cells to react in a drastically different manner to NP exposure. Results from experiments in a 3D setup therefore vary widely from those obtained in their 2D counterparts as most studies show hampered NP penetration through the ECM barrier and reduced cellular uptake. Subsequently toxicological profiles are significantly altered, as toxicity is mostly lower and limited to the outer layers of the culture. These 3D models are likely to have a higher predictive power, as they are a closer

representation of the *in vivo* environment. Thus, the development of 3D models is a recent milestone in bridging the *in vitro-in vivo* gap and these models are likely to greatly gain importance in the next few years.

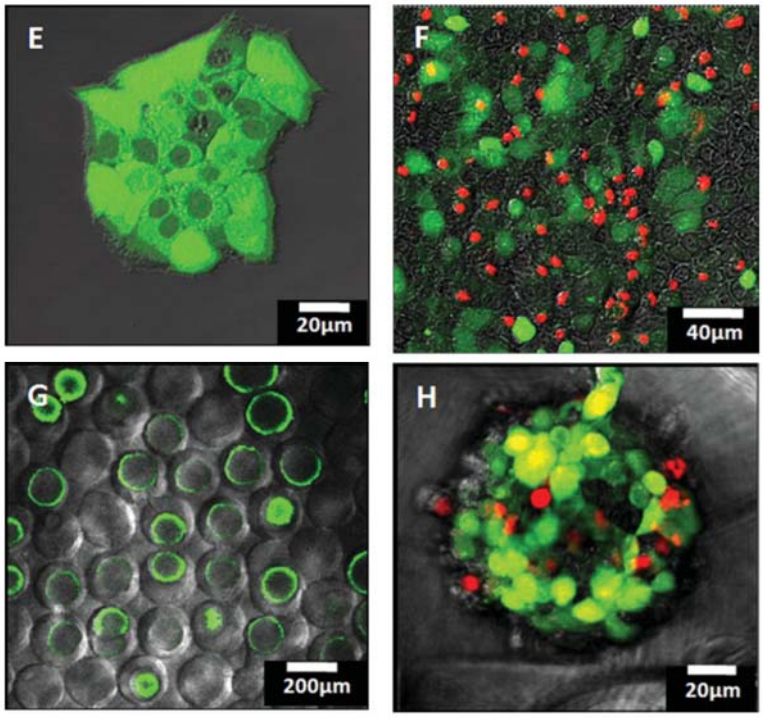


Figure 7: Confocal images of live/dead-stained normal E) 2D and G) 3D spheroid cultures and after CdTe NP exposure in F) 2D.and H) 3D spheroid cultures.

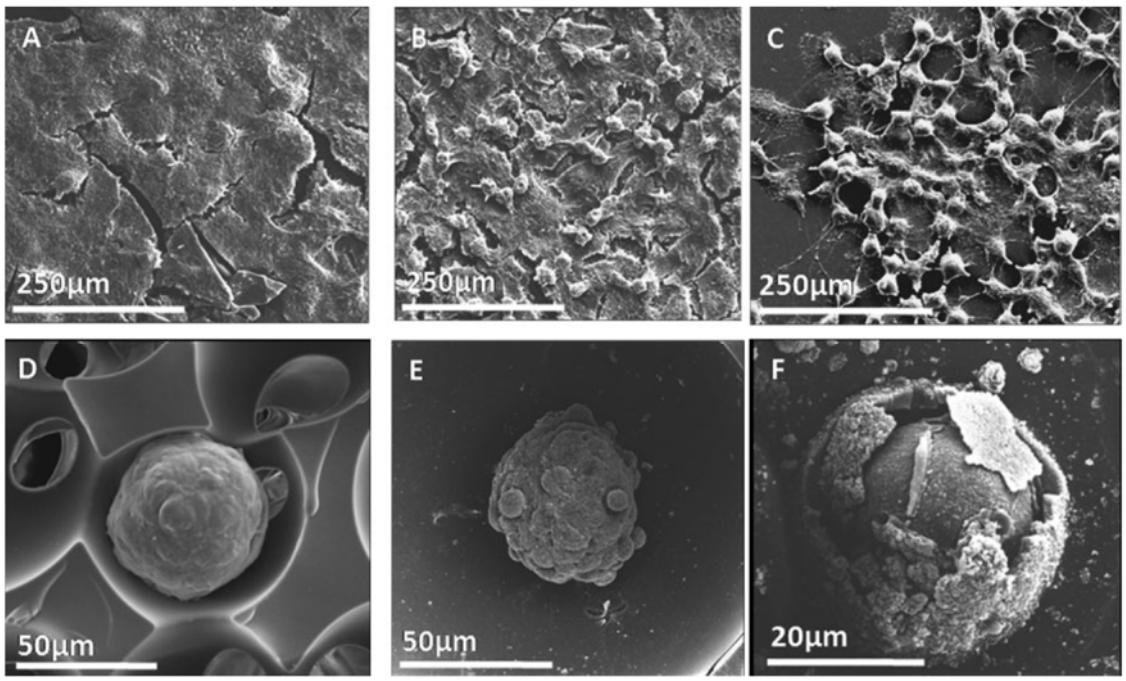


Figure 8: SEM images of 2D and 3D spheroid cultures before and after CdTe NP exposure. Typical morphology of A) 2D and D) 3D spheroid cultures after 5 days without CdTe NP exposure. Representative morphology of B) 2D and E) 3D spheroid cultures after 12 h of CdTe NP treatment. Morphological change of C) 2D and F) 3D spheroid cultures after 24 h of CdTe exposure.

7. Conclusion and outlook

The present review provides an overview of several strategies that have recently been put forward in order to try and optimize cell models for more robust and reliable *in vitro* analysis of NP uptake and toxicity in advance of any *in vivo* applications. Where the field of nanotoxicology is advancing fast, it is still lagging behind the rapid developments in the field of nanotechnology. The great number of different types of materials, each with their own specific features and the heavy impact of even the most miniature changes in a single physicochemical parameter of a NP on its interaction with biological entities make it very hard to thoroughly evaluate the interactions of nanomaterials with cells or tissues. Where *in vitro* studies are now often being performed by many research groups and are being optimized more and more in order to overcome some of the initial hurdles, *in vivo* studies remain scarce. The most used *in vitro* model, namely the classical 2D monoculture, is however a very reductionist approach were most of the complexity of the in vivo situation is lost. Therefore, results from *in vitro* studies often did not relate very well with the findings obtained in *in vivo* studies. Several groups have subsequently made substantial efforts in trying to optimize the current *in vitro* models to better mimic the *in vivo* conditions. As described in the various sections above, the use of inverted cell cultures, flow models, co-cultures or 3D cell cultures all have their advantages compared to the classical 2D monolayers used for NP uptake and toxicity studies.

As the field of nanotechnology keeps blooming and the safety of nanomaterials remains questionable as we are all being exposed more and more, it is to be expected that all these

models will gain more importance as more robust rapid screening tools. Based on the data obtained with these models, better predictions should be possible as well as a better selection of materials more interesting to further evaluate in *in vivo* studies. More optimisation is however needed to fully exploit the benefits of these models. One aspect that further needs to be looked into is how well the different models are suited for studying NP-cell interactions. A second aspect, for instance, is the possible combination of several of these models is a very interesting approach. Using co-culture models or flow models in settings that are suitable for HCS, would enable these methods to be used as rapid screening tools. Additionally, the use of 3D models with dynamic flow or co-cultures with dynamic flow would provide many opportunities to further bridge the *in vitro-in vivo* gap as well as to rapidly study the targeting efficacy of, for instance, anti-cancer agent-containing NP under more physiologically relevant conditions. The use of 3D models consisting of different cell types would also open up many opportunities and could serve as an important step towards the development of artificial organs for NP delivery/toxicity screening.

Given the rapid developments in the field of nanotoxicology and the on-going maturation of this niche area into a full scientific discipline, more relevant in vitro models such as the ones described in the present review will become increasingly important in various research areas that are linked to the use of nanomaterials in biological settings. Considering that most of these models have only recently been introduced, it is to be expected that more optimized models such as combinations of the ones mentioned will soon be set up and will have big impacts on our understanding of how nanosized materials interact with cells and tissues under physiologically relevant conditions.

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